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Rumen responses to dietary supplementation with cashew nut shell liquid and its cessation in sheep

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Abstract

Rumen responses to cashew nut shell liquid (CNSL) were evaluated in a feeding study. Four wethers were fed a hay and concentrate diet for 4 weeks (Pre-CNSL period), and then fed the same diet supplemented with low and high levels of CNSL for 2 weeks each (L-CNSL and H-CNSL periods, respectively). The diet was then reverted to the un-supplemented control diet for another 2 weeks (Post-CNSL period). Rumen parameters were monitored in each feeding period. CNSL, regardless of the two levels tested, did not show any adverse effects on total short chain fatty acid concentration and dry matter digestibility in the rumen. Propionate proportion increased in the H-CNSL period, while methane production potential, acetate and butyrate proportions, viscosity, foam formation and its stability, and ammonia concentration decreased. Values of these parameters returned to those in the un-supplemented control period after cessation of CNSL supplementation. Clone library analysis of 16S rRNA genes revealed the following shifts in the H-CNSL period. For bacteria, Firmicutes was frequently detected, while Bacteroidetes and Spirochetes were not. For archaea, *Methanobrevibacter wolinii* was predominant. These results indicate that CNSL could be a methane inhibitor and propionate enhancer by altering the rumen microbial community.

Keywords: cashew nut shell liquid, methane, microbiota, rumen fermentation, sheep

Introduction

Approximately 81–92 million tonnes of methane (CH_4) is emitted annually from ruminants, amounting to 23–27 % of total anthropogenic CH_4 and contributing to global warming (IPCC 2007). Methane from the rumen is considered to be a loss of gross energy intake (2–15 %), and thus reduces the potential conversion of feed energy to metabolizable

energy (Giger-Reverdin & Sauvant, 2000). According to Nkrumah et al. (2006), weight gain of growing animals or milk production can be increased based on the energy balance when CH_4 emission is deducted. Hence, the inhibition of methanogenesis has been viewed in the context of nutrition, and more recently from the perspective of mitigating greenhouse gas emissions. The mitigation of methane from ruminants, therefore, can theoretically improve feed conversion efficiency in ruminant animals as well as ease the environmental burden.

Several efforts have focused on decreasing enteric CH_4 production by the dietary addition of antibiotics, lipids, plant and other organic compounds, or by otherwise controlling dietary composition (Hook et al. 2010). In particular, the ionophore antibiotic monensin has been extensively used as an additive for beef as well as dairy cattle. Monensin increases propionate and decreases CH_4 production, deamination and biohydrogenation in the rumen, and prevents coccidiosis, lactic acidosis and feedlot bloat, all of which are favorable characteristics leading to efficient animal production (Russell & Strobel 1989).

However, the use of antibiotics in cattle farming is being reconsidered in terms of food safety. Growth-promoting antibiotics including ionophores were banned in the European Union in 2006. As alternatives to ionophore antibiotics, many natural products exerting ionophore-like actions have been actively explored. These alternatives include plant extracts such as tannins, saponins and essential oils containing cinnamaldehyde and eugenol (Calsamiglia et al. 2007; Kobayashi et al. 2016). The main action of these compounds is the reduction of ammonia and increase of propionate in the rumen. These activities could improve protein and energy utilization in ruminants. However, the literature is controversial as to the ability of these plant-originating compounds to decrease ruminant CH_4 production (Calsamiglia et al. 2007).

Cashew (*Anacardium occidentale*) nut shell liquid (CNSL) has the potential to mitigate rumen methane production (Watanabe et al. 2010; Shinkai et al. 2012). This material

is a byproduct of the cashew nut industry and has been used as a raw material for products such as paints and brake linings (Paramashivappa et al. 2001). This liquid contains antimicrobial compounds such as anacardic acid, cardanol and cardol, which are salicylic acid derivatives with an alkyl group. These compounds, especially anacardic acid, inhibit Gram-positive bacteria including those within the rumen (Watanabe et al. 2010). In fact, selection of rumen bacteria by CNSL is apparent in the artificial rumen, in which the fermentation pattern shifted to more propionate and less CH_4 production. Such shifts were indicated in feeding studies using dry cows (Shinkai et al. 2012) and milking cows (Shinkai et al. unpublished). The inhibitory action of CNSL against streptococci and lactobacilli (Watanabe et al. 2010) might prevent the occurrence of rumen lactic acidosis and feedlot bloat (Nagaraja & Titgemeyer 2007). In addition, the decrease of rumen ammonia concentration with CNSL supplementation (Watanabe et al. 2010; Shinkai et al. 2012) is indicative of efficient protein economy in ruminant animals. Based on the above, CNSL is considered to be a promising alternative to the ionophore monensin in terms of rumen modulatory functions including propionate enhancement, methane mitigation, and prevention of excess protein degradation, lactic acidosis and bloat.

In the present study, CNSL was further evaluated as a multi-functional additive for ruminant animals. The *in vivo* feeding study was conducted to elucidate how dietary CNSL supplementation and its cessation impact fermentation parameters and microbes in the rumen of sheep.

Materials and methods

Animals, diets, feeding and experimental design

The animal protocol used in the present study was in accordance with the Guidelines for Animal Experiments, Hokkaido University (2007) and the Act on Welfare and

101 Management of Animal (2005). We used four sheep 81.5±17.1 kg in body weight (BW) that
102 had been ruminally cannulated. All sheep were fed a basal diet consisting of orchard grass hay
103 and a concentrate (Lamb 76ME; Mercian, Tokyo, Japan) at a 3:7 ratio. The diet contained
104 16.0 % crude protein (CP), 40.5 % neutral detergent fiber (NDF) and 2.10 Mcal metabolizable
105 energy/kg on a dry matter (DM) basis and was given to the animals once daily (0900) to meet
106 the maintenance requirement of each animal (fed at 1.4 % of BW on a DM basis).
107 Sheep were fed the basal diet for 4 weeks (Pre-CNSL period), followed by the CNSL
108 supplementation period for 4 weeks. During the supplementation period, CNSL was fed at 2
109 g/day/100 kg BW for the first 2 weeks and at 4 g/day/100 kg for the second 2 weeks (L-CNSL
110 and H-CNSL periods, respectively). Then, the diet was reverted to the un-supplemented
111 control diet and fed for 2 weeks (Post-CNSL period). The experimental CNSL was extracted
112 from cashew nut shells (Indian origin) with compressing machinery without the use of
113 organic solvents at the Central Laboratory, Idemitsu Kosan Co. Ltd. (Sodegaura, Japan). The
114 CNSL was diluted with 25 ml of 99.5 % ethanol, mixed with the daily amount of concentrate,
115 air-dried overnight to remove ethanol, and fed to each sheep. The same procedure was
116 employed for the control diet but using ethanol without CNSL.
117
118 *Sampling*
119 Sampling of the rumen content was conducted on the 28th day of the Pre-CNSL period,
120 and the 7th and 14th days of each CNSL and Post-CNSL periods. On each sampling day, the
121 rumen content was removed by hand via the cannula of each sheep at 0, 4, 8 and 12 h post-
122 feeding (ca. 250g at 0h and ca.50g at other samplings). Ruminal pH was immediately
123 measured using a portable pH-temperature meter (HANNA Instruments HI 8424
124 Microcomputer, Singapore, Singapore). The rumen content was then strained through two
125 layers of surgical gauze and immediately used for gas and foam production measurements, or

126 frozen (-80 °C) until further analyses for short chain fatty acids (SCFA), ammonia, bacteria
127 and archaea. For protozoal analysis, the strained rumen fluid was fixed with a methylgreen-
128 formalin-saline (MFS) solution (Ogimoto & Imai 1981).

129 Nylon bags (15 cm x 10 cm, 50 μm pore size) containing the diet as fed to sheep
130 (mixture of 0.9 g hay and 2.1 g concentrate that were ground into 2mm) were suspended in
131 the rumen (4 bags in each sheep) before feeding on the above rumen sampling days (Orskov
132 & McDonald 1979). After incubation for 24 h, the bags were withdrawn from the rumen,
133 washed thoroughly with tap water and dried (at 100 °C for 6 h) to measure the remaining DM,
134 which was used to calculate ruminal DM disappearance. In the analysis, 0 h disappearance
135 was not recorded.

136

137 *Methane production potential and physical properties of rumen fluid*

138 The strained rumen fluid from each sheep taken at 0 h was mixed with an equal
139 volume of McDougal's buffer (McDougal 1948) and dispensed into a test tube (180 mm in
140 length and 10 mm in diameter), which was flushed with nitrogen (N_2) gas and fitted with a
141 butyl rubber stopper and a plastic screw cap. The tubes were incubated at 38 °C for 24 h
142 without substrate at 5 replicates for each sheep. Total gas produced in the headspace of the
143 tube was measured by a needle-attached pressure gauge and was employed for methane
144 analysis. These were performed as described by Watanabe et al. (2010). Using this data, CH_4
145 production from the rumen fluid was expressed as CH_4 production potential (CH_4 mL/mL of
146 rumen fluid/day).

147 Physical properties such as viscosity, ingesta volume index (IVI) and stable IVI were
148 assessed according to Sakauchi and Hoshino (1981) and Jacobson et al. (1957). In brief,
149 strained rumen fluid (0 h sample) from each sheep (50 mL) was dispensed into a gravimetric
150 cylinder (100 mL in volume) and incubated at 38 °C for 1 h (n=4). Volume increase was

recorded as IVI (foam formation potential), and then the content of the cylinder was mixed with a glass rod by gently rotating 3 times to break the foam on the surface and the final volume was recorded as stable foam formation potential (sIVI).

Chemical and microbiological analyses

Collected gas samples and SCFA were analyzed by gas chromatography (GC-14B; Shimadzu, Kyoto, Japan) fitted with a thermal conductivity detector and a flame ionization detector, respectively. The columns and operating conditions were as described by Ushida et al. (1985) and Suto (1973), respectively. Ammonia was analyzed by an indophenol reaction procedure (Weatherburn, 1967).

Bacterial and archaeal analysis using 0 h rumen samples (pooled samples from 4 sheep) obtained in the Pre-CNSL and H-CNSL periods was carried out by sequencing a 16S rRNA gene library as essentially described in Koike et al. (2003), except that PCR primers for the archaea library were according to Wright et al. (2007). Sequencing was performed by a commercial service (TAKARA Bio Inc., Kusatsu, Japan). Almost full length 16S rRNA genes (ca. 1300-1400 bases) were read, and BLAST was employed to define OTU sharing > 97 % sequence identity. Diversity indices were obtained from FastGroup II (http://biome.sdsu.edu/fastgroup/). Comparisons between libraries were performed using LIBSHUFF (http://libshuff.mid.uga.edu/). Rumen protozoa were enumerated (Ogimoto & Imai 1981) and counted by direct microscopic observation as described by Suto et al. (1973).

Statistical analysis

The data were subjected to ANOVA using the GLM procedure of SPSS (Version 16.0 J; Tokyo, Japan), in which the experimental period was a fixed effect and sheep was a random effect. Crossover design was avoided due to the possible residual effect of CNSL, while a

simple one-factorial design was adopted in which the effect of time was ignored. Multiple comparison was made by Tukey's test. The residual effect of CNSL was detected by simply comparing the data between the CNSL period and the post-CNSL period.

Results

Physical properties and gas production potential of rumen fluid

The effects of CNSL feeding on rumen physical properties and gas production potentials are shown in Table 1. Viscosity was reduced by CNSL supplementation, especially in the H-CNSL period. At the same time, IVI and sIVI were also decreased by CNSL feeding. All of these parameters were decreased in week 2 of the L-CNSL period compared with those in the Pre-CNSL period. This decrease lasted throughout the H-CNSL period. Meanwhile, the lowered values returned to the original control levels within the 2 weeks Post-CNSL period. Gas production potentials were also decreased by CNSL supplementation, except for H_2. Total gas, carbon dioxide (CO_2) and CH_4 decreased in week 1 of the H-CNSL period. However, these gas production potentials returned to the original levels once CNSL was removed from the diet.

Short chain fatty acids profile

Table 2 shows the effects of CNSL feeding on the SCFA profile. Total SCFA concentration was not affected by CNSL supplementation. However, the concentration of individual SCFA at 0 h feeding was differentially changed in the H-CNSL period, i.e., CNSL feeding increased the propionate proportion, while decreasing the acetate proportion without affecting the butyrate proportion. In the Post-CNSL period, the proportions of these acids returned to those in the Pre-CNSL period. These parameters did not show particular changes at 4-12 h after feeding.

201

202 *Ruminal pH, ammonia, lactate, DM disappearance and protozoa*

203 Effects of CNSL on ruminal pH, ammonia, DM disappearance and protozoa are
204 shown in Table 3. Ruminal pH was not affected by CNSL feeding. Ammonia concentrations
205 at 0 h after feeding were lower in the H-CNSL period, particularly in the second week of the
206 H-CNSL period, compared to the Pre-CNSL period. However, the values returned to original
207 levels in the Post-CNSL period. DM disappearance from the rumen and protozoal numbers
208 were not affected by CNSL feeding.

209

210 *Rumen bacteria and archaea*

211 Rumen bacterial and archaeal communities are shown in Table 4, as determined by the
212 analysis of the 16S rRNA gene library. LIBSHUFF analysis showed significant changes in
213 both communities with CNSL feeding. In addition, CNSL reduced the diversity of bacterial
214 and archaeal communities. Propionate-producing bacteria such as *Megasphaera elsdenii* and
215 *Selenomonas ruminantium* were more frequently detected in the H-CNSL period than in the
216 non-supplemented Pre-CNSL period. Meanwhile, a H_2 and formate producer, namely
217 *Treponema bryantii*, and the lactate producing *Lactobacillus mucosae* were detected more
218 often in the Pre-CNSL period. For methanogenic archaea, *Methanobrevibacter* was a major
219 genus, dominated by *Methanobrevibacter millerae* and *Methanobrevibacter ruminantium* in
220 the Pre-CNSL period, while these species were replaced by *Methanobrevibacter wolinii* in the
221 H-CNSL period.

222

223 **DISCUSSION**

224 This is the first feeding study of CNSL using sheep to monitor rumen responses to
225 CNSL under a fixed (restricted) feeding condition that controls for different feed intake

levels among animals. Responses were evaluated in sheep fed un-supplemented control diet, CNSL-supplemented diet at a low level followed by that at a high level, and then the un-supplemented control diet again. This design allows determination of how the responses to CNSL change in relation to addition and removal, which provides useful information for the practical use of CNSL.

Decrease in the production potential of methane gas (Table 1) together with enhanced propionate production (Table 2) by CNSL feeding was demonstrated. These results are in good agreement with the *in vitro* results of Watanabe et al. (2010) and the *in vivo* results using cattle of Shinkai et al. (2012), even though the methodology for methane measurement differed between the studies. Meanwhile, feed digestion was unchanged by CNSL feeding according to stable DM disappearance (Table 3). This is the most important characteristic when evaluating a new feed additive candidate to modulate rumen fermentation without negatively influencing animal growth and/or production performance.

Rumen ammonia level was decreased by CNSL feeding only in 0 h samples (Table 3), partly suggesting the possible occurrence of enhanced feed N economy, because the rapid release of ammonia in the rumen might cause inefficient N utilization (Wallace et al. 1997) and increase N output to excreta. In particular, the latter can cause emission of the strong greenhouse gas N_2O (Petersen 2017). Thus, the increase of propionate and decreases of methane and ammonia in the rumen are positive signs for improving feed utilization as well as easing the environmental burden.

The reason why changes of fermentation pattern (acetate, propionate and ammonia) are apparent at 0 h (or 24 h) (Tables 2 & 3) is not clear. However, these parameters are the reflection of rumen microbial composition and activity, which can be altered with CNSL exposure even in a single day. Such ecologic and metabolic changes could exist, depending

on dose level of CNSL and time after feeding. This possibility may explain interactions between treatment (period) and sampling time.

Physical parameters in the rumen fluid were changed in a direction favorable for ruminal health, i.e., viscosity, IVI, and sIVI were reduced in the H-CNSL period (Table 1). These parameters are potential indicators of rumen lactic acidosis and feedlot bloat (Sakauchi & Hoshino 1981; Russell & Strobel 1989). Watanabe et al. (2010) suggested that the addition of CNSL can prevent these disorders, by inhibiting the growth of lactate-producers as one of the known causes. In fact, the lactate producer *Lb. mucosae* was not detected in the rumen of sheep fed CNSL in the present study (Table 4). Kobayashi et al. (2012) showed SEM images of lactate-producing *Streptococcus bovis*, which is involved in lactic acidosis and feedlot bloat (Sakauchi & Hoshino 1981) and demonstrated the physical disruption of the bacterial cell surface by the surfactant action of CNSL. These indicate that CNSL could be a potential feed additive to prevent such disorders. Sakauchi & Hoshino (1981) reported that the ionophore monensin decreased these parameters, contributing to rumen health by minimizing foam formation and facilitating foam disappearance in the rumen. Therefore, CNSL could be suggested to have multiple functions for modulating rumen fermentation in terms of feed efficiency, environmental protection, and even animal health.

The present study clearly showed that the high dose (4 g/day/100 kg BW) of CNSL was effective in sheep rumen compared to the low dose (2 g/day/100 kg BW) (Tables 1-3), which is in agreement with the results in Holstein dry cows from Shinkai et al. (2012). Therefore, this CNSL supplementation level is recommended for further application studies. However, it is important to consider that rumen volume and digesta passage rate can affect the functionality of CNSL even in the same animal species, e.g., dairy cows ingest feed at a much higher level than dry cows, leading to faster passage and shortened exposure time of rumen microbes to CNSL. This can limit the action of CNSL. Thus, a higher CNSL supplementation

level could be considered for high-producing dairy cows. Another important finding is that fermentation shifts reverted once CNSL feeding ceased (Tables 1-3). This demonstrates that CNSL has no residual effect on rumen fermentation. In other words, CNSL would need to be fed continuously if long-term functional effects are expected. Similar results regarding the lack of residual effects on rumen fermentation were reported for supplementation with an ionophore antibiotic (Kobayashi et al. 1988).

The shifts in rumen fermentation observed in the present study are likely caused by changes in rumen microbiota. CNSL has been reported to alter microbial populations to support increased propionate and decreased methane (Watanabe et al. 2010; Shinkai et al. 2012; Oh et al. 2017). The effects include increases in propionate- and succinate-producers and decreases in hydrogen- and formate-producers as observed in the present study, i.e., greater detection of *Megasphaera* and *Selenomonas* and reduced detection of *Treponema*, respectively (Table 4). The archaeal community also showed clear shifts to decreased *M. ruminantium* and increased *M. wolinii* detection frequencies (Table 4). At present, it is not possible to speculate about these archaeal changes, because the methanogenic activity of the above two species has not yet been characterized. However, such a shift in community composition may contribute to decreased methanogenesis in the rumen. The present study provides limited information regarding the rumen microbiota because of the sparse microbial data and small coverage of the mini-clone library analysis. The above speculation requires confirmation using a more quantitative and comprehensive microbial dataset.

The mode of action for the microbial selection by CNSL is attributed to the surfactant action of alkylphenols, as represented by anacardic acid (Kobayashi et al. 2016). This compound should be kept intact to ensure stable function. In fact, heat-treated CNSL shows weakening or loss of antimicrobial function (Branco et al. 2015), thereby decreasing its rumen modulatory function (Kobayashi et al. 2016). Technological developments in processing

CNSL without heat treatment ensures the functionality of CNSL in ruminant livestock at practical levels.

CONCLUSION

Sheep rumen responses are apparent and are generally in good agreement with responses in cattle, which are caused by rumen microbial changes. However, once CNSL is removed, favorable changes regress. This indicates that CNSL works quickly and effectively when dosed at an appropriate level and has no residual effect once supplementation ceases. The main mode of action is microbial selection in the rumen, which quickly responds to the addition/removal of CNSL. In regards to the development of microbial tolerance to CNSL, a longer term feeding study is required.

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396
397

Table 1. Effect of cashew shell liquid feeding on rumen physical properties and gas production potentials obtained from 0h samples

Items	Pre-CNSL period	L-CNSL period		H-CNSL period		Post-CNSL period		SEM	*P*-value
		wk 1	wk 2	wk 1	wk 2	wk 1	wk 2		
Viscosity, *cp*	4.5^{a}	3.6^{ab}	2.8^{b}	2.4^{b}	2.8^{b}	2.8^{b}	3.6^{ab}	0.47	0.045
IVI, %	7.7^{a}	6.7^{ab}	3.5^{bc}	1.7^{c}	1.8^{c}	6.2^{ab}	6.8^{ab}	1.23	0.006
Stable IVI, %	6.3^{a}	5.8^{ab}	2.8^{bc}	1.3^{c}	1.2^{c}	4.5^{ab}	5.0^{ab}	0.98	0.004
Rumen gas, mL/day									
H_2	0.03	0.04	0.03	0.07	0.04	0.02	0.03	0.021	0.537
CO_2	3.4^{a}	3.4^{a}	2.4^{ab}	1.6^{b}	2.7^{ab}	2.8^{ab}	3.3^{a}	0.05	0.035
CH_4	0.83^{a}	0.69^{a}	0.46^{ab}	0.22^{b}	0.32^{a}	0.44^{ab}	0.73^{a}	0.092	0.030
Total gas	4.3^{a}	4.2^{a}	2.9^{ab}	1.9^{b}	3.0^{ab}	3.2^{a}	4.1^{a}	0.03	0.047

CNSL, cashew nut shell liquid; wk 1 and wk 2, samples at week 1 and week 2, respectively;
Pre-CNSL period, no supplement control;
L-CNSL period, CNSL was fed at 2 g/day/100kg BW; H-CNSL period, CNSL was fed at 4 g/day/100kg BW;
Post-CNSL period, no supplement control
cp, cPoise; measurement unit of dynamic viscosity in the centimeter gram second system of units
(1 centipoise = 0.01 gram per centimeter-second)
IVI, Increase volume index.
$^{a-c}$ Means (n = 4) within a row with different superscripts differ ($P < 0.05$).

Table 2. Effect of cashew shell liquid feeding on short chain fatty acid profile in the rumen

Items	Pre-CNSL period	L-CNSL period		H-CNSL period		Post-CNSL period		SEM	*P*-value
		wk 1	wk 2	wk 1	wk 2	wk 1	wk 2		
Total short chain fatty acids, mmol/dL									
0	4.1	3.8	3.7	3.1	2.9	2.8	3.4	0.48	0.612
4	8.4	9.1	8.7	8.6	8.9	8.1	7.6	1.17	0.947
8	7.9	7.9	7.6	6.8	7.0	6.0	6.5	1.03	0.860
12	6.6	6.2	7.3	5.3	5.4	5.9	6.5	0.79	0.624
Acetate, molar %									
0	56.2^{ab}	58.8^{ab}	54.3^{b}	47.2^{d}	51.4^{cd}	57.8^{ab}	62.5^{a}	1.72	0.001
4	55.8	57.7	56.9	53.3	61.8	64.3	60.0	2.76	0.145
8	57.5	60.5	58.2	56.2	61.9	63.0	60.4	2.85	0.667
12	58.6	59.7	57.6	53.5	58.7	60.3	58.7	1.99	0.374
Propionate, molar %									
0	22.0^{bc}	20.9^{bc}	22.8^{b}	32.1^{a}	30.0^{a}	19.9^{bc}	15.5^{c}	3.18	0.017
4	24.9	26.8	26.4	24.8	23.7	22.2	26.9	3.72	0.966
8	22.0	23.9	25.2	24.3	22.9	21.7	27.0	3.23	0.915
12	22.2	23.7	24.3	27.4	26.8	22.9	26.2	2.66	0.727
Butyrate, molar %									
0	14.7	14.3	12.2	11.1	10.2	13.8	14.6	1.88	0.543
4	15.7	12.3	12.2	16.8	11.6	11.0	10.4	1.80	0.144
8	17.0	13.0	12.2	15.3	12.1	12.0	9.7	1.75	0.136
12	15.9	13.3	13.1	14.4	11.4	12.6	10.9	1.89	0.633

CNSL, cashew nut shell liquid; wk 1 and wk 2, samples at week 1 and week 2, respectively;
Pre-CNSL period, no supplement control;
L-CNSL period, CNSL was fed at 2 g/day/100kg BW; H-CNSL period, SNSL was fed at 4 g/day/100kg BW;
Post-CNSL period, no supplement control.
[a-d] Means (n = 4) within a row with different superscripts differ ($P < 0.05$).

Table 3. Effects of cashew nut shell liquid feeding on ruminal pH, ammonia nitrogen, dry matter disappearance and protozoal numbers in the rumen

Items	Pre-CNSL period	L-CNSL period		H-CNS period		Post-CNSL period		SEM	*P*-value
		wk 1	wk 2	wk 1	wk 2	wk 1	wk 2		
pH									
0	7.1	7.0	7.0	7.0	7.1	6.9	6.8	0.06	0.111
4	5.5	5.5	5.4	5.5	5.3	5.2	5.6	0.16	0.711
8	5.9	5.9	5.8	5.8	6.0	5.8	5.9	0.21	0.989
12	6.1	6.3	5.9	6.3	6.4	6.3	6.0	0.14	0.317
Ammonia, mg of N/L									
0	24.1^{ab}	28.9^{ab}	17.9^{bc}	20.9^{bc}	14.2^{c}	27.9^{ab}	33.4^{a}	4.24	0.054
4	20.3^{ab}	39.3^{a}	26.0^{ab}	12.5^{b}	16.1^{b}	24.4^{ab}	25.8^{ab}	16.61	0.041
8	18.2^{ab}	30.0^{a}	25.1^{ab}	16.4^{b}	16.5^{b}	26.1^{ab}	31.3^{a}	4.40	0.011
12	21.1	33.5	29.5	20.0	20.0	32.5	32.7	4.61	0.125
DMd, %	60.7	58.3	63.3	47.6	58.6	64.7	70.0	7.46	0.535
Protozoa, log cell/mL	5.85	5.73	5.64	5.16	5.51	5.84	5.49	0.213	0.302

CNSL, cashew nut shell liquid; wk 1 and wk 2, samples at week 1 and week 2, respectively;
Pre-CNSL period, no supplement control;
L-CNSL period, CNSL was fed at 2 g/day/100kg BW; H-CNSL period, CNSL was fed at 4 g/day/100kg BW;
Post-CNSL period, no supplement control;
DMd, dry matter disappearance at 24h after incubation.
Protozoa were counted using 0h samples.
[a-c] Means (n = 4) within a row with different superscripts differ ($P < 0.05$).

438

439 Table 4. Changes in the rumen bacterial and archaeal community with cashew nut shell liquid
440 feeding as assessed by the analysis of 16S rRNA gene mini-library constructed from pooled
441 samples taken at 0h
442

Items	Pre-CNSL	H-CNSL
Eubacterial community		
Diversity index		
Chao I	259.5	174.0
Shanon	3.975	3.139
Total clones	143	121
Total OTU	79	46
Firmicutes	47	36
Bacteroidetes	26	8
Spirochetes	5	0
Proteobacteria	1	2
No of clones belonging to*		
Ruminococcus flavefaciens	1	0
Treponema bryantii	2	0
Lactobacillus mucosae	5	0
Lactobacillus bovis	0	1
Succinivibrio dextrinosolvens	0	1
Megasphaera elsdenii	1	8
Megasphaera hominis	0	1
Selenomonas ruminantium	0	3
Schwartia succinivorans	0	1
Eubacterium pyruvivorans	1	0
Archaeal community		
Diversity index		
Chao I	1.0	7.5
Shanon	1.887	1.391
Total clones	92	82
Total OTU	18	7
Methanobrevibacter	15	6
Thermoplasma	3	1
No of clones belonging to*		
Methanobrevibacter millerae	9	1
Methanobrevibacter ruminantium	44	8
Methanobrevibacter wolinii	0	44

443

444 CNSL, cashew nut shell liquid.
445 Pre-CNSL period, no supplement control;
446 H-CNSL, CNSL was fed at 4 g/day/100kg BW.
447 * Clones sharing > 97% sequence identity to known species were counted.