



HOKKAIDO UNIVERSITY

Title	Rumen responses to dietary supplementation with cashew nut shell liquid and its cessation in sheep
Author(s)	Kang, Sungchhang; Suzuki, Ryo; Suzuki, Yutaka et al.
Citation	Animal science journal, 89(11), 1549-1555 https://doi.org/10.1111/asj.13100
Issue Date	2018-11
Doc URL	https://hdl.handle.net/2115/75990
Rights	This is the peer reviewed version of the following article: Kang, Sungchhang; Suzuki, Ryo; Suzuki, Yutaka; Koike, Satoshi; Nagashima, Kyo; Kobayashi, Yasuo (2018). Rumen responses to dietary supplementation with cashew nut shell liquid and its cessation in sheep. Animal science journal, 89(11), 1549-1555., which has been published in final form at https://doi.org/10.1111/asj.13100 . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.
Type	journal article
File Information	Final_ASJ-2018-0229.pdf



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

Sungchhang Kang ^a, Ryo Suzuki ^b, Yutaka Suzuki ^b, Satoshi Koike ^b, Kyo Nagashima ^c and
Yasuo Kobayashi ^{b*}

^aNational Institute of Education, Phnom Penh, 12156, Cambodia

^bResearch Faculty of Agriculture, Hokkaido University, Hokkaido 060-8589, Japan

^cAgri-bio Business Division, Idemitsu Kosan Co. Ltd., Sodegaura 299-0293, Japan

*Corresponding author: kyas@anim.agr.hokudai.ac.jp

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 (CH₄) is emitted annually from ruminants, amounting to 23–27 % of total anthropogenic CH₄ 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 CH₄ 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 CH₄ 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 CH₄ 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 CH₄ 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 CH₄ 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

Management of Animal (2005). We used four sheep 81.5 ± 17.1 kg in body weight (BW) that had been ruminally cannulated. All sheep were fed a basal diet consisting of orchard grass hay and a concentrate (Lamb 76ME; Mercian, Tokyo, Japan) at a 3:7 ratio. The diet contained 16.0 % crude protein (CP), 40.5 % neutral detergent fiber (NDF) and 2.10 Mcal metabolizable energy/kg on a dry matter (DM) basis and was given to the animals once daily (0900) to meet the maintenance requirement of each animal (fed at 1.4 % of BW on a DM basis).

Sheep were fed the basal diet for 4 weeks (Pre-CNSL period), followed by the CNSL supplementation period for 4 weeks. During the supplementation period, CNSL was fed at 2 g/day/100 kg BW for the first 2 weeks and at 4 g/day/100 kg for the second 2 weeks (L-CNSL and H-CNSL periods, respectively). Then, the diet was reverted to the un-supplemented control diet and fed for 2 weeks (Post-CNSL period). The experimental CNSL was extracted from cashew nut shells (Indian origin) with compressing machinery without the use of organic solvents at the Central Laboratory, Idemitsu Kosan Co. Ltd. (Sodegaura, Japan). The CNSL was diluted with 25 ml of 99.5 % ethanol, mixed with the daily amount of concentrate, air-dried overnight to remove ethanol, and fed to each sheep. The same procedure was employed for the control diet but using ethanol without CNSL.

Sampling

Sampling of the rumen content was conducted on the 28th day of the Pre-CNSL period, and the 7th and 14th days of each CNSL and Post-CNSL periods. On each sampling day, the rumen content was removed by hand via the cannula of each sheep at 0, 4, 8 and 12 h post-feeding (ca. 250g at 0h and ca.50g at other samplings). Ruminal pH was immediately measured using a portable pH-temperature meter (HANNA Instruments HI 8424 Microcomputer, Singapore, Singapore). The rumen content was then strained through two layers of surgical gauze and immediately used for gas and foam production measurements, or

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

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

Methane production potential and physical properties of rumen fluid

The strained rumen fluid from each sheep taken at 0 h was mixed with an equal volume of McDougal's buffer (McDougal 1948) and dispensed into a test tube (180 mm in length and 10 mm in diameter), which was flushed with nitrogen (N₂) gas and fitted with a butyl rubber stopper and a plastic screw cap. The tubes were incubated at 38 °C for 24 h without substrate at 5 replicates for each sheep. Total gas produced in the headspace of the tube was measured by a needle-attached pressure gauge and was employed for methane analysis. These were performed as described by Watanabe et al. (2010). Using this data, CH₄ production from the rumen fluid was expressed as CH₄ production potential (CH₄ mL/mL of rumen fluid/day).

Physical properties such as viscosity, ingesta volume index (IVI) and stable IVI were assessed according to Sakauchi and Hoshino (1981) and Jacobson et al. (1957). In brief, strained rumen fluid (0 h sample) from each sheep (50 mL) was dispensed into a gravimetric 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 H₂. Total gas, carbon dioxide (CO₂) and CH₄ 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.

Ruminal pH, ammonia, lactate, DM disappearance and protozoa

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

Rumen bacteria and archaea

Rumen bacterial and archaeal communities are shown in Table 4, as determined by the analysis of the 16S rRNA gene library. LIBSHUFF analysis showed significant changes in both communities with CNSL feeding. In addition, CNSL reduced the diversity of bacterial and archaeal communities. Propionate-producing bacteria such as *Megasphaera elsdenii* and *Selenomonas ruminantium* were more frequently detected in the H-CNSL period than in the non-supplemented Pre-CNSL period. Meanwhile, a H₂ and formate producer, namely *Treponema bryantii*, and the lactate producing *Lactobacillus mucosae* were detected more often in the Pre-CNSL period. For methanogenic archaea, *Methanobrevibacter* was a major genus, dominated by *Methanobrevibacter millerae* and *Methanobrevibacter ruminantium* in the Pre-CNSL period, while these species were replaced by *Methanobrevibacter wolinii* in the H-CNSL period.

DISCUSSION

This is the first feeding study of CNSL using sheep to monitor rumen responses to 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 N₂O (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.

ACKNOWLEDGEMENTS

This study was supported in part by Grant-in-Aid for Scientific Research (B) to YK (No.18H02322) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The authors gratefully acknowledge the assistance of a Matsumae International Foundation Scholarship (Grant No. 17G05) to Dr. Sungchhang Kang to study in Hokkaido University as a post-doc fellow.

REFERENCES

- Branco AF, Giallongo F, Frederick T, Weeks H, Oh J, Hristov AN. 2015. Effect of technical cashew nut shell liquid on rumen methane emission and lactation performance of dairy cows. *Journal of Dairy Science* 98, 4030-4040.
- Calsamiglia S, Busquet M, Cardozo PW, Castillejos L, Ferret A. 2007. Essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science* 90, 2580–2595.

325 Giger-Reverdin S, Sauvant D. 2000. Methane production in sheep in relation to concentrate
 326 feed composition from bibliographic data. In: Ledin I, Morand-Fehr P (eds), *8th Seminar*
 327 *of the Sub-Network on Nutrition of the FAO-CIHEAM Inter-Regional Cooperative*
 328 *Research and Development Network on Sheep and Goats*, pp 43–46. Cahiers-Options-
 329 Mediterraneennes, Grignon, France.

330 Hook SE, Wright AG, McBride BW. 2010. Methanogens: methane producers of the rumen
 331 and mitigation strategies. *Archaea*. DOI:10.1155/2010/945785.

332 IPCC. 2007. Summary for policymakers. In: Solomon S, Qin D, Manning M, Chen Z,
 333 Marquis M, Averyt KB, Tignor M, Miller HL (eds), *Climate Change 2007: The Physical*
 334 *Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the*
 335 *Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge,
 336 UK and New York, USA.

337 Jacobson DR, Lindahl IL, McNeill JJ, Shaw JC, Doetsch RN, Davis RE, 1957. Feedlot Bloat
 338 Studies. II. Physical factors involved in the etiology of frothy bloat. *Journal of Animal*
 339 *Science* 16, 515-524.

340 Kobayashi Y, Wakita M, Hoshino S. 1988. Persistency of salinomycin effects on ruminal
 341 fermentation in wethers. *Nutrition Reports International* 38, 987-999.

342 Kobayashi Y. 2012. Cashew nut shell liquid formula as a new rumen modifier. *MP Agro*
 343 *Journal* 10, 15-18. (in Japanese)

344 Kobayashi Y, Oh S, Myint H, Koike S. 2016. Use of Asian selected agricultural by products
 345 to modulate rumen microbes and fermentation. *Journal of Animal Science and*
 346 *Biotechnology* 7(70). DOI:10.1186/s40104-016-0126-4.

347 Koike S, Yoshitani S, Kobayashi Y, Tanaka K. 2003. Phylogenetic analysis of fiber-
 348 associated rumen bacterial community and PCR detection of uncultured bacteria. *FEMS*
 349 *Microbiology Letters* 229, 23-30.

350 McDougall EI. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's
 351 saliva. *Biochemistry Journal* 43, 99–109.

352 Nagaraja TG, Titgemeyer EC. 2007. Ruminal acidosis in beef cattle: The current
 353 microbiological and nutritional outlook. *Journal of Dairy Science* 90 (Suppl.), E17–E38.

354 Nkrumah JD, Okine EK, Mathison GW, Schmid K, Li C, Basarab JA, Price MA, Wang Z,
 355 Moore SS. 2006. Relationships of feedlot feed efficiency, performance, and feeding
 356 behavior with metabolic rate, methane production, and energy partitioning in beef cattle.
 357 *Journal of Animal Science* 84, 145–153.

358 Ogimoto K, Imai S. 1981. *Atlas of Rumen Microbiology*. Japan Scientific Societies Press,
 359 Tokyo, Japan.

360 Oh S, Suzuki Y, Hayashi S, Suzuki Y, Koike S, Kobayashi Y. 2017. Potency of cashew nut
 361 shell liquid in rumen modulation under different dietary conditions and indication of its
 362 surfactant action against rumen bacteria. *Journal of Animal Science and Technology*
 363 59:27, doi: 10.1186/s40781-017-0150-8.

364 Orskov ER, McDonald I. 1979. The estimation of protein degradability in the rumen for
 365 incubation measurements weighted according to rate of passage. *Journal of Agricultural*
 366 *Science (Cambridge)* 92, 499-503.

367 Paramashivappa R, Phani Kumar P, Vithayathil PJ, Srinivasa Rao A. 2001. Novel method for
 368 isolation of major phenolic constituents from cashew (*Anacardium occidentale* L.) nut
 369 shell liquid. *Journal of Agriculture Food and Chemistry* 49, 2548–2551.

370 Petersen SO. 2017. Greenhouse gas emissions from liquid dairy manure: Prediction and
 371 mitigation. *Journal of Dairy Science*, doi: 10.3168/jds.2017-13301.

372 Russell JB, Strobel HJ. 1989. Effect of ionophores on ruminal fermentation. *Applied and*
 373 *Environmental Microbiology* 55, 1–6.

374 Sakauchi R, Hoshino S. 1981. Effects of monensin on ruminal fluid viscosity, pH, volatile
375 fatty acids and ammonia levels, and microbial activity and population in healthy and
376 bloated feedlot steers. *Journal of Animal Physiology and Animal Nutrition* 46, 21-33.

377 Shinkai T, Enishi O, Mitsumori M, Higuchi K, Kobayashi Y, Takenaka A, Nagashima K,
378 Mochizuki M, Kobayashi Y. 2012. Mitigation of methane production from cattle by
379 feeding cashew nut shell liquid. *Journal of Dairy Science* 95, 5308-5316.

380 Suto T. 1973. The Clinical Investigation of the Bovine. Nosangyoson Bunka Kyokai, Tokyo,
381 Japan.

382 Ushida K, Miyazaki A, Kawashima R. 1985. Effect of monensin on ruminal VFA and gas
383 production of sheep fed high concentrate diet. *Japanese Journal of Zootechnical Science*
384 56, 822-826.

385 Wallace RJ, Onodera R, Cotta MA. 1997. Metabolism of nitrogen-containing compounds. In:
386 Hobson PN, Stewart CS (eds), *The Rumen Microbial Ecosystem*, pp.283-328. Chapman
387 & Hall, London, UK.

388 Watanabe Y, Suzuki R, Koike S, Nagashima K, Mochizuki M, Forster RJ, Kobayashi Y. 2010.
389 *In vitro* evaluation of cashew nut shell liquid as a methane inhibiting and propionate-
390 enhancing agent for ruminants. *Journal of Dairy Science* 93, 5258-5267.

391 Weatherburn MW. 1967. Phenol-hypochlorite reaction for determination of ammonia.
392 *Analytical. Chemistry* 39, 971-974.

393 Wright AD, Auckland CH, Lynn DH. 2007. Molecular diversity of methanogens in feedlot
394 cattle from Ontario and Prince Edward Island, Canada. *Applied and Environmental*
395 *Microbiology* 73, 4206-4210.

396

397

398

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

400

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, <i>cp</i>	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

401

402 CNSL, cashew nut shell liquid; wk 1 and wk 2, samples at week 1 and week 2, respectively;

403 Pre-CNSL period, no supplement control;

404 L-CNSL period, CNSL was fed at 2 g/day/100kg BW; H-CNSL period, CNSL was fed at 4 g/day/100kg BW;

405 Post-CNSL period, no supplement control

406 *cp*, cPoise; measurement unit of dynamic viscosity in the centimeter gram second system of units

407 (1 centipoise = 0.01 gram per centimeter-second)

408 IVI, Increase volume index.

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

410

411

412

413

414

415

416

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

418

Items	Pre-CNSL period	L-CNSL period		H-CNSL period		Post-CNSL period		SEM	<i>P</i> -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

419

420 CNSL, cashew nut shell liquid; wk 1 and wk 2, samples at week 1 and week 2, respectively;

421 Pre-CNSL period, no supplement control;

422 L-CNSL period, CNSL was fed at 2 g/day/100kg BW; H-CNSL period, CNSL was fed at 4 g/day/100kg BW;

423 Post-CNSL period, no supplement control.

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

425

426

427

428

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	<i>P</i> - 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

429

430

431

432

433

434

435

436

437

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 ($P < 0.05$).

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

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*		
<i>Ruminococcus flavefaciens</i>	1	0
<i>Treponema bryantii</i>	2	0
<i>Lactobacillus mucosae</i>	5	0
<i>Lactobacillus bovis</i>	0	1
<i>Succinivibrio dextrinosolvens</i>	0	1
<i>Megasphaera elsdenii</i>	1	8
<i>Megasphaera hominis</i>	0	1
<i>Selenomonas ruminantium</i>	0	3
<i>Schwartia succinivorans</i>	0	1
<i>Eubacterium pyruvivorans</i>	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*		
<i>Methanobrevibacter millerae</i>	9	1
<i>Methanobrevibacter ruminantium</i>	44	8
<i>Methanobrevibacter wolinii</i>	0	44

CNSL, cashew nut shell liquid.

Pre-CNSL period, no supplement control;

H-CNSL, CNSL was fed at 4 g/day/100kg BW.

* Clones sharing > 97% sequence identity to known species were counted.