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Effects of Cashew Nut Shell Extract on Ruminal Fermentation and Nutrient Digestibility under Continuous Culture

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Abstract: The overall objective of this study was to determine the dose response to four levels of cashew nut shell extract in a granulated form (CNSE, containing 59% anacardic acid and 18% cardol) on culture pH, rumen fermentation metabolites, and apparent nutrient digestibility in continuous culture fermenters. The study was conducted as a generalized randomized complete block design with four treatments and four replications per treatment. The four treatments were randomly assigned to eight fermenters for two incubation runs of 10 d. Treatments consisted of (1) Control (CO, no CNSE), (2) Control plus 100 ppm of CNSE, (3) Control plus 200 ppm of CNSE, and (4) Control plus 300 ppm of CNSE. Fermenters were fed 52 g/d (DM basis) of a total mixed ration (TMR; 17.0% crude protein (CP), 29.7% NDF, and 29.9% starch), divided between two feedings at 0800 and 2000 h. The apparent digestibility of dry matter (DM), organic matter (OM), and neutral detergent fiber (NDF) were not affected by CNSE supplementation. Similarly, CNSE had no effect on culture pH, total volatile fatty acids (VFA) or individual VFA molar proportions. These results suggest that at the dosages evaluated in this study, CNSE has no impact on the rumen fermentation profile and the apparent nutrient digestibility under continuous culture conditions.

Keywords: anacardic acid; continuous culture; digestibility; fermentation; feed additive

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1. Introduction

Diet supplementation with some feed additives (e.g., antibiotics), can provide a competitive advantage for specific ruminal microbial populations, thus improving the overall feed utilization efficiency by changing fermentation patterns in the rumen [1,2]. However, there is growing concern about the usage of antibiotics in food animal production due to potential antibiotic resistance and drug residues. As a result, many countries are considering restrictions on the use of antibiotics in feed, and the European Union has banned the use of antibiotics as growth promoters in feed (Regulation 1831/2003/EC). Therefore, researchers are becoming increasingly interested in using natural alternatives such as tannins, essential oils, cashew nut shell extract, and other organic compounds as a way to replace antibiotics to lessen worries about human health risks [3,4].

Cashew nut shell liquid (CNSL) is a by-product of the processing of cashew nuts and is used in various industrial applications [5]. Furthermore, the phenolic compounds found in CNSL, such as anacardic acid, cardanol, and cardol, have been shown to have antimicrobial properties [6]. In particular, anacardic acids have been shown to inhibit gram-positive rumen bacteria [7]. Watanabe et al. [6] reported a decrease in active hydrogen and formate producers (e.g., *Ruminococcus flavefaciens*, *Eubacterium ruminantium*, *Ruminococcus albus*, and *Butyrivibrio fibrisolvens*) but increased propionate and succinate producing bacteria species (e.g., *Succinivibrio dextrinosolvens* and *Megasphaera elsdenii*).]. Furthermore, CNSL inhibited CH_4 formation and increased propionate production in a dose-dependent manner under in vitro conditions [6]. The reduction in methanogenesis has been attributed to a

reduction in hydrogen and formate, which are the most important electron donors used by methanogenic bacteria [8]. Although the effect of CNSL on CH_4 formation was also observed in vivo [9], the authors also reported a reduction on total VFA production when the diet fed to non-lactating cows was supplemented with CNSL. Interestingly, technical CNSL (cashew nut shell extract that contains cardanol and cardol as the main active ingredients, but no anacardic acids) had no effect on methane emission in lactating dairy cows but tended to increase NDF digestibility [10]. The variable effects of cashew nut shell byproducts on nutrient digestibility reported in the literature are probably associated with the type and amounts of phenolic compounds found in the additive, as a result of the extraction method, and the dose fed to the animals. Thus, CNSE fed at a level that does not compromise animal performance might be used as a dietary strategy to shift rumen fermentation to improve the efficiency of feed energy utilization in cows. However, there are only a few dose-response studies investigating the effects of CNSE on nutrient digestibility and rumen metabolism [6].

Based on these studies, we hypothesized that incorporating incremental levels of CNSE would not affect nutrient digestibility, but it will shift the production of VFA towards propionate. Thus, the objective of this study was to determine the dose response to four levels of CNSL in a granulated form (cashew nut shell extract, CNSE) on culture pH, rumen fermentation metabolites, and apparent nutrient digestibility in continuous culture fermenters.

2. Materials and Methods

2.1. Experimental Design and Treatments

The study was conducted as a generalized randomized complete block design with four treatments fed to continuous culture fermenters. Treatments consisted of (1) Control (CO, no CNSE), (2) Control plus 100 ppm of CNSE (L), (3) Control plus 200 ppm of CNSE (M), and (4) Control plus 300 ppm of CNSE (H). The granulated CNSE (Agri-Bio Business Department, Idemitsu Kosan Co., Ltd., Tokyo, Japan) contained 50% CNSE (59% anacardic acid and 18% cardol) and was further diluted by premixing it with corn grain before dietary treatment mixing. The content of CNSE in each dietary treatment was not measured to verify that CNSE was added to each fermenter at the intended level. However, in a recent study comparing two ionophore sources, premixes prepared with the same methodology averaged 105% of the expected ionophore content [11]. The CO diet was formulated to meet NRC (2001) requirements for a multiparous cow with 150 DIM, 40 kg/d of milk production, and 26.6 kg/d of predicted DMI (Table 1). The CNSE added to the CO in stepwise increments was equivalent to 2.5, 5.0, and 7.5 g/d per cow with a DMI of 26.6 kg/d for L, M, and H treatments, respectively. For each incubation run, treatments were randomly assigned to 1 of 8 fermenters and run for a 10-d period with 7 d for adaptation and 3 d for sample collection. Fuentes et al. [12] suggested a minimum of 5 d of adaptation to stabilize the microbial population within the cultures. We conducted two incubation runs (block), resulting in a total of 4 replicates per treatment. A total of 52.0 g of diet DM was fed to each fermenter daily in 2 equal portions at 0800 and 2000 h. Dietary treatments and treated corn were mixed using a commercial mixer (Commercial Series 8, KitchenAid, Benton Harbor, MI, USA).

Table 1. Ingredient composition of control diet.

Item	Control Diet (CO)
Ingredient, % of DM	
Corn silage [1]	37.0
Alfalfa hay [2]	15.0
Ground corn	23.7
Solvent soybean meal	9.5
Expeller soybean meal [3]	4.0
Soyhulls	8.0
Energy Booster 100	1.0
Mineral premix [4]	1.8

[1] Chemical composition (DM basis) was 7.2% CP, 38.6% NDF, and 30.2% starch. [2] Chemical composition (DM basis) was 26.5% CP, 30.3% NDF, and 3.1% starch. [3] SoyPlus, West Central Coop. (Ralston, IA). [4] Premix composition: guaranteed minimum concentration (as-fed basis): Ca 17.9%, Zn 7.2%, Mn 5.8%, Cu 1.5%, Fe 1.2%, I 1440 ppm, Co 768 ppm, and Se 288 ppm.

2.2. Continuous Culture Conditions

The care and handling of animals used for collecting rumen contents and in situ incubations were conducted as outlined in the guidelines of the Clemson University Committee on Animal Use (AUP2019-074). On day 0 of each incubation run, rumen fluid and solids were collected from two ruminally fistulated lactating dairy cows that were fed a diet containing 44% corn silage, 4.1% barley silage, and 51.9% concentrate mix (DM basis).

Within 20 min of collection, large particles were removed from the whole ruminal contents by filtration through 2 layers of cheesecloth, and the filtrate containing the microbial population was transferred immediately to the laboratory in a sealed container. With constant stirring, the filtered ruminal inoculum was diluted 1:1 with the buffer [13] and then added to completely fill (approximately 800 mL) each dual-flow fermenter that was modified in construction and operation from the design described by Teather and Sauer [14]. The main modifications were a reconfigured overflow sidearm that was angled downward at approximately 45° to facilitate emptying, a faster stirring rate (45 rpm) that still allowed stratification of particles into an upper mat, a middle liquid layer of small feed particles, a lower layer of dense particles, and a higher feeding rate [15,16]. The buffer solution [13] was delivered continuously using a peristaltic pump to achieve a 0.10/h fractional dilution rate. Each morning, the buffer solution was prepared and adjusted using 6 N NaOH or 3 N HCL to maintain the buffer's pH levels. All fermenters received the same buffer solution, thus treatment effects of CNSE had the opportunity to alter pH. Anaerobic conditions were maintained by purging the cultures with CO_2 at a rate of 20 mL/min and were checked nightly to verify consistency. The temperature of the fermenters was held at 39 °C by a circulating water bath. The culture pH was monitored daily by taking pH readings just before each feeding (Symphony H10P, VWR, Radnor, PA, USA).

2.3. Sample Collection and Analysis

On d 10 of each incubation run, a 5 mL sample of mixed culture contents was collected at 0 (before feeding), 2, 4, 6, 8, 10, and 12 h after feeding into polycarbonate centrifuge tubes containing 1 mL of 25% (*wt*/*v*) metaphosphoric acid and frozen at −20 °C until further analysis. The culture pH was determined immediately after sample collection using a calibrated portable pH meter (Symphony H10P, VWR, Radnor, PA, USA). The culture contents were thoroughly mixed (100 rpm) during the sampling collection and pH measurements to ensure proper mixing. On d 8, 9, and 10 of each incubation run, overflow from each fermenter was collected in 2-L Erlenmeyer flasks submerged in an ice bath and containing 10 mL of H_2SO_4 (50% solution) to prevent further microbial activity. The volume of the overflow flask was measured twice daily (before each feeding). After recording the total volume, a 20% sample of the overflow was collected and immediately frozen at a −20 °C. Frozen overflow samples were later thawed and composited by fermenter and incubation run, and a subsample was dried for 48 h at 55 °C and ground through a

1-mm screen using a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA, USA). A second subsample was centrifuged at 40,000× *g* for 20 min at 4 °C. The supernatant was carefully removed using 3 mL plastic transfer pipettes, and the remaining sample was immediately dried for 48 h at 100 °C. The resulting dry matter (DM) concentration was used to estimate daily overflow mass.

Ground feed and overflow samples were dried at 105 °C for 24 h to determine analytical DM. Ash concentration was determined after combusting samples in a furnace for 3 h at 600 °C (Method 942.05) [17]. Neutral detergent fiber (aNDFom) and ADFom concentrations were determined using an Ankom200 Fiber Analyzer (Ankom Technology, Faiport, NY, USA) and corrected for ash concentration. Sodium sulfite and α-amylase (Sigma no. A3306: Sigma Chemical Co., St. Louis, MO, USA) were included for NDF analysis [18]. For each ground feed and culture overflow sample, a subsample was submitted to Cumberland Valley Analytical Services (Waynesboro, PA, USA) to determine the concentrations of N (Method 990.03, AOAC) [19] and starch [20]. The feeds and diets' crude protein concentration was calculated as a percentage of N × 6.25. Samples of mixed culture contents were thawed and centrifuged at 40,000× *g* for 20 min at 4 °C. After centrifugation, the supernatant was filtered, diluted with 0.5 mL distilled H_2O, and combined with 100 µL of internal standard (86 µmol of 2 ethylbutyric acid/mL), in a 2 mL GC vial. Samples were injected into a Hewlett-Packard 6980 gas chromatograph (San Jose, CA, USA) fitted with a custom packed column for VFA-flame-ionization detection.

2.4. Statistical Analysis

Data were analyzed with the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) as a generalized randomized complete block design. For apparent digestibility data, the statistical model included the fixed effect of treatment, the random effect of incubation run (block), the random effect of fermenter, and the residual error. The above model was expanded to include the fixed effect of time as a repeated measure, and the interaction between treatment and time, to determine treatment effects on pH, TVFA and VFA molar proportions. The first-order autoregressive covariance structure was used to fit a time series-type covariance structure in which the correlation declines as a function of time. Pre-planned orthogonal contrasts were used to test linear and quadratic effects of treatments (CNSE level). Significant differences and tendencies to differ were declared at $p < 0.05$ and $p \leq 0.10$, respectively.

3. Results and Discussion

As polyphenols were the main contributions to the granulated CNSE, as expected, their increasing concentration in the diet had a limited effect on dietary composition (Table 2). Thus, dietary OM, CP, NDF, and starch content were similar between dietary treatments and averaged 93.9, 17.0, 29.3, and 29.5% (DM basis), respectively.

Table 2. Chemical composition of dietary treatment containing different levels of CNSE fed to continuous culture fermenters.

Item	CNSE (ppm)			
	0	**100**	**200**	**300**
DM, %	92.1	92.4	92.3	91.6
OM, % DM	94.2	93.6	94.1	93.8
CP, % DM	16.7	17.2	17.2	16.9
NDF, % DM	29.2	30.0	28.9	28.9
ADF, % DM	19.1	18.5	19.7	18.5
Starch, % DM	29.9	29.7	29.1	29.4

3.1. Culture Fermentation Measurements

Table 3 presents the least square mean on the effects of CNSE supplementation on culture pH, total VFA concentration and molar proportion of VFAs, and acetate-to-propionate

ratio. Averaged culture pH values were not different between CO and the increasing levels of CNSE (5.76 ± 0.18). As expected, there was a significant ($p < 0.01$) effect of pH over time and the pattern of change was similar between all treatments (Figure 1). The culture pH pre-feeding (time 0) was 5.79; it declined to a nadir on time 6 (5.40) and reached the highest pH at time 12 (6.03). These results are consistent with an in vivo [8] that observed no effect of CNSE on rumen pH. Watanabe et al. [6] observed a lower, albeit negligible, pH value (6.45) on the diets supplemented with different levels of CNSE compared with the control (6.59) under semicontinuous culture conditions.

Table 3. Effect of different levels of CNSE supplementation on continuous culture fermenters metabolites.

Item	CNSE (ppm)				SEM [2]	*p*-Value [1]	
	0	100	200	300		L	Q
pH	5.78	5.72	5.75	5.80	0.20	0.51	0.47
Total VFA, mM	76.8	70.5	72.3	71.2	10.9	0.46	0.51
VFA, mol/100 mol							
Acetate	37.5	39.2	35.8	38.1	1.57	0.96	0.51
Propionate	27.8	26.6	28.6	28.5	8.12	0.67	0.92
Butyrate	17.8	17.6	18.4	16.7	1.81	0.61	0.57
Isovalerate	0.82	1.12	1.09	0.82	0.44	0.82	0.26
Valerate	8.70	8.71	9.30	8.52	1.56	0.93	0.58
A:P [3]	1.24	1.29	1.17	1.21	1.29	0.64	0.78

[1] Probability of a linear (L) or quadratic (Q) effect of CNSE level in the diet. [2] Standard error of the mean (highest when uneven samples). [3] Acetate-to-propionate ratio.

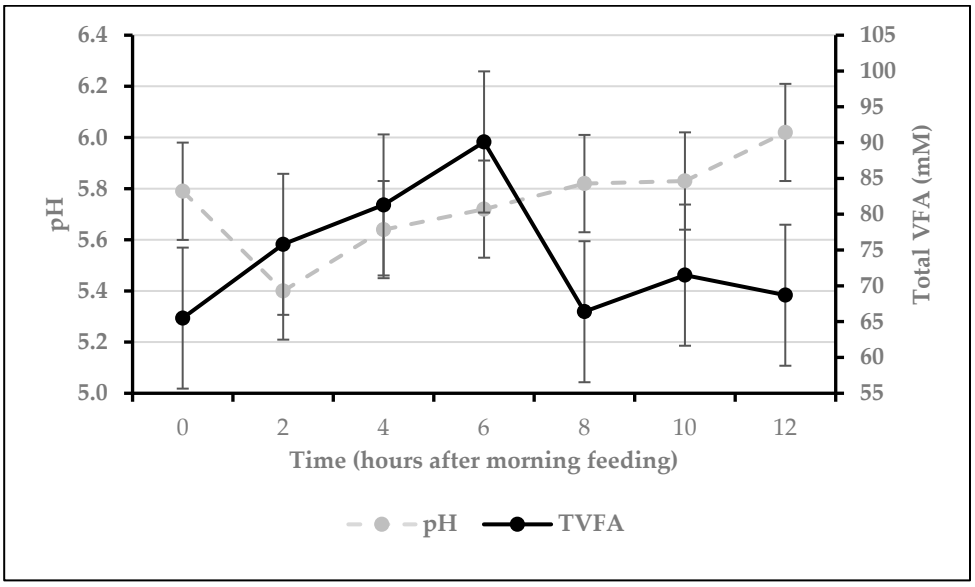

Figure 1. Changes in pH and total VFA of fermenter contents of all treatments (combined) before (0 h) and every two hours after the a.m. feeding on d 10. Bars indicate SEM.

Increasing levels of CNSE in the diet had no effect on total VFA concentration and ranged between 70.5 to 76.8 mM across all treatments. These numbers were similar to those previously reported studies on continuous cultures. For instance, total VFA concentrations were 77.1 mM [15] and 62.9 mM [16] in continuous cultures with no additives. Watanabe et al. [6] observed a small increase in total VFA concentration when CNSL was supplemented to the diet. Conversely, Shinkai et al. [9] reported a significant reduction in VFA concentration in two in vivo studies. Under the condition of this study, CNSE supplementation had no effect on the molar proportions of acetate (37.7 ± 2.35), propionate (27.8 ± 0.94), butyrate (17.6 ± 2.48), isovalerate (0.96 ± 0.18), valerate (8.81 ± 0.59), or A:P

ratio. Results reported in the literature on the effect of CNSE on the VFA profile are inconsistent. For example, both batch culture and continuous culture trials have found a consistent shift in VFA molar proportions towards lower acetate and higher propionate content when CNSL or CNSE were added to the diet [6,21]. Furthermore, similar changes on the molar proportions of acetate and propionate have been reported in an in vivo study [9]. However, when the same product as the one evaluated in this study was fed at 2.5 and 5.0 g/d per day to dairy cows during the transition period, the authors reported no treatment effect on the VFA profile [22]. Also, Sarmikasoglou et al. [23] observed no changes in VFA production or in the molar proportions of acetate and propionate when CNSE (the same product as in the current study) was fed at two different levels (100 or 200 ppm) and under continuous culture conditions.

When taken together, data from this study and from Sarmikasoglou et al. [23] and Goetz et al. [22] suggest that when CNSE with 59% anacardic acid and 18% cardol is fed at a level between 100 and 300 ppm, there is no shift in VFA molar proportion towards propionate. The inconsistent results between studies using CNSL or CNSE on propionate production highlights the importance that the phenolic composition and dose level of CNSE in the diet probably have on the rumen microbial population.

3.2. Apparent Digestibility of Nutrients

Table 4 summarizes the effects of CNSE supplementation on apparent digestibility. Under the condition of this study, apparent digestibility of DM (54.7% ± 7.6), OM (63.8% ± 5.9), NDF (58.8% ± 8.2), and starch (97.2% ± 1.3) were similar among the treatments. Average digestibility values were similar or higher to those previously reported in continuous cultures studies fed dairy diets [24–26]. Sarmikasoglou et al. [23] observed no effect on nutrient digestibility under continuous culture conditions. Shinkai et al. [9] reported a decrease in DM and OM digestibility in one of two feeding trials, when approximately 22 g/d per cow of CNSL (pellet form) was fed to dry cows. This level of supplementation was 4.6 times higher than the highest dose used in the current trial. Furthermore, Shinkai et al. [9] hypothesized that the discrepancy in DM and OM digestibility between the two trials was that a CNSL pellet with greater rumen diffusion was fed in the trial and digestibility was reduced by CNSL supplementation. In their recent study, Goetz et al. [27] observed no effects of CNSE supplementation on DM, OM, NDF, ADF, and starch digestibility. Branco et al. [10], reported that technical CNSL, rich in cardanol and cardol, had no effect on total-tract apparent digestibility of nutrients, except for a tendency to increase digestibility of NDF digestibility. Taken together, data from the current and previous in vitro and in vivo studies suggest that regardless of the level of supplementation, CNSE has little impact on apparent nutrient digestibility.

Table 4. Effect of different levels of CNSE supplementation on nutrient apparent digestibility from continuous cultures.

Item	CNSE (ppm)				SEM [2]	*p*-Value [1]	
	0	100	200	300		L	Q
DM, %	54.2	59.2	53.4	56.2	4.27	0.57	0.57
OM, %	63.3	66.2	62.6	65.5	3.33	0.74	0.85
NDF, %	57.8	62.1	55.7	59.9	4.72	0.71	0.71
Starch, %	97.8	97.5	96.8	97.4	0.65	0.47	0.15

[1] Probability of a linear (L) or quadratic (Q) effect of CNSE level in the diet. [2] Standard error of the mean (highest when uneven samples).

4. Conclusions

In this study, CNSE (containing 59% anacardic acid and 18% cardol) was added at incremental levels to continuous cultures of mixed ruminal microorganisms to determine how they affected the volatile fatty acid profile and apparent nutrient digestibility. Although CNSE supplementation did not influence the overall nutrient digestibility, the results of this

study do not support our hypothesis that increasing levels of CNSE in the diet will promote the production of propionate. Future studies should evaluate CNSE supplementation at a level not evaluated in this study and with a different diet composition before considering CNSE as an additive for ruminants.

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