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Effect of Cyanide-Utilizing Bacteria and Sulfur Supplementation on Reducing Cyanide Concentration and In Vitro Degradability Using In Vitro Gas Production Technique

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Abstract: The objective of this research was to supplement the cyanide-utilizing bacteria and sulfur in the HCN-rich diet, affecting the gas production and fermentation of rumen in vitro, and lowering the HCN content and the digestion of nutrients. A 2 × 2 × 3 factorial experiment in a completely randomized design was applied during the test. In the experiments, three factors were used. Factor A was the level of CUB at 0 and 10^8 CFU/mL. Factor B was the level of sulfur in the diet at 0% and 3% of dry matter (DM). Factor C was the three levels of potassium cyanide (KCN) at 0, 300, and 600 ppm. The interaction of CUB × sulfur × KCN affected gas production from the immediately soluble fraction (a) ($p < 0.05$). However, the greatest ruminal cyanide concentration was found when CUB (with and without), sulfur (3%), and KCN (600 ppm) were introduced at 0 h ($p < 0.05$). It revealed that the addition of CUB and sulfur had a significant impact on gas accumulation at 96 h ($p < 0.05$). The addition of CUB with sulfur had an effect on pH at 2 h and ruminal cyanide levels at 6 h ($p < 0.05$). At 2 h, sulfur supplementation with KCN had an effect on NH_3-N ($p < 0.01$). The addition of sulfur (3%) and KCN (300 ppm) produced the highest ammonia nitrogen. However, the combination of sulfur (3%) and KCN (600 ppm) produced the lowest level of ammonia nitrogen ($p < 0.01$). CUB supplementation increased the in vitro dry matter digestibility (IVDMD) by 11.16% compared to the without-CUB supplemented group ($p < 0.05$). Supplementation with 3% sulfur increased the in vitro neutral detergent fiber (IVNDFD) by 16.87% but had no effect on IVDMD or in vitro acid detergent fiber (IVADFD) ($p < 0.05$). The volatile fatty acid (VFA) such as acetate, propionate, and butyrate were not different when CUB, sulfur, and KCN were added. Doses above 600 ppm had the lowest concentrations of TVFA and propionate ($p < 0.01$). Based on the results of this investigation, supplementing with CUB and sulfur (3%) may improve cumulative gas, digestibility, and TVAF. Supplementing with CUB, on the other hand, reduced HCN the most, by 54.6%.

Keywords: cyanide-utilizing bacteria; sulfur; cyanide; detoxify; in vitro; rumen fermentation

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1. Introduction

Several tropical feed sources, such as mimosine and hydrogen cyanide (HCN), contain anti-nutritional factors that might negatively impact ruminant animals. Mimosine is found in the *Leucaena leucocephala* (Leucaena) plant, while HCN is found in the cassava plant (root and leaves). Tropical regions can use the legume tree Leucaena as a source of protein. Mimosine is a toxic amino acid that is converted by leaf enzymes into 3-hydroxy-4[1H]-pyridone, limiting its use (3,4-DHP) [1]. The rumen bacteria that degrade mimosine metabolites are responsible for ruminants' tolerance to *L. leucocephala* intake. *Synergistes jonesii* is a rumen bacterium capable of digesting the isomers 3,4- and 2,3-DHP, as well as using arginine as an

energy source [2,3]. Mimosine and its major rumen metabolite, dihydroxypyridone (DHP), produce several negative effects when *S. jonesii* is removed from the rumen, including the inhibition of live weight growth [4]. When *S. jonesii* was first introduced to Australian ruminant animals in the 1980s, it helped inoculated animals to digest significant proportions of Leucaena and virtually completely degrade DHP, resulting in higher production [5]. The ability of a cyanogenic plant to produce highly deadly HCN when consumed determines its potential toxicity. The intake of HCN has been associated with ruminant mortality [6]. Cassava root is often used as a source of energy by ruminant animals; however, it does contain a significant amount of HCN (90–114 mg/kg), which can be toxic to them [7]. Rumen bacteria efficiently reduce the lower HCN content in ruminants by using rhodanese and -mercaptopyruvate sulfur transferase, sulfurtransferase [8,9]. When provided with available sulfur sources, microorganisms could be able to produce a synthesis of rhodanese enzymes, which can break down HCN. According to the NRC [10], bacteria could require the supply of a high-sulfur source to activate and convert dietary HCN into thiocyanate and eliminate it from the body. The HCN from animals fed fresh cassava root has been successfully detoxified using elemental sulfur. According to Supapong and Cherdthong [11], reducing the levels of HCN by 99% requires adding 2.0% sulfur to a fermented total mixed ration (FTMR) containing fresh cassava root. Additionally, supplementing the high sulfur pellets (PELFUR) at 3.0% decreased the amount of HCN by 37.06–40.8% compared to the group that received no sulfur [7]. On the other hand, high levels of elemental sulfur may have detrimental effects such as reduced feed intake, diarrhea, twitching muscles, and polioencephalomalacia, all of which can reduce ruminant performance. Therefore, a potential alternate technique to HCN detoxification should be investigated.

In tropical developing countries, current biological therapies for cyanide poisoning remain a challenge [12]. For biological protection, free cyanide limits have been drawn and established [13]. While mammalian rhodanese can detoxify cyanide [14], physical and biochemical procedures such as soaking, drying, and ensiling fresh plant materials are required to remove a significant amount of HCN from the diet. The rumen microbial system has also evolved to convert cyanide to a less hazardous thiocyanate or to directly break down cyanide to -cyanoalanine (intermediate), formic acid, and ammonia [15]. Ruminants are typically fed cyanogenetic plants such as cassava, sorghum cultivars, maize, alfalfa, and para-rubber seeds [16]. In the rumen, biological and chemical hydrolysis of the glycoside (cyanogenesis) is swift, and HCN is swiftly converted into thiocyanate, resulting in detoxification [17]. Gene transfer for adaption to the cyano group with enzymatic action is used in microbial cyanide detoxification [18]. Microorganisms and cyanide intake in non-rumen habits are becoming more prevalent [17,19], and more than 35 types of microorganisms have been found as cyanide predators from various sources (soil, wastewater, sludge, and plants) [20]. However, no cyanide-using bacteria from bovine rumen fluid have been grown, so the detoxification abilities of this genus are untested [17].

It was hypothesized that adding cyanide-using bacteria and sulfur to the feed would lower HCN levels in the feed and increase nutrient digestion and ruminal fermentation. The aim of the study was to investigate how the addition of cyanide-using bacteria and sulfur to an HCN-rich diet affects gas kinetics, rumen fermentation in vitro, HCN content, and nutrient digestion.

2. Materials and Methods

2.1. Design of Experiments and Treatments

A 2 × 2 ×3 factorial experiment in a completely randomized design was applied during the test. In the experiments, three factors were used. Factor A was the level of cyanide-utilizing bacteria (CUB) at 0 and 10^8 CFU/mL. Factor B was the level of sulfur in the diet at 0% and 3% of dry matter (DM). Factor C was the three levels of potassium cyanide (KCN) (Potassium cyanide, Merck KGaA, Darmstadt, Germany) at 0, 300 and 600 ppm, which was suggested by Prachumchai et al. [7]. The roughage-to-concentrate ratio in the 0.5 g substrate was 40:60, and the forage diet was rice straw. Table 1 shows the

ingredients and chemical composition of the concentrate feed utilized as substrate in the study. Concentrate diets contained DM, CP, NDF, and ADF at 93%, 16% DM, 58% to 59% DM, and 15% DM, respectively. In the experimental diets, corn meal was utilized as the primary energy source, whereas palm kernel meal, soybean meal, and urea were employed as protein sources, with sulfur supplementation at 0% and 3%.

Table 1. Ingredient and chemical composition of concentrate in the experiment.

Item	0% Sulfur	3% Sulfur	Rice Straw
Ingredients, % dry matter			
Soybean meal	11.85	11.85	
Palm kernel meal	20.00	20.00	
Rice bran	9.43	7.83	
Corn	55.97	54.50	
Salt	1.00	1.00	
Urea	1.50	1.57	
Mineral and vitamins *	0.25	0.25	
Sulfur powder	0.00	3.00	
Chemical composition			
Dry matter (DM), %	93.00	93.00	92.50
Organic matter (OM), %DM	96.00	96.00	89.50
Ash, %DM	4.00	4.00	10.50
Crude protein (CP), %DM	16.00	16.00	2.3
Neutral detergent fiber (NDF), %DM	59.00	58.00	71.20
Acid detergent fiber (ADF), %DM	15.00	15.00	44.2

* Contains per kilogram premix: 10,000,000 IU of vitamin A; 70,000 IU of vitamin E; 1,600,000 IU of vitamin D; 50 g of iron; 40 g of zinc; 40 g of manganese; 0.1 g of cobalt; 10 g of copper; 0.1 g of selenium; 0.5 g of iodine.

2.2. Rumen Inoculum Preparation and Animals

Two dairy bulls with rumen fistulas and body weights of 450 ± 30 kg were used to collect rumen fluid. Separate pens were used to house the animals. For a period of 21 days, the cattle were fed at 0.5 % BW in two equal parts at around 8:00 a.m. and 5:00 p.m. The concentration of CP and total digestible nutrients (TDN) in the concentrate diet was 14% and 75%, respectively [21]. Rice straw, minerals, and water were available ad libitum. Before morning feeding, ruminal fluid from the animals was collected, filtered through by using cloth into a warmed thermos flask before being transported to the laboratory. The Menke and Steingass technique [22] was used to manufacture artificial saliva. The inoculum was prepared by combining ruminal fluid and synthetic saliva in a thermos flask, warming it to 39 °C, and then constantly supplying it with carbon dioxide.

2.3. Substances and Incubation

In the in vitro gas production procedure, flasks were closed with rubber and aluminum cap stoppers and incubated at 39 °C. The flasks were gently shaken every 2 h during each sampling interval. Five of the flasks included only the rumen inoculums. The mean gas production values of these bottles were employed as a control. By removing the blank values from each measured value, the net gas production was computed. The 12 treatments, each with 3 flasks, were individually prepared for pH, ammonia-nitrogen (NH_3-N), and volatile fatty acids (VFA) analyses. For the digestibility, an additional set of 36 flasks was employed. A second set of 108 flasks was used to prepare the HCN concentration analysis. Cyanide-utilizing bacteria were cultured for 24 h in Lactobacilli MRS Broth medium (Difco Laboratories, Detroit, MI, USA) and absorbance at 660 nm was measured, obtaining approximately 10^8 CFU/mL [23].

2.4. Samples and Analyses

For the chemical analysis and the in vitro gas test, the feed was dried for 48 h at 60 °C before being ground to pass through a milling grid of 1 mm. (Cyclotech Mill, Tecator, Hoganas, Sweden). The samples were chemically analyzed for dry matter (DM), crude protein (CP), ash, and organic matter (OM), in accordance with the AOAC procedure [24]. The amount of neutral detergent fiber (NDF) and acid detergent fiber (ADF) in the samples was determined according to Van Soest et al. [25]. Data on gas production were taken directly after incubation at 0, 0.5, 1, 2, 4, 6, 8, 12, 18, 24, 48, 72, and 96 h using a calibrated syringe and a pressure transducer. The Sommart et al. [26] model was used as an equation to calculate cumulative gas production. As an example, consider the following:

$$Y = a + b\ (1 - e^{-ct}) \quad (1)$$

where a represents gas production from the immediately soluble fraction, b denotes gas production from the insoluble fraction, c denotes the insoluble fraction (b) gas production rate constant, "t" denotes incubation time, and (a + b) denotes the possible extent of gas production. The amount of gas produced at time "t" is denoted by the letter Y.

Using this digital pH meter (HANNA Instrument (HI) 8424 microcomputer, Singapore), fermentation liquid was sampled at 2 and 4 h after inoculation for pH measurement. Before analyzing NH_3-N by the Kjeldahl methods [24] and VFA by gas chromatography (Wilmington, DE 5890A Series II gas chromatograph and a glass column (180 cm, 4 mm) packed with 100 g/L SP-1200/10 g/L H3PO4 on 80/100 mesh Chromosorb WAW; Supelco, Bellefonte, PA, USA), the samples were centrifuged at 16,000× *g* for 15 min. To measure the concentrations of HCN in fermentation liquid, a modified variation of Fisher and Brown's picric acid method [26] was utilized (0, 2, 4, and 6 h after incubation). A linear calibration curve was generated using the standard KCN solutions by mixing 0.1 mL aliquots of a solution containing 0.5% (*w*/*v*) picric acid and 0.25 M of Na_2CO_3 using KCN solutions in 0.05 mL aliquots (after centrifugation at 15,000× *g* for 10 min at 4 °C). The solutions were simmered for 5 min, then diluted to 1 mL with 0.85 mL of distilled water and chilled in tap water for 30 min. Using a spectrophotometer and a blank of distilled water and picric acid reagent, the absorbance at 520 nm was measured. The degradation efficiency (DE) of HCN was calculated using the following formula:

$$DE\ (\%) = [(Ic - Rc)/Ic] \times 100 \quad (2)$$

where Ic = initial concentration of HCN (ppm) and Rc = residual concentration of HCN (ppm). The in vitro dry matter digestibility (IVDMD) was measured after a 24-h incubation period; calculation of the weight loss percentage, and IVDMD was used to visualize it. In vitro neutral detergent fiber (IVNDFD) and in vitro acid detergent fiber (IVADFD) digestibility were also investigated [27].

2.5. Analytical Statistics

Using SAS (Cary, NC, USA) software, the data were statistically analyzed as a 2 × 2 × 3 factorial in a completely randomized design. The level of cyanide-utilizing bacteria (CUB), level of sulfur, and level of potassium cyanide (KCN) were incorporated into the statistical model. The following model was used:

$$yijkl = \mu + ai + bj + ck + abij + acik + bcjk + abcijk + \varepsilon ijkl \quad (3)$$

where yijkl is the observation; μ is the overall mean; ai is the level of CUB (i, with and without); bj is the level of sulfur (j, 0–3); ck is the level of KCN at 0, 300 and 600 ppm (k, 1–3); abij, acik, bcjk, abcijk is the interaction effect; and εijkl is the error. To discover significant differences between treatments, Tukey's Multiple Comparison Test was performed. The normality of data was evaluated. A *p*-value of less than 0.05 ($p \leq 0.05$) was used to evaluate if the differences between the means were statistically significant.

3. Results

3.1. Gas Kinetics

The effect of CUB, sulfur, and KCN on gas kinetics and cumulative gas at 96 h after incubation is shown in Table 2. Interaction between CUB × sulfur × KCN had no effect on gas production from the insoluble fraction (b), the gas potential extent of gas production ($|\mathbf{a}|$ + b), or cumulative gas at 96 h, but gas production from the immediately soluble fraction (a) was affected ($p < 0.05$). CUB supplementation in combination with sulfur (0, 3%) and KCN (0, 300, and 600 ppm) improved gas production from the immediately soluble fraction (a) and was higher than without CUB, sulfur, and KCN ($p < 0.01$). It showed that the addition of CUB and sulfur (0.3%) positively impacts on gas production from an insoluble fraction (b) ($p < 0.05$), gas production rate constant for an insoluble fraction (c) ($p < 0.05$), and cumulative gas ($p < 0.05$). Supplementation without CUB and sulfur (0%) resulted in the lowest gas production from an insoluble fraction (b) and cumulative gas, but the highest gas production from an insoluble fraction (b) and cumulative gas occurred when using without CUB and sulfur (3%) ($p < 0.05$). The gas production rate constant for an insoluble fraction (c) was highest when added without CUB and sulfur (0%) in the diet, but it was lowest when CUB and sulfur (0%) were added ($p < 0.01$). The increased gas potential extent of gas production ($|\mathbf{a}|$ + b) was possible with supplementation at 3% in the diet ($p < 0.05$).

3.2. Rumen Fermentation and Ruminal Cyanide Concentration

The pH, NH_3-N, and ruminal cyanide concentration responses to the effect of CUB, sulfur, and KCN are presented in Table 3. CUB × sulfur × KCN had no effect on pH and NH_3-N at 2 and 4 h and ruminal cyanide levels at 2, 4, and 6 h ($p > 0.05$). However, the greatest rumen cyanide content was found when CUB (with and without), sulfur (3%), and KCN (600 ppm) were introduced at 0 h ($p < 0.05$). The addition of CUB with sulfur had an effect on pH at 2 h and ruminal cyanide levels at 6 h ($p < 0.05$). The highest pH was achieved by supplementing without CUB and sulfur (0%), whereas the lowest pH was found when supplementing CUB and sulfur (3%) ($p < 0.05$). The degradation efficiency was highest when CUB and sulfur (0%) were added ($p < 0.05$). At 2 and 4 h, CUB dosing with KCN (0, 300, and 600 ppm) had an impact on rumen pH changes ($p < 0.05$). Supplementation without CUB and KCN (300 ppm) produced the highest pH at 2 h, while supplementation with CUB and KCN (300, 600 ppm) produced the lowest pH ($p < 0.05$). Addition without CUB and KCN (300 ppm) or CUB and KCN (0, 300 ppm) produced the highest pH at 4 h, while CUB and KCN (600 ppm) produced the lowest pH ($p < 0.05$). At 2 h, sulfur supplementation with KCN had an effect on NH_3-N ($p < 0.01$). The addition of sulfur (3%) and KCN (300 ppm) produced the highest ammonia nitrogen. However, the combination of sulfur (3%) and KCN (600 ppm) produced the lowest ammonia nitrogen ($p < 0.01$). The quantity of NH_3-N decreased during 4 h of incubation time with increasing the dosages of KCN ($p < 0.05$). KCN increased the amount of ruminal cyanide at 2 and 4 h after incubation ($p < 0.01$).

Table 2. Effect of cyanide-utilizing bacteria, sulfur, and potassium cyanide (KCN) on gas kinetics and cumulative gas at 96 h after incubation.

Enterococcus faecium **(10^8 CFU/mL)** [1]	**Sulfur (%)** [2]	**KCN (ppm)** [3]	**Gas Kinetics**				**Cumulative Gas (mL)**
			a	**b**	**c**	**| a | + b**	
without-*E. faecium*	0% Sulfur	0	$-3.95^{cde} \pm 0.07$	104.67 ± 3.61	0.040 ± 0.00	108.63 ± 3.71	95.60 ± 7.65
		300	$-5.40^{ef} \pm 0.36$	96.44 ± 0.00	0.045 ± 0.00	101.63 ± 0.00	93.50 ± 3.54
		600	$-5.14^{ef} \pm 0.85$	96.69 ± 14.21	0.048 ± 0.01	101.83 ± 13.38	89.35 ± 14.35
	3% Sulfur	0	$-5.90^{f} \pm 0.22$	107.46 ± 2.57	0.043 ± 0.00	111.69 ± 0.00	99.87 ± 9.07
		300	$-4.57^{def} \pm 0.86$	106.91 ± 2.19	0.039 ± 0.00	111.48 ± 3.03	101.50 ± 1.00
		600	$-5.63^{f} \pm 0.63$	111.63 ± 4.43	0.039 ± 0.00	117.26 ± 3.83	107.25 ± 2.05
E. faecium	0% Sulfur	0	$-3.37^{bcd} \pm 3.09$	103.12 ± 5.60	0.036 ± 0.00	101.90 ± 8.00	105.40 ± 2.55
		300	$-2.39^{abc} \pm 1.69$	107.23 ± 5.77	0.031 ± 0.00	116.28 ± 0.00	95.35 ± 4.31
		600	$-1.88^{ab} \pm 0.14$	100.34 ± 2.02	0.038 ± 0.00	102.82 ± 2.30	100.90 ± 71.35
	3% Sulfur	0	$-1.47^{a} \pm 1.04$	105.09 ± 10.20	0.042 ± 0.00	117.53 ± 0.00	108.85 ± 6.29
		300	$-2.74^{abc} \pm 0.78$	101.75 ± 1.90	0.035 ± 0.00	108.07 ± 1.21	98.25 ± 6.29
		600	$-2.53^{abc} \pm 1.79$	99.36 ± 1.40	0.044 ± 0.00	100.90 ± 0.00	96.10 ± 0.07
SEM			0.34	3.84	0.003	4.21	3.73
p-value Interaction							
A × B × C			0.01	0.45	0.25	0.21	0.35
A × B			0.70	0.04	<0.01	0.40	<0.05
A × C			0.23	0.68	0.16	0.43	0.27
B × C			<0.05	0.39	0.20	0.59	0.56
Main Effects							
E. faecium (CFU/mL)	without *E. faecium*		$-5.10^{a} \pm 0.72$	103.97 ± 6.16	$0.043^{a} \pm 0.00$	108.75 ± 6.12	97.84 ± 6.36
			$-2.40^{b} \pm 0.52$	102.82 ± 2.97	$0.038^{b} \pm 0.00$	107.92 ± 7.40	100.81 ± 21.24
	p-value		<0.01	0.48	<0.01	0.54	0.14
Sulfur (%)	0		-3.69 ± 1.43	101.42 ± 4.37	0.040 ± 0.01	$105.51^{b} \pm 5.91$	96.68 ± 19.27
	3		-3.80 ± 1.67	105.37 ± 4.37	0.041 ± 0.00	$111.15^{a} \pm 6.21$	101.97 ± 5.06
	p-value		0.13	0.13	0.70	0.05	0.11
Level of KCN	0		-3.67 ± 1.58	105.09 ± 1.79	$0.041^{ab} \pm 0.00$	109.94 ± 6.51	102.43 ± 5.87
	300		-3.77 ± 1.44	103.08 ± 5.09	$0.038^{c} \pm 0.01$	109.36 ± 6.16	97.15 ± 3.50
	600		-3.79 ± 1.87	102.00 ± 6.60	$0.043^{a} \pm 0.00$	105.70 ± 7.74	98.40 ± 24.69
	p-value		0.59	0.63	0.04	0.70	0.55

[a–f] value on the same column with different superscripts differ $p < 0.05$, $p < 0.01$. SEM = standard error of the mean. 1 = factor A: *Enterococcus faecium*; *E. faecium* (10^8 CFU/mL). 2 = factor B: level of sulfur in diet. 3 = factor C: level of potassium cyanide. a = the gas production from the immediately soluble fraction; b = the gas production from the insoluble fraction; c = the gas production rate constant for the insoluble fraction (b); **| a | + b** = the gas potential extent of gas production.

Table 3. Effect of cyanide-utilizing bacteria, sulfur, and potassium cyanide (KCN) on pH, ammonia-nitrogen (NH_3-N), and ruminal cyanide concentration.

Enterococcus faecium **(10^8 CFU/mL) [1]**	**Sulfur (%) [2]**	**KCN (ppm) [3]**	**pH**		**NH_3-N (mg/dL)**		**Degradation Efficiency (%)**			
			H2	**H4**	**H2**	**H4**	**H0**	**H2**	**H4**	**H6**
without-*E. faecium*	0% Sulfur	0	4.44 ± 0.02	6.80 ± 0.04	25.33 ± 0.64	22.26 ± 1.73	0.00 ^{c} ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		300	4.54 ± 0.04	6.82 ± 0.01	19.52 ± 0.00	16.27 ± 1.25	1.76 ^{c} ± 2.15	3.64 ± 3.04	13.67 ± 16.71	17.02 ± 0.00
		600	4.43 ± 0.06	6.81 ± 0.00	16.02 ± 1.70	13.70 ± 1.78	3.29 ^{c} ± 0.51	8.97 ± 1.71	13.90 ± 2.22	22.36 ± 0.00
	3% Sulfur	0	4.41 ± 0.01	6.82 ± 0.02	23.23 ± 5.95	23.39 ± 0.18	0.00 ^{c} ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		300	4.41 ± 0.08	6.84 ± 0.01	25.85 ± 0.00	14.71 ± 1.93	16.53 ^{b} ± 3.29	19.40 ± 14.69	20.65 ± 7.34	45.27 ± 10.26
		600	4.28 ± 0.04	6.82 ± 0.01	15.14 ± 0.00	14.09 ± 0.00	22.63 ^{a} ± 0.00	23.79 ± 0.00	28.14 ± 1.71	52.98 ± 11.14
E. faecium	0% Sulfur	0	4.28 ± 0.04	6.83 ± 0.00	18.98 ± 0.00	25.06 ± 1.37	0.00 ^{c} ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		300	4.24 ± 0.01	6.81 ± 0.01	15.21 ± 2.08	15.86 ± 0.66	11.70 ^{b} ± 3.55	27.10 ± 15.45	27.90 ± 2.41	54.76 ± 17.35
		600	4.24 ± 0.01	6.79 ± 0.01	17.08 ± 1.42	11.33 ± 1.58	12.96 ^{b} ± 0.63	19.94 ± 18.36	28.67 ± 8.17	54.33 ± 0.00
	3% Sulfur	0	4.27 ± 0.01	6.84 ± 0.01	12.66 ± 0.00	22.18 ± 3.56	0.00 ^{c} ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		300	4.21 ± 0.01	6.84 ± 0.00	21.63 ± 1.55	16.79 ± 0.61	2.12 ^{c} ± 1.39	5.07 ± 5.57	24.77 ± 28.11	29.51 ± 26.72
		600	4.22 ± 0.03	6.81 ± 0.01	13.54 ± 3.99	13.03 ± 0.8	17.26 ^{ab} ± 6.33	20.97 ± 4.88	36.33 ± 13.80	37.99 ± 10.07
SEM			0.03	0.01	1.53	1.15	1.79	6.25	7.62	8.65
p-value Interaction										
A × B × C			0.32	0.50	1.00	0.15	0.03	0.11	0.89	0.27
A × B			0.01	0.69	0.58	0.59	<0.01	0.06	0.54	0.01
A × C			0.01	0.04	0.07	0.32	0.83	0.92	0.55	1.00
B × C			0.17	0.79	<0.01	0.62	<0.01	0.51	0.57	1.00
Main effects										
E. faecium (CFU/mL)		without *E. faecium*	4.42 ^{a} ± 0.08	6.83 ± 0.02	20.80 ^{a} ± 4.66	17.7 ± 4.30	7.37 ± 9.73	9.30 ± 10.17	12.73 ± 11.19	23.59 ± 22.31
			4.24 ^{b} ± 0.03	6.81 ± 0.02	16.65 ^{b} ± 3.41	17.38 ± 5.30	7.34 ± 7.54	12.18 ± 11.89	19.61 ± 15.66	29.43 ± 24.77
p-value			<0.01	0.89	0.01	0.64	0.40	0.28	0.14	0.52
Sulfur (%)		0	4.36 ^{a} ± 0.13	6.81 ^{b} ± 0.01	18.58 ± 3.65	17.66 ± 5.22	4.95 ^{b} ± 5.86	9.94 ± 11.25	14.02 ± 12.65	24.74 ± 24.76
		3	4.30 ^{b} ± 0.12	6.83 ^{a} ± 0.01	18.40 ± 5.59	17.41 ± 4.39	9.76 ^{a} ± 10.17	11.54 ± 11.04	18.31 ± 15.09	27.63 ± 22.77
p-value			<0.01	<0.01	0.87	0.72	<0.01	0.90	0.35	0.37
Level of KCN		0	4.35 ^{a} ± 0.09	6.82 ± 0.02	21.11 ^{a} ± 5.59	23.22 ^{a} ± 1.34	0.00 ^{c} ± 0.00	0.00 ^{b} ± 0.00	0.00 ^{b} ± 0.00	0.00 ^{b} ± 0.00
		300	4.35 ^{a} ± 0.16	6.83 ± 0.01	19.84 ^{a} ± 4.43	15.91 ^{b} ± 0.89	8.03 ^{b} ± 7.30	13.80 ^{a} ± 11.37	21.75 ^{a} ± 6.15	39.45 ^{a} ± 16.72
		600	4.29 ^{b} ± 0.10	6.81 ± 0.01	15.49 ^{b} ± 1.50	12.89 ^{b} ± 1.22	14.04 ^{a} ± 8.18	18.42 ^{a} ± 8.18	26.76 ^{a} ± 9.35	41.91 ^{a} ± 14.99
p-value			0.02	0.06	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

$^{a–c}$ value on the same column with different superscripts differ $p < 0.05$, $p < 0.01$. SEM = standard error of the mean. 1 = factor A: *Enterococcus faecium*; *E. faecium* (10^8 CFU/mL). 2 = factor B: level of sulfur in diet. 3 = factor C: level of potassium cyanide. H0: 0 h of incubation. H2: 2 h of incubation. H4: 4 h of incubation. H6: 6 h of incubation.

3.3. In Vitro Digestibility

Table 4 shows the impact of CUB, sulfur, and KCN on IVDMD, IVNDFD, and IVADFD. In vitro digestibility was unaffected by the interactions of two and three variables ($p > 0.05$). However, CUB supplementation increased IVDMD by 11.16% compared to the without-CUB supplemented group ($p < 0.05$). Supplementation with 3% sulfur increased IVNDFD by 16.87% but had no effect on IVDMD or IVADFD ($p < 0.05$). The level of KCN had no effect on the in vitro digestibility value ($p > 0.05$).

Table 4. Effect of cyanide-utilizing bacteria, sulfur, and potassium cyanide (KCN) on in vitro degradability.

Enterococcus faecium **(10^8 CFU/mL)** [1]	**Sulfur (%)** [2]	**KCN (ppm)** [3]	**IVDMD, %**	**IVNDFD, %**	**IVADFD, %**
without-*E. faecium*	0% Sulfur	0	67.89 ± 19.99	49.31 ± 11.70	23.87 ± 0.70
		300	59.39 ± 2.80	49.63 ± 11.54	29.24 ± 5.81
		600	54.79 ± 1.32	37.55 ± 0.19	27.90 ± 11.56
	3% Sulfur	0	67.45 ± 1.57	48.81 ± 4.87	29.28 ± 0.59
		300	64.51 ± 3.04	59.37 ± 3.06	29.30 ± 5.10
		600	66.68 ± 0.24	56.95 ± 8.17	32.07 ± 4.12
E. faecium	0% Sulfur	0	71.72 ± 9.88	44.18 ± 8.38	24.35 ± 0.79
		300	64.28 ± 5.18	50.48 ± 5.61	27.78 ± 1.58
		600	70.15 ± 4.03	55.46 ± 2.29	29.03 ± 2.72
	3% Sulfur	0	69.03 ± 5.05	45.81 ± 19.44	21.69 ± 3.29
		300	71.80 ± 0.11	60.77 ± 10.46	29.68 ± 1.04
		600	76.17 ± 6.62	63.25 ± 22.83	31.47 ± 1.86
SEM			5.13	6.37	3.14
p-value Interaction					
A × B × C			0.85	0.71	0.55
A × B			0.75	0.69	0.48
A × C			0.42	0.23	0.67
B × C			0.35	0.36	0.86
Main effects					
E. faecium (CFU/mL)		without *E. faecium*	63.45 [b] ± 5.27	50.27 ± 7.65	28.61 ± 2.69
			70.53 [a] ± 3.91	53.34 ± 7.83	27.33 ± 3.65
p-value			0.03	0.42	0.50
Sulfur (%)		0	64.27 ± 6.57	47.77 [b] ± 6.16	27.03 ± 3.34
		3	69.27 ± 4.17	55.83 [a] ± 6.97	28.92 ± 3.73
p-value			0.15	<0.05	0.32
Level of Cyanide		0	69.02 ± 1.92	47.03 ± 2.45	24.80 ± 3.21
		300	64.99 ± 5.11	55.07 ± 5.82	29.00 ± 0.83
		600	66.95 ± 9.00	53.30 ± 11.03	30.12 ± 1.98
p-value			0.56	0.21	0.08

[a–b] value on the same column with different superscripts differ $p < 0.05$, $p < 0.01$. SEM = standard error of the mean. 1 = factor A: *Enterococcus faecium*; *E. faecium* (10^8 CFU/mL). 2 = factor B: level of sulfur in diet. 3 = factor C: level of potassium cyanide. IVDMD: in vitro dry matter digestibility. IVNDFD: in vitro neutral detergent fiber digestibility. IVADFD: in vitro acid detergent fiber digestibility.

3.4. Volatile Fatty Acid Content in the Rumen

Table 5 displays the impacts of CUB, sulfur, and KCN levels on VFA profiles and TVFAs. For all these data points, there was no evidence of an interaction effect between the three variables. When CUB, sulfur, and KCN were introduced, the VFA remained the same. ($p > 0.05$). The concentrations of lowered TVFAs and C3 were at their lowest when KCN was introduced in doses of 600 ppm ($p < 0.01$).

Table 5. Effects of cyanide-using bacteria, sulfur, and potassium cyanide (KCN) on total volatile fatty acids (TVFAs) and VFA profiles in vitro.

Enterococcus faecium (10^8 CFU/mL) [1]	Sulfur (%) [2]	KCN (ppm) [3]	Total VFA, mmol/L	VFA Profiles, mol/ 100 mol		
				Acetic Acid	Propionic Acid	Butyric Acid
without-*E. faecium*	0% Sulfur	0	111.79 ± 12.91	87.59 ± 3.78	10.91 ± 0.57	3.93 ± 0.23
		300	89.35 ± 6.45	87.11 ± 0.36	8.95 ± 0.21	3.95 ± 0.15
		600	86.73 ± 20.37	86.98 ± 2.69	9.00 ± 1.80	4.02 ± 0.88
	3% Sulfur	0	102.94 ± 7.46	83.09 ± 1.05	11.83 ± 1.86	4.57 ± 0.09
		300	108.83 ± 2.87	85.93 ± 2.94	10.20 ± 2.32	3.88 ± 0.62
		600	79.57 ± 4.02	87.05 ± 0.85	9.36 ± 1.78	3.90 ± 0.03
E. faecium	0% Sulfur	0	113.01 ± 14.96	88.32 ± 4.99	12.55 ± 0.97	3.70 ± 0.66
		300	93.12 ± 2.36	84.48 ± 1.16	10.94 ± 0.98	4.58 ± 0.18
		600	66.98 ± 1.55	85.77 ± 0.51	9.63 ± 0.40	4.60 ± 0.11
	3% Sulfur	0	108.19 ± 42.16	95.25 ± 16.69	13.74 ± 1.84	4.84 ± 1.04
		300	95.43 ± 32.33	85.29 ± 0.34	10.36 ± 0.19	4.35 ± 0.53
		600	76.51 ± 12.94	86.65 ± 0.39	9.17 ± 0.28	4.19 ± 0.11
SEM			12.79	3.75	0.94	0.36
p-value Interaction						
A × B × C			0.65	0.56	0.74	0.71
A × B			0.94	0.29	0.48	0.97
A × C			0.73	0.28	0.52	0.55
B × C			0.63	0.97	0.71	0.08
Main effects						
E. faecium (CFU/mL)		without *E. faecium*	96.53 ± 13.12	86.29 ± 1.66	10.04 ± 1.16	4.04 ± 0.27
			92.21 ± 17.79	87.63 ± 3.69	11.06 ± 1.76	4.37 ± 0.40
p-value			0.57	0.55	0.08	0.1300
Sulfur (%)		0	93.49 ± 17.21	86.71 ± 1.37	10.33 ± 1.40	4.13 ± 0.37
		3	95.25 ± 14.20	87.21 ± 4.18	10.78 ± 1.73	4.29 ± 0.38
p-value			0.82	0.82	0.42	0.46
Level of Cyanide		0	108.98 [a] ± 4.52	88.56 ± 5.02	12.26 [a] ± 1.20	4.26 ± 0.53
		300	96.68 [ab] ± 8.48	85.70 ± 1.11	10.11 [b] ± 0.84	4.19 ± 0.33
		600	77.45 [b] ± 8.19	86.61 ± 0.59	9.29 [b] ± 0.27	4.18 ± 0.31
p-value			0.01	0.56	<0.01	0.94

[a–b] value on the same column with different superscripts differ $p < 0.05$, $p < 0.01$. SEM = standard error of the mean. 1 = factor A: *Enterococcus faecium*; *E. faecium* (10^8 CFU/mL). 2 = factor B: level of sulfur in diet. 3 = factor C: level of potassium cyanide. H2: 2 h of incubation. H4: 4 h of incubation.

4. Discussion

The high volume of KCN had a negative impact on the kinetics of gas production and gas storage. However, the gas kinetics and cumulative gas production can be improved by adding CUB and sulfur. Rumen bacteria that utilize HCN can reduce the appropriate level in the rumen. When HCN is broken down by CUB, it can be made available as a N source for bacterial growth through enzymes that catalyze the conversion of sulfur compounds to rhodanese and mercaptopyruvate sulfurtransferase [7]. However, a high concentration of HCN might be too much for the rumen microorganisms to metabolize, which would limit feed digestion and gas production. HCN may limit the growth of bacteria by limiting the cytochrome respiratory chain and the electron transport chain, which lessens the likelihood that HCN will be eliminated from rumen fluid [28]. According to earlier studies by Sumadong et al. [9], increasing fresh cassava root from 300 mg/L to 400 mg/L as an HCN source decreases gas production from the insoluble fraction (b) and gas production after 96 h of incubation by 12.3% and 15.0%, respectively. Gas production is reduced when KCN is used at 600 ppm. At lower doses, however, this has no effect. This experiment showed that the addition of CUB to sulfur and HCN results in an increase in the gas production from the immediately soluble fraction (a) value because sulfur may promote CUB activity, affecting carbohydrate metabolism, where gas production from the immediately soluble fraction (a) value was the immediate gas production from the soluble substance. Therefore, CUB supplementation with sulfur and HCN increased the gas production from the immediately soluble fraction (a) value and reduced the toxicity of HCN as well. This experiment showed that sulfur may improve CUB function, as sulfur

contains the amino acids necessary for the growth of CUB and other microorganisms, thereby increasing digestibility and resulting in gas production from insoluble fractions (b), and constant gas production rates for insoluble fractions (c) when CUB is used with increased sulfur.

After 4 h, adding CUB to a feed HCN-containing compound kept the pH in a range where microbes in the rumen could influence feed digestion. Ruminal pH ranged from 6.79 to 6.84, similar to Supapong and Cherdthong [29], where rhodanese was supplemented at 1.0–1.35 mg/10^4 ppm. KCN was studied to reduce HCN in feed with a pH in the range of 6.99 to 7.06. Furthermore, the concentration of NH_3-N ranged from 13.03 to 25.33 mg%, indicating that it might be utilized as a source of nitrogen for bacterial development. Sulfur supplementation causes KCN breakdown, enhancing ammonia supply in the rumen fluids. The 3-cyanoalanine synthase, which uses cysteine as a substrate, breaks down cyanide, which in sulfur contains cysteine [30]. This method generates 3-cyanoalanine, which can subsequently be converted to NH_3 and aspartate either directly or by the use of asparagine as an intermediary [31]. The concentration of NH_3-N decreases with an increase in KCN levels of more than 300 ppm. As a result, microbial protein synthesis may necessitate the usage of NH_3-N in conjunction with sulfide [32].

There are approaches involved in the use of chemical and biological procedures, such as bacterial and sulfur dosing, although these have not been thoroughly studied in ruminants. These guidelines are for lowering HCN toxins from animal feed. Bacteria capable of digesting thiocyanate have been found in soils, soda lakes, gold mine tailings, and activated sludge, among other anaerobic and aerobic environments [33]. Such microorganisms could use thiocyanate as a source of energy, carbon, sulfur, or nitrogen as a source of energy [33–35]. Most thiocyanate-degrading chemolithotrophic bacteria oxidize the sulfide released during thiocyanate degradation aerobically, but some species, such as *Thioalkalivibrio thiocyanodenitrificans*, are facultative anaerobes capable of anaerobic growth using thiocyanate as an electron donor and nitrate or nitrite as an electron acceptor [36]. The function of the rhodanese enzyme in the rumen, which transforms HCN into a less harmful chemical (thiocyanate) and excretes it via urine [37,38], could explain the lowering of HCN. Promkot et al. [38] reported that increasing sulfur addition to 0.5 and 1 % in the fresh cassava leaf results in a significant in vitro decrease in HCN as compared to 0.2% sulfur supplementation. Similarly, Dagaew et al. [32], found a significant decrease in in vitro HCN content when sulfur was introduced to the feed-block at 2 and 4% with FCR supplementation. The addition of CUB and sulfur may have influenced the decrease in cyanide in the rumen. Sulfur transferase rhodanese lowers cyanide and thiosulfate levels, which lessens thiocyanate toxicity. According to Supapong et al. [6], the hypothesized purpose of 1.2 g of sulfur transferring to thiocyanate is to replace 1 g of cyanide. CUB can reduce the concentration of HCN by using HCN as a nitrogen source and rhodanese enzyme synthesis. It works best after 6 h or more of HCN treatment. Furthermore, according to Latif et al. [39], the Multifect® enzyme (cellulase, hemicellulase, xylanase, and beta-glucanase), which is made by Trichoderma reesei, reduced the HCN level of cassava leaves by 82%. Likewise, Sornyotha et al. [40] discovered that the linamarin content in cassava root decreased by 90.3% after being incubated with xylanase and cellulase. Additional rhodanese enzyme at 1.0–1.35 mg/10^4 ppm KCN increased the degradation efficiency rate by 70%, according to Supapong and Cherdthong [29].

Additionally, compared to the group not supplemented with CUB, the rate of in vitro DM digestion rose by 11.16 %. It is possible that CUB has an indirect effect on digestion, thereby increasing IVDMD as CUB reduces the HCN toxin in food and produces ammonia [30]. The resulting ammonia synthesizes into microorganisms in the rumen as the number of microorganisms increases, resulting in an increase in IVDMD [41,42]. Sulfur increased IVNDFD digestibility by 15.85% compared to the non-sulfur-enriched group. This is consistent with a report by Sumadong et al. [9] that the rise in IVDMD, IVNDFD, and IVADFD could be attributed to sulfur's beneficial effect in increasing rumen microbial activity associated with digestion. Therefore, enough sulfur would increase the growth of

microorganisms and enhance nutrient digestion [43,44]. Ruminants rely on bacteria to convert sulfate into hydrogen sulfite, which is subsequently utilized to manufacture necessary amino acids for the creation of microbes in the rumen (cysteine and methionine) [45,46].

Ruminal fermentation produces a large quantity of TVFA, which is important since it provides over 70% of the ruminant's energy [47–49]. However, when animals are exposed to HCN toxins, TVFA and C3 values are reduced. At 600 ppm, an increase in KCN causes reduced TVFAs and VFA profiles. According to earlier research by Supapong and Cherdthong [29], raising KCN to 600 ppm produces the lowest TVFA when compared to 300 and 450 ppm KCN, respectively. Among the most important factors in TVFA reduction is the negative effects of high-dose KCN on microbial toxicity and feed digestion.

5. Conclusions

Based on the results of this investigation, it can be concluded that the increase in KCN dose in an animal's diet has a negative impact on gas kinetics and ruminal fermentation, particularly NH_3-N, TVFA, and VFA profiles. Therefore, to minimize the impact of KCN, it is necessary to introduce the use of CUB in combination with sulfur. Supplementing with CUB (10^8 CFU/mL) and sulfur (3%) in the animal's diet may improve cumulative gas, digestibility, and maintain ruminal pH. In vivo experiments are required to examine the effect of CUB and sulfur in combination with fresh cassava in a practical feeding program.

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