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Isolation of Cyanide-Utilizing Ruminal Bacteria and Mitigation of Cassava Cyanide Toxicity by High-Sulfur Pellets In Vitro

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ABSTRACT

This research involved two distinct experiments: the first (Experiment 1) aimed to identify ruminal bacteria capable of breaking down cyanide, while the second (Experiment 2) assessed how fresh cassava root (FCR) and sulfur-enriched pellets (PELFUR) affect cyanide levels, gas generation traits, in vitro degradation, and ruminal fermentation. In Experiment 1, a completely randomized design (CRD) was used to test bacterial tolerance at cyanide concentrations of 0, 150, 300, and 450 ppm. Experiment 2 followed a 5 × 3 factorial CRD design. Factor A represented the inclusion of FCR at 0, 260, 350, 440, and 530 g/kg of dry matter (DM), and Factor B denoted PELFUR at 0, 15, and 30 g/kg DM.

In Experiment 1, varying cyanide doses significantly influenced the growth of cyanide-metabolizing bacteria in the rumen ($p < 0.05$). Raising cyanide from 0 to 150 ppm and 150 to 300 ppm led to bacterial growth increases of 38.2% and 15.0%, respectively. For Experiment 2, no significant interaction between FCR and PELFUR was found regarding gas production ($p > 0.05$). FCR inclusion above 260 g/kg DM promoted higher total gas output ($p < 0.05$). Likewise, elevating PELFUR from 15 to 30 g/kg DM enhanced cumulative gas yield compared with 0 g/kg ($p < 0.05$). PELFUR also lowered cyanide concentration in rumen fluid ($p < 0.05$). Dry matter and organic matter digestibility rose after 12 and 24 h incubation, especially when 15 g PELFUR/kg DM was combined with 440 g FCR/kg DM ($p < 0.05$). The distribution of volatile fatty acids—acetic (C2), propionic (C3), and butyric acids—varied significantly among FCR levels ($p < 0.05$). In summary, this study presents the first evidence of ruminal bacteria utilizing cyanide as a nitrogen source for growth. Using 530 g FCR/kg DM along with 30 g PELFUR/kg DM enhanced cumulative gas output, bacterial population, digestibility, propionate proportion, and cyanide degradation rate.

Keywords: Thiocyanate, Rumen microbes, Cyanide metabolism, Fermentation, Degradability

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Introduction

Cassava root serves as a vital carbohydrate source for livestock, particularly in tropical climates [1]. It is consumed by both humans and animals. Typically, roots are chopped and sun-dried into chips before feeding. The dried form shows high rumen degradability (95–99%) [2]. Feeding cassava roots fresh is practical year-round [3]. Cherdthong *et al*. [2] noted that using fresh cassava root (FCR) bypasses the need for drying but presents a challenge due to cyanogenic compounds such as linamarin and lotaustralin. These are converted to cyanohydrins through enzymatic hydrolysis by linamarase, increasing toxicity as hydrolysis proceeds [4].

The danger of cyanide poisoning depends on intake amount and animal health. Diets with over 200 mg/kg cyanide (wet weight) are risky, and those with above 500 mg/kg (dry matter) are potentially lethal [5]. According to Cherdthong *et al*. [2], FCR contains about 85–114 mg/kg cyanide (fresh basis). Promkot and Wanapat [6]

proposed that rumen microorganisms might neutralize cyanide's toxic impact. Likewise, Jones and Megarrity [7] found that Synergistes jonesii bacteria can detoxify the mimosine compound from Leucaena. However, microbial cyanide degradation in the rumen has not been clearly demonstrated. Two enzymes—rhodanese and mercaptopyruvate sulfurtransferase—are key in detoxification. Rhodanese converts cyanide into thiocyanate, a harmless compound eliminated through urine [8]. This conversion requires sulfur, usually derived from amino acids containing this element; hence, additional sulfur is advised in low-sulfur diets [9].

Cherdthong *et al*. [2] showed that feeding FCR at 10 and 15 g/kg of body weight with sulfur-rich feed blocks (20 and 40 g/kg) did not harm digestibility or rumen activity and transformed cyanide into the less toxic thiocyanate. Supapong *et al*. [10] further observed that adding 20 g sulfur/kg in fermented total mixed rations (FTMR) containing FCR enhanced nutrient digestibility, microbial protein synthesis, and concentrations of total volatile fatty acids (VFAs), propionate, and thiocyanate in blood. Sulfur-based pellets also provide a practical, value-added feed source for farmers.

Pelleting is a widely adopted feed process that increases density, palatability, and uniformity [11]. Pelletized feeds can improve nutrient availability and fermentation efficiency in the rumen [12, 13]. To date, the combined effects of FCR and sulfur-enriched pellets on ruminal fermentation remain unexplored. It was postulated that using both FCR and PELFUR could decrease cyanide content while improving fermentation—particularly through higher propionate (C3) production.

Thus, this study was conducted to isolate cyanide-degrading ruminal bacteria and to investigate how FCR and PELFUR supplementation influence cyanide reduction, gas generation, degradability, and fermentation in vitro.

Materials and Methods

Experiment 1

Isolation of rumen microbes capable of cyanide utilization

Animals and feeding protocol

Two fistulated dairy cows, averaging 370 ± 10 kg body weight, were used as rumen fluid donors. Each animal received a concentrate containing 140 g/kg dry matter (DM) crude protein (CP), 410 g/kg DM ether extract (EE), 220 g/kg DM neutral detergent fiber (NDF), 110 g/kg DM acid detergent fiber (ADF), and 756 g/kg DM total digestible nutrients (TDN). Feed was offered twice daily at 07:00 and 15:30, at a rate of 5 g/kg of body weight, with unrestricted access to rice straw. Clean water and mineral blocks were available at all times. Before the morning feeding, 100 mL of rumen fluid was withdrawn from each cow, strained through cheesecloth, placed in pre-warmed thermos flasks (39 °C), flushed continuously with CO_2, and transferred immediately to the laboratory in sealed containers.

Sample preparation and enrichment of cyanide-degrading bacteria

A 500-mL Erlenmeyer flask containing 90 mL of saline with 1 g/L Tween 80 was used to detach microbial cells from rumen fluid. Then, 10 mL of rumen liquor was added and incubated at 39 °C with shaking at 150 rpm for one hour. Enrichment was achieved by subculturing 10% (v/v) inoculum into fresh media every four days. After three transfers, 0.1 mL aliquots were streaked onto nutrient agar supplemented with 1 mM cyanide as the only nitrogen source. The plates were stored at 4 °C for colony development. Medium pH was adjusted to 6.5–7.0 with 0.1 M NaOH, and 10 mL of H_2SO_4 per 100 g/L culture was added to test cyanide metabolism [14].

Culture media and growth conditions

Following Kandasamy *et al*. [14], sodium cyanide (NaCN) was used as the nitrogen source for isolating cyanide-metabolizing microbes. The mineral enrichment medium contained 10 g/L (w/v) NaCN and 10 g/L glucose (w/v), along with 0.1 M NaOH, 4.35 g/L K_2HPO_4, and 10 mL of a salt mix containing: 300 mg $FeSO_4 \cdot 7H_2O$, 180 mg $MgSO_4 \cdot 7H_2O$, 130 mg $CoCl_2$, 40 mg $CaCl_2$, 40 mg $MnCl_2 \cdot 4H_2O$, and 20 mg MoO_3 per liter of deionized water. The pH was maintained between 6.5 and 7.0, and media were sterilized by autoclaving at 121 °C, 15 psi for 20 minutes.

A completely randomized design (CRD) was employed with four cyanide concentrations: 0, 150, 300, and 450 ppm. NaCN (1 g/L) and glucose (1 g/L) solutions were filter-sterilized (0.2 µm pore size; Advantec Toyo Kaisha, Tokyo, Japan) and introduced aseptically via syringe through a silicone septum [15].

Bacterial enumeration

Colony counts were performed using the standard plate count procedure [16] and expressed as colony-forming units (cfu/g fresh matter). Ten grams of fresh matter were mixed with 90 mL of sterile saline (8.5 g/L NaCl), hand-shaken, and serially diluted (10^{-1}–10^{-5}). From each dilution, 20 µL were inoculated onto agar plates. Under anaerobic conditions (Sugiyama-gen Co., Tokyo, Japan), plates were incubated at 39 °C for 96 h. Agar medium contained peptone (0.5 g), beef extract (0.3 g), sodium chloride (0.8 g), and agar (15.0 g) per liter.

Experiment 2

In vitro fermentation assay

Preparation of Sulfur-Enriched Pellets (PELFUR) and Experimental Setup

All ingredients used in the study were first milled through a 0.1-mm sieve. Components were mixed thoroughly, formed into pellets, and sun-dried for roughly three days to obtain proper moisture levels [12]. Fresh cassava roots (FCR, Kasetsart 50 cultivar) were purchased from a local grower in Khon Kaen, Thailand. Roots were harvested at 12 months of age, washed, chopped, and processed immediately.

The experimental design followed a 5 × 3 factorial arrangement under a completely randomized layout. Factor A represented five inclusion levels of FCR (0, 260, 350, 440, and 530 g/kg DM substrate), while Factor B comprised three PELFUR concentrations (0, 15, and 30 g/kg DM substrate). Each 0.5 g sample had a roughage-to-pellet ratio of 70:30, with rice straw as the roughage source.

Samples of FCR, PELFUR, and rice straw were ground to 1-mm particle size for chemical analysis. Parameters such as DM, CP, and ash content were determined according to AOAC [17]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed following the method of Van Soest *et al*. [18]. The cyanide concentration of FCR was quantified spectrophotometrically using the method of Bradbury *et al*. [19]; absorbance readings were multiplied by 396 to express cyanide concentration in mg/kg. The composition of PELFUR, FCR, and rice straw used in the trial is summarized in **Table 1**.

Table 1. Ingredient and nutrient profile of pellets containing high sulfur (PELFUR) used in the study (g/kg DM).

Item	PELFUR 0	PELFUR 15	PELFUR 30	FCR	Rice Straw
Ingredients, g/kg DM					
Cassava chips	556	556	556	-	-
Soybean meal	110	110	110	-	-
Rice bran	110	100	105	-	-
Coconut meal	89	89	70	-	-
Palm kernel meal	78	73	72	-	-
Urea	10	10	10	-	-
Salt	10	10	10	-	-
Sulfur powder	0	15	30	-	-
Mineral premix	10	10	10	-	-
Molasses, liquid	27	27	27	-	-
Chemical composition					
DM, g/kg as basis	946	943	941	351	925
Organic matter, g/kg DM	925	921	932	924	895
Ash, g/kg DM	75	79	68	76	105
Crude protein, g/kg DM	132	130	130	21	23
Neutral detergent fiber, g/kg DM	228	231	226	156	712
Acid detergent fiber, g/kg DM	104	106	104	89	442
Cyanide, ppm	-	-	-	106	-

FCR = fresh cassava root, PELFUR = pellets containing high sulfur.

Inoculum

Two rumen-cannulated dairy cows, averaging 370 ± 10 kg body weight, served as donors. The animals were offered a mixed concentrate diet containing 140 g/kg DM crude protein (CP), 410 g/kg DM ether extract (EE),

220 g/kg DM neutral detergent fiber (NDF), 110 g/kg DM acid detergent fiber (ADF), and 756 g/kg DM total digestible nutrients. Feed was provided twice daily (at 07:00 and 15:30 h) at a rate of 5 g/kg body weight for 21 consecutive days. Each cow was housed separately with unrestricted access to drinking water and a mineral block. Prior to the morning meal, approximately 3500 mL of rumen content was collected from each cow, strained through multiple layers of cheesecloth, and transferred into preheated insulated flasks. The samples were then conveyed to the laboratory under CO_2 flushing to maintain anaerobiosis.

Substrate

For each incubation unit, 350 mg of rice straw and 150 mg of PELFUR (maintaining a 70:30 straw-to-PELFUR proportion) were placed in 50 mL glass bottles. Fresh cassava root (FCR) was added at dry matter (DM) inclusion rates of 0, 131, 175, 219, and 262 mg DM. Each treatment was performed in triplicate, and the entire trial was conducted across three separate runs on different days. Blank bottles (without substrate) were incorporated into each run as controls. Artificial saliva was prepared following the Menke and Steingass [20] protocol. A mixture of 2 mL rumen liquor and 1 mL saliva was incubated at 39 °C under continuous CO_2 flow. Subsequently, 40 mL of this inoculum blend was injected into each bottle, sealed with rubber stoppers and aluminum caps, and prewarmed to 39 °C. Gas production readings were taken at 0, 1, 2, 4, 6, 8, 10, 12, 18, 24, 48, 72, and 96 hours of incubation.

Analysis of samples

Gas accumulation was quantified using the method of Schofield [21], and results were fitted to the equation $V_t = V_f \times (1\text{-}\exp^{(-k(t-L))})$, where V_t represents gas volume at time t, V_f is the maximal gas volume (mL/g DM), k denotes the gas production rate constant (units $time^{-1}$),, and L the lag phase (h).

At 2 and 4 h, pH readings were recorded for each bottle. Immediately after, 18 mL of fermentation fluid was taken and preserved in 2 mL H_2SO_4 for later determination of ammonia nitrogen (NH_3–N) and volatile fatty acids (VFAs). The Kjeldahl method [17] was applied for NH_3–N quantification, while VFA profiles—acetate (C_2), propionate (C_3), and butyrate (C_4)—were assessed by HPLC using a Nova-Pak C18 column (4 × 150 mm; Waters, USA), as described by Samuel *et al*. [22]. Total VFA concentration was calculated as the sum of individual VFAs. For microbial enumeration, 1 mL of inoculum was combined with 6 mL of formalin to count bacteria and protozoa [23]. Cyanide content in the fermentation medium was determined spectrophotometrically following the method of Bradbury *et al*. [24].

To assess substrate degradability, samples were retrieved after 12 and 24 h incubation. Each sample was transferred to Gooch crucibles (40 mm pore size), weighed, and washed thoroughly with distilled water. The residues were oven-dried to estimate dry matter digestibility (IVDMD), corrected using blank values. The dried crucibles were then combusted at 550 °C for 6 h, and organic matter digestibility (IVOMD) was determined from the ash-free residue following AOAC [25] guidelines.

Statistical analysis

Data for cyanide-degrading bacterial activity were evaluated through the PROC GLM procedure in SAS [26], employing a completely randomized design (CRD) model.

$$Y_{ij} = \mu + A_i + \varepsilon_{ij} \quad (1)$$

where Y_{ij} denotes the recorded observation for cyanide-metabolizing bacteria, μ stands for the grand average, A_i represents the impact of cyanide concentration (i = 1–4), and ε_{ij} is the random variation term. Differences among treatment averages were evaluated using Duncan's post-hoc comparison, with significance considered at $p < 0.05$.

For the in vitro analysis, data were processed through the PROC GLM function in SAS [26], structured as a 5 × 3 factorial layout within a completely randomized experimental setup. The analytical framework was defined as follows:

$$Y_{ijk} = \mu + a_i + b_j + ab_{ij} + \varepsilon_{ijk} \quad (2)$$

where Y_{ijk} refers to the measured dependent variable, μ designates the general population mean, a_i reflects the influence of FCR inclusion levels (0, 260, 350, 440, and 530 g/kg of 0.5 g DM) (i = 1–5), and bj expresses the influence of PELFUR supplementation (0, 15, and 30 g/kg DM substrate) (j = 1–3). The ab_{ij} term represents the combined interaction between both factors, while ε_{ijk} accounts for the unexplained experimental error. Average responses were summarized together with their standard errors (SEM). Statistical significance among treatment groups was determined using Duncan's multiple range test at $p < 0.05$.

Results and Discussion

Cyanide-degrading bacteria in the rumen

Figure 1 depicts how rumen bacteria capable of metabolizing cyanide responded to different cyanide concentrations. The addition of cyanide significantly influenced bacterial growth ($p < 0.05$). Measured populations of these bacteria were 525 × 10³, 850 × 10³, 1000 × 10³, and 100 × 10³ cfu/g FM at 0, 150, 300, and 450 ppm cyanide, respectively. Raising cyanide from 0 to 150 ppm and 150 to 300 ppm led to growth increases of 38.2% and 15.0%, suggesting that cyanide can be utilized by rumen microbes, possibly serving as a nitrogen source for their growth.

These results are consistent with findings by Razanamahandry *et al*. [27], who reported that certain bacterial enzymes, including rhodanese and mercaptopyruvate sulfurtransferase, enable cyanide utilization. However, further increasing cyanide to 450 ppm caused a dramatic drop (90%) in bacterial numbers, likely due to toxic effects on cellular respiration and key enzymes, such as cytochrome oxidase, catalase, and oxidase, which interfere with electron transport and oxygen reduction [28].

The data indicate that rumen microbes can safely degrade cyanide up to 450 ppm in 0.5 g of substrate, implying that feeds containing cyanide below this level could be used for ruminants. This aligns with Kang and Kim [29], who showed that mixed bacterial cultures maximized growth and cyanide degradation at concentrations up to 300 ppm.

This study represents the first report of cyanide-metabolizing bacteria in the rumen, highlighting a potential role for cyanide as a nitrogen source in microbial cell synthesis. Previous studies also demonstrate that rumen microbes can detoxify other antinutritional compounds. For instance, Jones and Megarrity [7] found that microbial consortia from Hawaiian goats could neutralize leucaena toxicity by breaking down mimosine, 3,4-DHP, and 2,3-DHP. Similarly, Intanoo *et al*. [30] isolated rumen yeast capable of degrading aflatoxin in dairy cattle diets.

Overall, the in vitro cyanide degradation observed here suggests that rumen bacteria may mitigate cyanide toxicity when cyanogenic feeds are included in diets. Further research should aim to identify the specific bacterial species responsible for this activity.

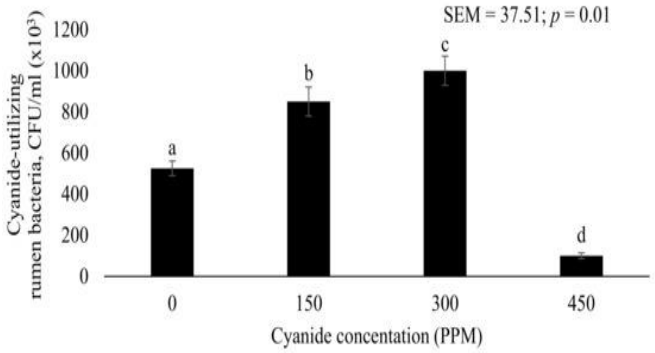

Figure 1. Growth trajectories of cyanide-utilizing rumen bacteria at 0, 150, 300, and 450 ppm initial cyanide levels.

In vitro gas production study

Gas output characteristics and total gas formation

Figure 2 shows the progression of cumulative gas formation over incubation time, while **Table 2** lists the measured parameters and total gas output after 96 h. There was no significant interactive effect between FCR and PELFUR inclusion levels on the final asymptotic gas volume (V_f), rate constant (k), lag phase (L), or cumulative gas yield at 96 h ($p > 0.05$). However, increasing the FCR content in the diet (from 0 to 530 g/kg) significantly

influenced Vf, L, and the total gas volume after 96 h ($p < 0.05$), which varied from 91.85 to 193.70 mL, 0.50 to 2.10 h, and 75.43 to 161.09 mL, respectively. Raising FCR levels above 260 g/kg of DM substrate promoted higher gas accumulation. Similarly, increasing PELFUR inclusion from 0 to 30 g/kg affected the final gas volume ($p < 0.05$) but not the lag phase ($p > 0.05$). These outcomes indicate that both FCR and PELFUR additions stimulate in vitro rumen fermentation [21]. Furthermore, supplementing 15–30 g PELFUR/kg led to a rise in cumulative gas output compared with 0 g PELFUR/kg, possibly because of enhanced microbial proliferation [4], improving feed breakdown and fermentation efficiency. Since FCR is rich in starch, substituting cassava carbohydrates with starch increases nutrient accessibility, enhancing total gas production [3].

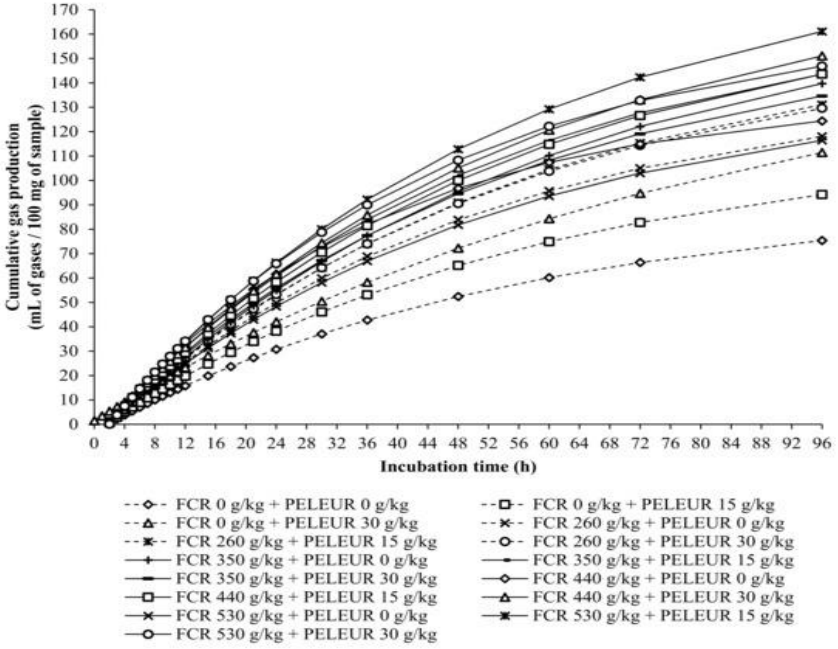

Figure 2. Influence of fresh cassava root (FCR) combined with sulfur-enriched pellets (PELFUR) on cumulative gas output across incubation periods.

Table 2. Influence of fresh cassava root (FCR) and high-sulfur pellets (PELFUR) on gas parameters and cumulative gas yield after 96 h of incubation.

FCR (g/kg DM)	PELFUR (g/kg DM)	Gas Production Parameters			Cumulative Gas (mL)
		Vf	k	L	
0	0	91.85	0.02	1.60	75.43
0	15	115.80	0.02	1.45	94.16
0	30	153.95	0.02	0.50	111.40
260	0	139.30	0.02	1.95	114.82
260	15	159.75	0.02	1.90	131.04
260	30	156.75	0.02	1.60	129.49
350	0	173.65	0.02	2.10	139.68
350	15	169.50	0.02	1.70	142.94
350	30	161.40	0.02	1.75	133.71
440	0	134.45	0.03	1.70	124.40
440	15	173.65	0.02	2.10	143.45
440	30	183.50	0.02	1.75	150.62
530	0	139.75	0.02	1.55	116.05
530	15	193.70	0.02	1.80	161.09
530	30	166.05	0.02	2.00	146.27
SEM		13.19	0.01	0.95	9.08
Main Effects – FCR (g/kg DM)					

FCR	Vf	k	L	Cumulative Gas (mL)
0	120.53[a]	0.02	1.18[a]	93.66[a]
260	151.93[b]	0.02	1.82[b]	125.12[b]
350	168.18[b]	0.02	1.85[b]	138.78[bc]
440	163.87[b]	0.02	1.85[b]	139.49[bc]
530	166.50[b]	0.02	1.78[b]	141.14[c]
Main Effects – PELFUR (g/kg DM)				
PELFUR	Vf	k	L	Cumulative Gas (mL)
0	135.80[a]	0.02	1.78	114.07[a]
15	162.48[b]	0.02	1.79	134.53[b]
30	164.33[b]	0.02	1.52	134.30[b]
Significance of Effects				
Source	Vf	k	L	Cumulative Gas
FCR	0.01	0.64	0.01	0.01
PELFUR	0.01	0.82	0.16	0.01
FCR × PELFUR	0.15	0.99	0.15	0.29

[a,b,c] Means within a column with distinct superscripts differ ($p < 0.05$). FCR = fresh cassava root; PELFUR = pellets containing high sulfur; SEM = standard error of mean; V_f = final asymptotic gas volume (mL/500 mg DM); k = rate constant (units time^{-1}),; L = lag time (h).

In vitro fermentation and ruminal microbial counts

Table 3 summarizes the effects of FCR and PELFUR supplementation on in vitro fermentation parameters and microbial populations. No significant interaction was detected between FCR and PELFUR treatments for any variable ($p > 0.05$). With higher FCR inclusion, rumen pH tended to decline, likely due to a greater proportion of fermentable carbohydrates rapidly converted to volatile acids, which reduced pH. The pH values dropped slightly (6.90–6.86), probably due to ongoing carbohydrate fermentation. Despite this decrease, the pH levels remained within the optimal range for microbial activity and ruminal fermentation processes [31].

Table 3. Effects of fresh cassava root (FCR) and sulfur-enriched pellets (PELFUR) on ruminal pH, ammonia-nitrogen (NH_3-N), microbial populations, and cyanide content.

FCR (g/kg DM)	PELFUR (g/kg DM)	pH	NH_3-N (mg/dL)	Protozoa (×10⁶ cells/mL)	Bacteria (×10⁸ cells/mL)
0	0	6.91	16.86	7.00	9.50
0	15	6.89	14.96	6.00	11.00
0	30	6.90	15.21	6.00	11.50
260	0	6.89	15.71	6.00	10.50
260	15	6.86	11.51	6.00	11.50
260	30	6.87	12.91	5.00	12.00
350	0	6.87	14.31	7.00	11.00
350	15	6.86	12.21	7.00	12.00
350	30	6.85	12.21	7.00	12.50
440	0	6.85	15.71	6.00	11.50
440	15	6.83	12.96	9.00	12.50
440	30	6.83	12.41	8.00	12.50
530	0	6.82	17.26	8.50	12.00
530	15	6.83	16.01	8.00	13.00
530	30	6.83	15.01	9.00	13.00
SEM		0.01	0.53	1.85	0.73
Main Effects – FCR (g/kg DM)					
FCR	pH	NH_3-N (mg/dL)	Protozoa (×10⁶ cells/mL)		Bacteria (×10⁸ cells/mL)
0	6.90[a]	15.68[a]	6.30		10.70
260	6.87[b]	13.38[b]	5.70		11.30
350	6.86[c]	12.91[b]	7.00		11.80
440	6.84[d]	13.69[b]	7.70		12.20

530	6.83[d]	16.09[a]	8.50	12.70
Main Effects – PELFUR (g/kg DM)				
PELFUR	pH	NH_3-N (mg/dL)	Protozoa (×10⁶ cells/mL)	Bacteria (×10⁸ cells/mL)
0	6.87[a]	15.97[a]	6.90	10.90
15	6.85[b]	13.53[b]	7.20	12.00
30	6.86[b]	13.55[b]	7.00	12.30
Significance of Effects				
Source	pH	NH_3-N	Protozoa	Bacteria
FCR	0.01	0.01	0.40	0.22
PELFUR	0.04	0.01	0.97	0.65
FCR × PELFUR	0.28	0.23	0.98	0.90

a–j Means with different superscripts in the same column are statistically distinct ($p < 0.05$). FCR = fresh cassava root; PELFUR = pellets with high sulfur; SEM = standard error of mean.

Ammonia-nitrogen (NH_3-N) levels were significantly affected ($p < 0.05$), showing a decline as the FCR proportion increased. This may result from the enhanced energy availability from rapidly fermentable carbohydrates that facilitate the utilization of peptides, amino acids, and NH_3-N for microbial protein synthesis [10]. In line with this, Cherdthong *et al*. [2] observed an increase in ruminal NH_3-N concentration with higher FCR intake. The addition of 15 and 30 g PELFUR/kg also lowered NH_3-N concentrations by 2.44 and 2.42 mg/dL compared to control diets, possibly due to microbial incorporation of NH_3-N and sulfide during protein synthesis [3]. Supapong *et al*. [10] reported a similar trend, noting slightly reduced NH_3-N (15.20 mg/dL) with sulfur supplementation at 20 g/kg. Protozoa and bacterial counts were not significantly affected by FCR or PELFUR ($p > 0.05$), ranging between 5.00×10^6–9.00×10^6 cells/mL and 9.50×10^8–13.00×10^8 cells/mL, respectively.

Cyanide levels in rumen fluid, influenced by FCR and PELFUR treatments, are shown in **Table 3**. The inclusion of PELFUR markedly lowered cyanide concentrations ($p < 0.05$). Supplementation at 15 and 30 g PELFUR/kg decreased cyanide by 30.35–36.96% and 37.06–40.08%, respectively, relative to the control. This reduction is attributed to sulfur's role in microbial detoxification processes. The enzyme rhodanese converts cyanide to thiocyanate, mitigating toxicity by reducing cyanide and thiosulfate concentrations. Supapong *et al*. [10] explained that approximately 1.2 g sulfur is required to neutralize 1 g cyanide through this conversion. This aligns with Cherdthong *et al*. [2], who found that supplementing 20 or 40 g sulfur/kg in feed blocks for cattle fed FCR effectively reduced cyanide through thiocyanate formation. Moreover, experiments on cyanide-utilizing rumen bacteria confirmed that higher cyanide availability from FCR may activate detoxifying bacterial strains. Hence, ensuring adequate supplies of energy, amino acids, and nitrogen sources may promote bacterial growth and further reduce cyanide levels.

In vitro degradability

Table 4 outlines the data on in vitro degradability obtained at varying incubation intervals. A significant interactive influence between FCR and PELFUR treatments was found ($p < 0.05$). Both in vitro dry matter degradability (IVDMD) and in vitro organic matter degradability (IVOMD) showed marked improvement after 12 and 24 hours of incubation when FCR was combined with PELFUR. The most pronounced increases (651.7 g/kg DM and 672.1 g/kg DM; 690.0 g/kg DM and 725.9 g/kg DM, respectively) occurred at 15 g PELFUR/kg inclusion level in 440 g FCR/kg ($p < 0.05$). This enhancement likely originates from the elevated content of nonstructural carbohydrates present in FCR, which are readily fermentable in the rumen, leading to greater microbial proliferation and consequently higher DM and OM degradability [3]. Sulfur, being a critical nutrient for rumen microorganisms, enables the microbial conversion of sulfate to hydrogen sulfide — a precursor for the synthesis of sulfur-containing amino acids such as cysteine and methionine — which supports microbial protein formation [6]. Thus, adequate sulfur availability stimulates microbial development and promotes nutrient digestion. These findings are comparable with those of Cherdthong *et al*. [2], who reported improved DM and OM digestibility in cattle provided with 40 g sulfur/kg compared with those receiving 20 g sulfur/kg in feed blocks containing 10 or 15 g/kg BW of FCR.

Table 4. Influence of fresh cassava root (FCR) with sulfur-rich pellets (PELFUR) on nutrient digestibility in vitro.

FCR (g/kg DM)	PELFUR (g/kg DM)	IVDMD (g/kg DM)			IVOMD (g/kg DM)
		12 h	24 h	Mean	12 h
0	0	562.6^{a}	598.8^{a}	580.7^{a}	600.8^{a}
0	15	569.5^{a}	603.7^{ab}	586.6^{a}	612.7^{ab}
0	30	559.6^{a}	613.9^{bc}	586.7^{a}	601.6^{a}
260	0	582.9^{c}	611.7^{abc}	597.3^{b}	623.1^{bc}
260	15	601.7^{dh}	621.7^{c}	611.7^{c}	644.6^{de}
260	30	597.2^{d}	637.2^{de}	617.2^{cd}	637.9^{d}
350	0	603.9^{bdh}	616.2^{bc}	610.1^{c}	630.5^{cd}
350	15	616.4^{e}	649.3^{ef}	632.9^{e}	653.2^{e}
350	30	608.6^{beh}	654.7^{fg}	631.7^{e}	644.0^{de}
440	0	612.3^{be}	635.7^{d}	624.0^{de}	637.8^{d}
440	15	647.8^{g}	668.5^{h}	658.1^{gh}	684.3^{gh}
440	30	629.0^{f}	664.2^{gh}	646.6^{f}	668.4^{f}
530	0	617.5^{e}	643.5^{def}	630.5^{e}	642.2^{de}
530	15	651.7^{g}	672.1^{h}	661.9^{h}	690.0^{h}
530	30	633.7^{f}	665.9^{gh}	649.8^{fg}	672.4^{fg}
SEM		3.4	4.3	3.1	4.9
Main Effects – FCR (g/kg DM)					
FCR	IVDMD 12 h	IVDMD 24 h	IVDMD Mean		IVOMD 12 h
0	563.9^{a}	605.4^{a}	584.7^{a}		605.0^{a}
260	593.9^{b}	623.5^{b}	608.7^{b}		635.2^{b}
350	609.6^{c}	640.1^{c}	624.9^{c}		642.6^{c}
440	629.7^{d}	656.1^{d}	642.9^{d}		663.5^{d}
530	634.3^{d}	660.5^{d}	647.4^{d}		668.2^{d}
Main Effects – PELFUR (g/kg DM)					
PELFUR	IVDMD 12 h	IVDMD 24 h	IVDMD Mean		IVOMD 12 h
0	605.6^{a}	621.2^{a}	608.5^{a}		626.9^{a}
15	617.4^{b}	643.0^{b}	630.2^{b}		657.0^{b}
30	595.8^{c}	647.2^{b}	626.4^{b}		644.9^{c}
Significance of Effects					
Source	IVDMD 12 h	IVDMD 24 h	IVDMD Mean		IVOMD 12 h
FCR	0.01	0.01	0.01		0.01
PELFUR	0.01	0.01	0.01		0.01
FCR × PELFUR	0.01	0.01	0.01		0.01

$^{a–j}$ Mean values within a column marked by different superscripts differ significantly ($p < 0.05$). FCR = fresh cassava root; PELFUR = sulfur-enriched pellets; SEM = standard error of the mean; IVDMD = in vitro dry matter digestibility; IVOMD = in vitro organic matter digestibility.

Volatile fatty acid (VFA) concentrations

No interaction effects were detected between FCR and PELFUR in total VFA yield, VFA profiles, or C2:C3 ratio **(Table 5)**($p > 0.05$). However, significant variations ($p < 0.05$) were observed in total VFA proportions, C2, C3, and C4 fractions, as well as in the C2:C3 ratio across the FCR treatments. When the FCR content was raised to 530 g/kg of DM substrate, the C3 concentration increased by 14.6%. This likely results from the higher supply of fermentable carbohydrates in FCR, which ferment rapidly and boost overall VFA and C3 production in the rumen. Elevated C3 levels are advantageous, as they provide the main substrate for glucose generation through gluconeogenesis. These outcomes are consistent with the observations of Cherdthong *et al*. [2], who found that Thai native cattle fed diets with higher FCR levels had elevated C3 compared to those fed lower levels. Dagaew *et al*. [3] also reported that adjusting the FCR-to-rice straw ratio to 100:0 and including 20–40 g sulfur/kg in a high-sulfur feed block (FBS) led to a 16.7% rise in C3 compared to the control. Furthermore, the sulfur concentration in PELFUR affected C3 content, with higher sulfur inclusion increasing ruminal C3, likely acting

as a hydrogen sink under excess sulfide conditions [10]. Promkot *et al*. [4] similarly documented higher C3 concentrations in fresh cassava foliage supplemented with 10 g sulfur/kg, noting sulfur's role in microbial protein formation. Supapong and Cherdthong [8] also observed a 10.9% increase in propionic acid when 20 g sulfur/kg FTMR containing FCR was used, compared with the sulfur-free control.

Table 5. Effect of fresh cassava root (FCR) and sulfur-enriched pellets (PELFUR) on volatile fatty acid concentrations.

FCR (g/kg DM)	PELFUR (g/kg DM)	Total VFAs (mmol/L)	Acetate (C2)	Propionate (C3)	Butyrate (C4)
			(mol/100 mol)		
0	0	91.40	67.26	23.44	9.30
0	15	91.60	67.41	23.37	9.22
0	30	92.24	66.91	23.61	9.49
260	0	93.07	66.19	24.82	8.98
260	15	93.54	65.41	25.47	9.12
260	30	94.83	65.06	25.49	9.45
350	0	95.13	64.99	25.18	9.84
350	15	98.08	64.78	25.70	9.52
350	30	96.15	64.71	25.52	9.76
440	0	99.42	64.56	25.64	9.81
440	15	100.71	63.53	27.01	9.46
440	30	103.88	63.89	26.67	9.43
530	0	106.45	63.86	26.93	9.21
530	15	108.82	63.48	28.27	8.25
530	30	107.35	64.16	27.32	8.52
SEM		2.53	0.33	0.34	0.32
Main Effects – FCR (g/kg DM)					
FCR	Total VFAs	Acetate (C2)	Propionate (C3)	Butyrate (C4)	
0	91.75^{a}	67.19^{a}	23.47^{a}	9.33^{b}	
260	93.81^{ab}	65.55^{b}	25.26^{b}	9.18^{ab}	
350	96.45^{b}	64.83^{c}	25.47^{b}	9.71^{b}	
440	101.34^{c}	63.99^{d}	26.44^{c}	9.57^{b}	
530	107.54^{d}	63.83^{d}	27.51^{d}	8.66^{a}	
Main Effects – PELFUR (g/kg DM)					
PELFUR	Total VFAs	Acetate (C2)	Propionate (C3)	Butyrate (C4)	
0	97.09	65.37	25.20^{a}	9.43	
15	98.55	64.92	25.97^{b}	9.11	
30	98.89	64.95	25.72^{b}	9.33	
Significance of Effects					
Source	Total VFAs	Acetate (C2)	Propionate (C3)	Butyrate (C4)	
FCR	0.01	0.01	0.01	0.01	
PELFUR	0.51	0.09	0.01	0.32	
FCR × PELFUR	0.98	0.41	0.48	0.74	

$^{a–d}$ Mean values in the same column with different superscripts are significantly different ($p < 0.05$). FCR = fresh cassava root; PELFUR = sulfur-enriched pellets; SEM = standard error of the mean.

Conclusion

This study identified rumen bacteria capable of both tolerating and degrading cyanide, withstanding concentrations up to 300 ppm in culture medium; however, their populations declined beyond 450 ppm. It can be inferred that supplementing 530 g FCR/kg DM substrate together with 30 g PELFUR/kg improves total gas output,

microbial abundance, in vitro degradability, propionic acid proportion, and cyanide disappearance rate, without negatively influencing in vitro fermentation. Nonetheless, further in vivo research is necessary to confirm these effects under real feeding conditions.

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