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Research article

Detoxification and fermentation of fresh cassava roots using sulfur and cyanide-utilizing bacteria for FTMR: An *in vitro* study

Kannika Saisombut, Suphakon Pramotchit, Molthida Rungchaicharoenphai, Anusorn Cherdthong and Chanon Suntara*

Tropical Feed Resource Research and Development Center (TROFREC), Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand

* **Correspondence:** Email: Chansun@kku.ac.th; Tel: +66840351217.

Abstract: This study aimed to evaluate the effects of sulfur and cyanide-utilizing bacteria (CUB) supplementation, along with varying fermentation durations, on the nutritional quality, gas production kinetics, digestibility, and ruminal fermentation characteristics of fermented total mixed rations (FTMR) based on fresh cassava tubers. A completely randomized design (CRD) was applied in a 3 × 3 factorial arrangement consisting of three additive treatments (no additive, 1.5% sulfur, and CUB at 10^8 CFU/g) and three fermentation periods (0, 7, and 14 days), resulting in nine dietary treatments. The CUB strain used was *Enterococcus faecium* KKU-BF7, previously isolated from rumen fluid for its cyanide-degrading capability. Results revealed that both additives and fermentation time significantly ($p < 0.01$) affected feed chemical composition, particularly crude protein (CP), either extract (EE), acid detergent fiber (ADF), and acid detergent lignin (ADL). The highest NH_3-N concentration (22.43 mg/dL; $p < 0.01$) was observed in the CUB group fermented for 14 days. In vitro dry matter digestibility (IVDMD) was highest in the CUB group, similar to the control, while sulfur reduced it ($p < 0.01$). In vitro organic matter digestibility (IVOMD) is highest on day 14 of fermentation, regardless of additive ($p < 0.05$). Sulfur supplementation, particularly after 14 days of fermentation, markedly reduced cumulative gas production over the 96-hour incubation period. As the fermentation time increased, acetate decreased, while propionate and butyrate increased. At the onset of fermentation (day 0), HCN concentrations were similar among treatments (≈81–83 ppm). After 7 days, CUB reduced HCN to 11.9 ppm, compared with 29.0 ppm for sulfur and 46.51 ppm for the control ($p < 0.01$). By day 14, HCN in the CUB group further declined to 4.12 ppm, the lowest among treatments, compared with 10.11 ppm for sulfur and 22.77 ppm for the control ($p < 0.01$). These findings demonstrate that CUB is a promising additive for improving cassava-based FTMR through

enhanced detoxification, fermentation efficiency, and nutrient utilization. Optimization of additive type and fermentation duration can significantly enhance feed safety and energy availability in ruminant diets.

Keywords: cyanide reduction; cassava fermentation; cyanide-utilizing bacteria (CUB); rumen fermentation; *in vitro* gas production

1. Introduction

Cassava (*Manihot esculenta* [L.] Crantz) is a pivotal tropical crop of economic significance that is extensively cultivated across Asia, Africa, and South America. In Thailand, cassava plays a dual role as both an industrial raw material and an energy-rich feed ingredient for ruminants, primarily due to its high starch content, which contributes to increased fermentable carbohydrate supply in the rumen [1,2].

Despite its nutritional potential, the utilization of fresh cassava roots in animal feeding is constrained by the presence of cyanogenic glycosides, mainly linamarin and lotaustralin. These compounds undergo enzymatic hydrolysis during mastication and digestion, leading to the release of HCN, a potent cellular toxin [3,4]. HCN inhibits cytochrome oxidase activity in mitochondria, resulting in impaired oxidative phosphorylation and tissue hypoxia. Acute cyanide poisoning in ruminants may manifest as respiratory distress, neurological dysfunction, and sudden death. The minimum lethal dose for cattle has been reported as low as 2 mg HCN per kg of body weight [5,6]. The concentration of cyanide varies among different plant parts, with the highest levels typically found in cassava peels (804 ppm), followed by leaves (655 ppm), and root pulp (305 ppm) [7]. Therefore, effective detoxification strategies are critical before cassava-based materials can be safely incorporated into livestock diets.

Given the rapid postharvest deterioration of cassava roots within 48 to 72 hours [8], preservation through sun-drying or fermentation is often employed. However, sun-drying exposes cassava to environmental contaminants, such as dust, insects, and soil particles, which can compromise the safety and quality of the final product [9]. While fermentation offers a safer process, it also alters the microbial community in cassava silage, promoting beneficial bacteria that contribute to the stability and quality of the feed [10,11]. Additionally, fermentation facilitates the microbial degradation of cyanogenic compounds, thereby enhancing the safety and palatability of cassava-based feeds [12].

Recent studies have demonstrated the potential of biological detoxification using rumen-derived microorganisms [13,14]. Specifically, strains of *Enterococcus faecium* and E. *gallinarum*, isolated from the rumen of cattle and buffalo, have been shown to convert cyanide to thiocyanate through the enzymatic action of rhodanese, thereby significantly reducing the toxicity of cassava substrates. These microbial interventions not only detoxify cyanide but may also contribute to enhanced rumen fermentation efficiency [15].

Simultaneously, chemical detoxification using sulfur compounds, such as sodium thiosulfate, has been explored. Sulfur serves as a donor of sulfane sulfur groups in the rhodanese-mediated conversion of cyanide to thiocyanate, a less toxic metabolite [16]. Although this method is effective, excessive sulfur inclusion can disrupt rumen microbial ecology, suppress fermentation, and reduce gas production [17,18]. Hence, its application requires careful dosage management.

Considering the above considerations, this study was designed to evaluate and compare the efficacy of sulfur supplementation and cyanide-utilizing bacteria (CUB) in reducing HCN concentrations in fresh cassava root during fermentation. Additionally, the research investigates the impact of these treatments on in vitro gas production kinetics, nutrient degradability, and ruminal fermentation characteristics. The outcomes are expected to provide insights into safe and efficient strategies for enhancing the utilization of cassava in ruminant nutrition.

2. Materials and methods

2.1. Animal ethics

The Institutional Animal Care and Use Committee of Khon Kaen University approved all methods and procedures (record No. IACUC-KKU 32/67).

2.2. Cassava sourcing and environmental context

Fresh cassava roots (*Manihot esculenta* var. Kasetsart 50) were sourced directly from local farmers in Khon Kaen Province; specific cultivation or harvest methods were not available. The study site is located on the Khorat Plateau, which is underlain by loess-derived silty sand/dust soils common to northeastern Thailand. Meteorological data for May in Khon Kaen indicate hot and humid conditions, with average daytime temperatures around 35 ℃, nighttime temperatures around 26–27 ℃, and monthly precipitation of approximately 174–180 mm.

2.3. CUB

The cyanide-utilizing bacterium used in this study, *Enterococcus faecium* KKU-BF7, was a product previously co-developed by our research team and Khota et al. [13]. In the original work, the strain was isolated from rumen fluid of healthy ruminants under anaerobic conditions, enriched in nutrient broth with stepwise increases in potassium cyanide (KCN) concentration (10–50 g/L) at 39 ℃, 120 rpm, and subsequently cycled in mineral–glucose medium (pH 7.0, 10–30 g/L KCN). Cultures were serially diluted, plated on nutrient agar, and incubated anaerobically at 39 ℃ for 48 h. Purified colonies were cryopreserved at −80 ℃ and regrown in nutrient broth containing 10 g/L KCN to an OD_{600} of 1.0 (~10^8 CFU/mL). Rhodanese activity was assayed at 39 ℃ using sodium thiosulfate and KCN, with thiocyanate formation quantified colorimetrically at 460 nm; strain KKU-BF7 showed the highest CN^- to SCN^- transformation rate. Genetic identification via 16S rRNA sequencing (primers 27F/1492R) confirmed the species as *E. faecium* KKU-BF7, with sequences deposited in GenBank under accession numbers MZ959826–MZ959831 (available to access from https:// www. ncbi. nlm. nih. gov/ nucco re/? term= MZ959 826: MZ959 831).

2.4. Preparation of fermented total mixed ration (FTMR)

Fresh cassava root was chopped into approximately 1 cm pieces. The FTMR was prepared by thoroughly mixing the concentrate diet with rice straw chopped to 3–5 cm, after which water was added to adjust the moisture content to 55%. The mixture was tightly packed into plastic bags and

allowed to ferment anaerobically at ambient temperatures (25–32 ℃) for 7 days prior to use. Detailed compositions of the feed ingredients, as well as the chemical compositions of both the raw materials and the concentrate diet, are presented in Table 1.

Table 1. Ingredients of FTMR used in the experiment.

Ingredients	DM (%)	CP (%)	EE (%)	NDF (%)	ADF (%)	ADL (%)	TDN (%)	Ca (%)	P (%)
Soybean meal	10	4.40	0.15	129.	0.83	0.24	8.40	0.03	0.03
Fresh cassava	15	0.51	0.07	1.26	0.60	0.23	11.91	-	-
Palm kernel meal	7	0.70	0.83	3.36	2.31	0.20	4.59	0.05	0.03
Rice bran	13	1.69	1.88	2.08	0.91	0.27	10.66	0.01	0.06
Rice straw	40	1.00	0.70	24.00	14.40	1.36	17.58	0.07	0.02
Corn meal	14	1.26	0.60	2.52	0.42	0.18	11.90	-	0.03
Urea	0.5	1.44	-	-	-	-	-	-	-
Premix[1]	0.5	-	-	-	-	-	-	0.08	0.04
Total.	100	12.53	4.23	34.51	19.47	2.48	65.03	0.24	0.2

Note: [1]Premix (per kg): Vitamin A = 10,000,000 IU, Vitamin E = 70,000 IU, Vitamin D = 1,600,000 IU, Fe = 50 g, Zn = 40 g, Mn = 40 g, Co = 0.1 g, Cu = 10 g, Se = 0.1 g, I = 0.5 g, DM = dry matter, CP = Crude protein, EE = Ether Extract, NDF = Neutral Detergent Fiber, ADF = Acid Detergent Fiber, ADL = Acid Detergent Lignin, TDN = Total Digestible Nutrients, Ca = Calcium, P = Phosphorus.

2.5. Experimental design and dietary treatments

This study was designed to evaluate the effectiveness of sulfur (1.5%) and CUB (10^8 CFU/g) in reducing HCN concentrations in feed containing fresh cassava tubers as a primary ingredient. The inclusion rate of 1.5% sulfur was selected based on previous reports indicating that this level provides sufficient sulfur for rhodanese-mediated conversion of HCN to the less toxic thiocyanate, while avoiding adverse effects on rumen fermentation or feed intake [19]. Fermentation durations of 7 and 14 days were selected to represent the early, rapid lactic phase and a subsequent stabilization phase. Prior studies on cassava silages show that lactic acid production peaks around day 7 with the lowest pH, whereas fermentations exceeding 14 days tend to have higher pH and slower changes; moreover, substantial HCN detoxification occurs within the first week of ensiling cassava materials [20]. A completely randomized design (CRD) was employed, consisting of nine dietary treatments arranged in a 3 × 3 factorial format: three levels of additive supplementation (no additive, sulfur, and CUB) and three durations of FTMR fermentation (0, 7, and 14 days).

2.6. Animal requirements for in vitro study

Feed intake and nutrient requirements for Thai native beef cattle with an average body weight of approximately 300 ± 50 kg were estimated according to the guidelines of the WTSR [21]. Dry matter intake (DMI) was calculated using the predictive equation for Thai native beef cattle:

$$\text{DMI (kg/d)} = 0.02887 \times \text{BW} - 0.5778,$$

where BW is body weight in kilograms.

The metabolizable energy intake (MEI) requirement, expressed in kJ/kgBW^0.75/d, was determined from the equation:

$$MEI = 30.294 \times ADG + 489.367,$$

The crude protein intake (CPI) requirement, expressed in gCP/kgBW^0.75/d, from:

$$CPI = 0.380 \times ADG + 5.0262,$$

where ADG is the average daily gain in g/kgBW^0.75/d. Based on an expected ADG of 650 g/d, the calculated CPI corresponded to approximately 12% crude protein in the total mixed ration (FTMR), which was formulated to meet both the maintenance and growth requirements of the cattle.

2.7. Determination of dietary nutrient composition

The chemical compositions analyzed in the experimental treatment samples included dry matter (DM), ash, organic matter (OM), crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL). Before analysis, samples were dried at 60 ℃ for 48 h in a forced-air oven and ground to pass through a 1 mm mesh screen.

The contents of DM (ID 967.03), CP (ID 984.13), EE (ID 920.39), and ash were determined following AOAC guidelines [22]. The detergent fiber method described by Van Soest et al. [23] was employed to analyze NDF and ADF, and ADL content.

2.8. Collecting rumen fluid and preparing artificial saliva

Rumen fluid was collected from Thai native beef cattle weighing approximately 300 ± 50 kg, which were fed FTMR containing 12% crude protein, formulated based on the nutrient requirements for beef cattle as specified by the Department of Livestock Development [21]. These cattle were housed at the Ruminant Nutrition Laboratory, Faculty of Agriculture, Khon Kaen University, Thailand. Rumen fluid was obtained before the morning feeding by inserting a stomach tube and collecting approximately 1,000 mL of rumen liquor. The collected fluid was immediately filtered through four layers of cheesecloth and stored in a pre-warmed thermos flask (maintained at 39 ℃) flushed with CO_2 to ensure anaerobic conditions and transported to the laboratory within 15 minutes. Artificial saliva was prepared following the method described by Sommart et al. [24], comprising buffer solution, macro and micro-mineral solutions, resazurin indicator, and a reducing solution. Each solution was mixed under a CO_2 stream to maintain anaerobic conditions, and the mixture was warmed to 39 ℃ before inoculation.

2.9. In vitro gas production and nutrient degradability

Experimental bottles containing 0.5 g of the designated treatment feed were filled with 40 mL of the buffered rumen inoculum mixture under CO_2 flushing to maintain anaerobic conditions. Bottles were sealed using butyl rubber stoppers and aluminum caps and incubated in a hot air oven at 39℃. Gas volume was measured at regular intervals (0, 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 h) using a glass syringe attached via an 18-gauge needle. Cumulative gas production and kinetic parameters (a, b, c) were estimated using the Ørskov and McDonald [25] equation.

For each treatment, triplicate bottles were prepared for three experimental groups: (1) gas kinetics and total gas production, (2) rumen fermentation parameters (pH, NH_3-N), volatile fatty acids (VFAs) at 24 and 48 h, and (3) degradability analysis (in vitro dry matter degradability [IVDMD] and in vitro organic matter degradability [IVOMD]) at 24 and 48 h. pH was measured using a digital pH meter (HANNA Instruments), and NH3-N concentration was determined using the method of Fawcett and Scott [26], and VFA profiles (C2, C3, C4) were analyzed via gas chromatography (GC) using the method of Samuel et al. [27]. The final set of bottles was dedicated to assessing IVDMD and IVOMD.

2.10. Determination of hydrogen cyanide (HCN) content

The HCN concentration in feed samples was determined using a colorimetric method as described by Sommart et al. [28]. An alkaline picrate solution was prepared by dissolving 25 g of sodium carbonate and 5 g of picric acid in 1 L of distilled water. The standard cyanide solution was made by dissolving 0.255 g of potassium cyanide (KCN; AR grade, 98%) in 1 L of distilled water after drying at 100 ℃ for 30 minutes, yielding a concentration of 0.1 mg/mL HCN.

Alkaline picrate filter papers (10 × 2 cm) were soaked in the prepared solution and air-dried. For standard curve generation, diluted standard solutions were added to 500 mL flasks along with 1 mL of 3 M HCl and two picrate papers. The flasks were sealed, shaken, and left at room temperature for 24 hours. The picrate strips were then eluted in 10 mL of distilled water, and absorbance was measured at 515 nm to generate the standard curve.

For sample analysis, 1 g of either fresh or dried ground feed was mixed with 25 mL of distilled water in a 500 mL flask. Two alkaline picrate strips were suspended inside the flask, which was sealed and left at room temperature for 18 hours, followed by heating at 60℃ for 30 minutes. The color-changed picrate strips were eluted in 20 mL of distilled water, and the absorbance was recorded at 515 nm. HCN concentration was calculated using the linear regression equation derived from the standard curve. This method is particularly suitable for fresh feed samples.

2.11. Statistical analyses

All data were analyzed using the general linear model (GLM) procedure in a 3 × 3 factorial arrangement under a completely randomized design (CRD), utilizing SAS software (SAS, 2002). The statistical model used was as follows:

$$Yij = \mu + M_i + A_j + (MA)_{ij} + e_{ij,}$$

where:

- Y_{ij} is the observed value of the response variable for the i_{th} additive type and j_{th} fermentation duration,
- μ is the overall mean,
- Mi is the effect of additive type (i = 1, 2, 3),
- Aj is the effect of fermentation time (j = 1, 2, 3),
- $(MA)_{ij}$ is the interaction effect between additive and fermentation duration,
- e_{ij} is the residual error term.

The results are presented as means with standard deviations (mean ± SD). Differences between treatment means were compared using Duncan's multiple range test, with statistical significance declared at $p < 0.05$.

Table 2. Effect of sulfur or CUB supplementation and fermentation time on the chemical composition of FTMR.

Items	Control			Sulfur			CUB			SEM	P-value		
	0 day	7 days	14 days	0 day	7 days	14 days	0 day	7 days	14 days		Additives	TIME	Additives x TIME
Dry Matter (%)	29.83^{b}	28.87^{d}	27.27^{g}	29.25^{c}	28.55^{e}	27.71^{f}	30.49^{a}	29.85^{b}	29.64^{b}	0.05	<0.01	<0.01	<0.01
Organic matter (%DM)	93.65^{b}	93.72^{bc}	93.58^{bc}	93.83^{b}	93.23^{c}	93.2^{c}	93.34^{b}	93.67^{bc}	94.55^{a}	0.2	<0.01	0.21	<0.05
Ash (%DM)	0.06^{e}	0.13^{bc}	0.13^{b}	0.06^{e}	0.14^{a}	0.13^{bc}	0.06^{e}	0.12^{c}	0.11^{d}	0.0012	<0.01	<0.01	<0.01
Crude protein (%DM)	11.50^{e}	13.01^{c}	14.04^{a}	12.04^{d}	13.4^{b}	13.2^{bc}	11.32^{e}	12.00^{d}	12.96^{c}	0.09	<0.01	<0.01	<0.01
Ether Extraction (%DM)	1.31^{f}	2.73^{d}	2.8^{d}	3.17^{c}	4.63^{a}	4.27^{b}	1.34^{f}	2.62^{e}	2.62^{e}	0.04	<0.01	<0.01	<0.01
Neutral detergent fiber (%DM)	46.62	45.66	43.18	41.41	41.24	41.72	47.29	46.21	45.16	0.5	<0.01	<0.01	0.059
Acid detergent fiber (%DM)	29.57^{a}	27.80^{b}	25.73^{c}	28.04^{b}	28.47^{ab}	28.56^{ab}	26.58^{c}	26.52^{c}	26.04^{c}	0.31	<0.01	<0.01	<0.01
Acid detergent lignin (%DM)	15.08^{a}	11.08^{bc}	9.93^{c}	10.25^{c}	7.72^{d}	12.52^{b}	9.42^{c}	10.65^{c}	7.70^{d}	0.4	<0.01	<0.01	<0.01
pH	6.91^{a}	3.83^{d}	3.77^{d}	6.79^{a}	3.84^{d}	3.85^{d}	6.04^{b}	3.81^{d}	4.14^{c}	0.35	<0.01	<0.01	<0.01

Note: Additives = FTMR with sulfur or CUB added, without fermentation, TIME = day of fermentation, CUB = cyanide-utilizing bacteria, DM = dry matter, different superscripts in the same column mean the significant difference at $p < 0.01$.

3. Results

3.1. Chemical composition

According to Table 2, both supplementation and fermentation duration significantly affected the chemical composition of the fermented feed. While all parameters showed significant differences ($p < 0.01$) for the main effects, the interaction effect (supplementation × time) was not significant for NDF ($p = 0.059$). The highest DM content (30.49%) was observed in the treatment supplemented with CUB without fermentation, whereas the lowest value (27.27%) was recorded in the control group fermented for 14 days ($p < 0.01$). The highest OM content (94.55%) was found in the group supplemented with CUB and fermented for 14 days. In contrast, OM values were lowest (93.23% and 93.20%) when sulfur was used as a supplement and the feed was fermented for 7 and 14 days, respectively ($p < 0.05$). The highest CP content (14.04%) was found in the control group after 14 days of fermentation, while the lowest values were in the control at 0 days (11.5%) and the unfermented CUB group (11.32%) ($p < 0.01$). Regarding EE, the highest value (4.63%) was found in the group supplemented with sulfur and fermented for 7 days, while the lowest EE values (1.31% and 1.34%) were observed in the control and CUB groups without fermentation, respectively ($p < 0.01$). The highest values of ADF and ADL were found in the control group without additives or fermentation ($p < 0.01$), indicating a reduction in fiber fractions due to both supplementation and fermentation processes. In terms of rumen fermentation characteristics, the highest pH values were recorded in the control group (6.91) and the group supplemented with sulfur without fermentation (6.79).

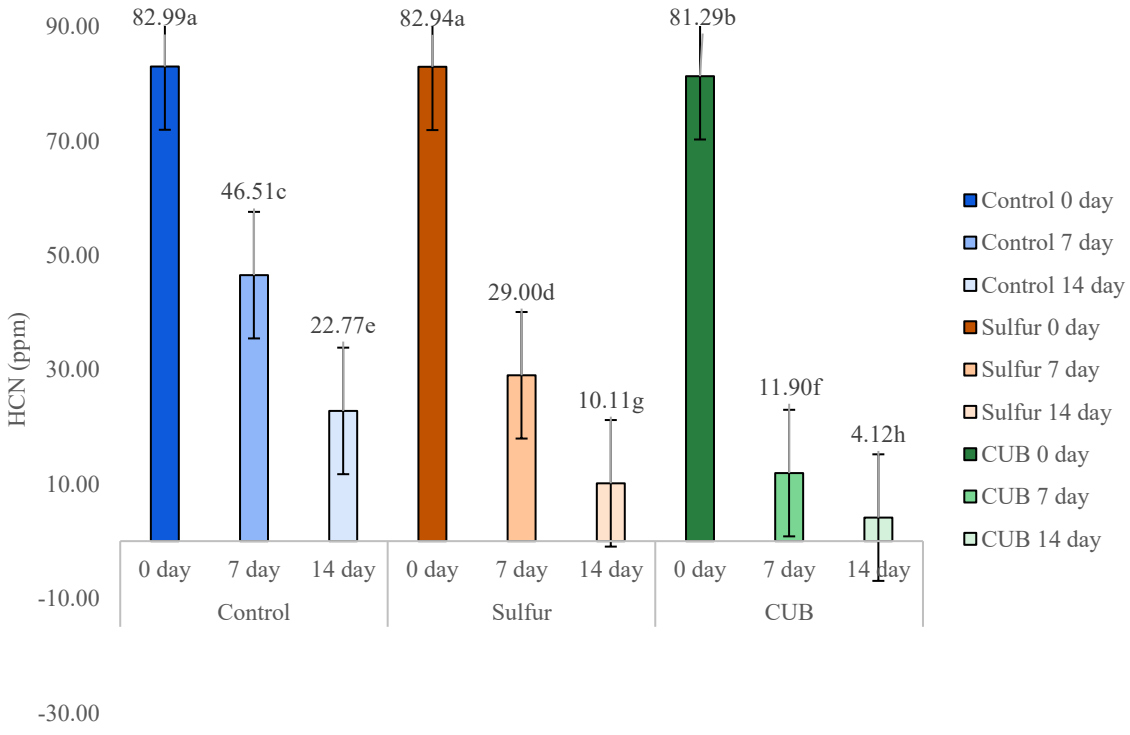

Figure 1. HCN concentration in different treatments over time.

Table 3. Effect of sulfur or CUB supplementation and fermentation time on the kinetics of gas production of fermented total mixed rations FTMR.

Treatment	Fermentation (day)	Gas Kinetics				Cumulative gas 96 h (mL)
		a	b	c	|a| + b	
Control	0	-3.56^{cd}	96.16^{a}	0.030^{bc}	99.05^{a}	90.96^{a}
	7	-2.30^{ab}	78.32^{c}	0.041^{ab}	83.73^{c}	77.30^{b}
	14	-2.93^{bc}	61.30^{d}	0.025^{c}	63.52^{de}	56.46^{cd}
Sulfur	0	-4.14^{de}	84.20^{b}	0.041^{ab}	85.44^{c}	77.15^{b}
	7	-1.67^{a}	54.65^{e}	0.049^{a}	57.37^{e}	52.70^{d}
	14	-2.51^{b}	37.61^{f}	0.037^{abc}	39.80^{f}	34.95^{e}
CUB	0	-4.37^{e}	91.93^{a}	0.035^{bc}	93.31^{ab}	88.10^{a}
	7	-2.77^{bc}	86.43^{b}	0.031^{bc}	89.21^{bc}	77.10^{b}
	14	-2.65^{b}	64.55^{d}	0.041^{ab}	67.38^{d}	59.86^{c}
SEM		0.20	1.46	0.00	3.92	2.25
Main effect						
Additives	Control	-2.93	78.59	0.032	82.10	74.91
	Sulfur	-2.77	58.82	0.042	60.87	54.93
	CUB	-3.26	80.97	0.036	83.30	75.02
TIME	0	-4.02	90.76	0.035	92.60	85.40
	7	-2.53	73.13	0.036	76.77	63.12
	14	-2.42	54.49	0.038	56.90	56.34
Significance of main effects and interaction						
Additives		0.08	< 0.01	< 0.05	< 0.01	< 0.01
TIME		< 0.01	< 0.01	0.75	< 0.01	< 0.01
Additives x TIME		< 0.05	< 0.01	< 0.05	< 0.01	< 0.01

Note: Additives = FTMR with sulfur or CUB added, without fermentation, TIME = day of fermentation, CUB = cyanide-utilizing bacteria, SEM = standard error of the means, a = the gas production from the immediately soluble fraction (mL/g DM), b = the gas production from the in soluble fraction (mL/g DM), c = the gas production rate constant from the insoluble fraction (mL/h), (|a|+b) = the gas potential extent of gas production (mL/g DM), different superscripts in the same column mean the significant difference at $p < 0.01$.

The lowest pH values were detected in the groups without supplementation (3.77), supplemented with sulfur and fermented for 14 days (3.85), and supplemented with CUB and fermented for 7 days (3.81) ($p < 0.01$). At the onset of fermentation (day 0), the HCN concentrations were similar across treatments, with values of 82.99 ppm in the sulfur-supplemented group, 82.94 ppm in the control, and 81.29 ppm in the CUB-supplemented group. After 7 days of fermentation, a substantial reduction in HCN concentration was observed in all treatments, with the CUB group showing the most pronounced decrease to 11.9 ppm, followed by the sulfur group (29.0 ppm) and the control group (46.51 ppm) ($p < 0.01$). By day 14, HCN levels in the CUB group had further declined to 4.12 ppm, representing the lowest concentration among treatments ($p < 0.01$). In comparison, the sulfur and control groups recorded concentrations of 10.11 ppm and 22.77 ppm, respectively. These results demonstrate the superior cyanide-degrading capability of CUB during fermentation compared with sulfur treatment

and the untreated control.

3.2. In vitro kinetics of gas production

The interaction between additive supplementation and fermentation time significantly influenced the kinetics of gas production and the cumulative gas volume, as shown in Table 3. The volume of gas produced from the immediately soluble fraction (gas a) was highest (–1.67 mL) when sulfur was supplemented and the feed was fermented for 7 days.

Conversely, the lowest gas a (–4.37 mL) was recorded in the treatment supplemented with CUB without fermentation ($p < 0.05$). For the insoluble fraction (b), the highest gas production was observed in control and CUB-supplemented groups without fermentation, with values of 96.16 mL and 96.93 mL, respectively ($p < 0.01$). The gas production rate constant (c), ranging from 0.025 to 0.049 mL/h, was highest (0.049 mL/h) in the sulfur-supplemented group fermented for 7 days, while the lowest value (0.025 mL/h) was found in the control group fermented for 14 days ($p < 0.05$). The potential gas production, calculated as the sum of the absolute value of a and b ($|a| + b$), was highest (99.05 mL) in the control group without supplementation or fermentation. In contrast, the lowest value (39.80 mL) was obtained when the sulfur-supplemented group fermented for 14 days ($p < 0.01$). Cumulative gas production after 96 hours of incubation was also significantly affected. The highest gas volumes were recorded in control and CUB-supplemented groups without fermentation, yielding 90.96 mL and 88.10 mL, respectively. The lowest cumulative gas production (34.95 mL) was observed in the sulfur-supplemented group fermented for 14 days ($p < 0.01$).

3.3. Alterations in vitro feed degradability and rumen fermentation

3.3.1. *In vitro* degradability

As shown in Table 4, no significant interaction effects were observed on IVDMD or IVOMD at 24 or 48 hours of incubation. However, at 48 hours, IVDMD was significantly higher ($p < 0.01$) in the control (60.57%) and the CUB-supplemented group (61.07%), compared to the sulfur-supplemented group (57.93%). Fermentation time had a significant effect on both IVDMD and IVOMD, except for IVOMD at 48 hours. The highest IVDMD values at 24 and 48 hours (53.82% and 62.39%, respectively) were observed in the unfermented group. Conversely, fermentation for 7 and 14 days reduced IVDMD values to 51.89% and 59.03%, and 51.00% and 58.15%, respectively ($p < 0.01$). The highest average IVDMD was recorded in the unfermented group (58.11%), whereas the 7- and 14-day fermented groups showed lower averages (55.46% and 54.57%, respectively; $p < 0.01$). For IVOMD, the highest value at 24 hours (89.17%) occurred in the 14-day fermented group, while the lowest values were found in the 0 and 7-day fermentation groups (87.10% and 87.98%, respectively; $P<0.01$). The average IVOMD was highest in the 14-day fermentation group (87.92%) and lowest in the 0-day group (86.90%). The 7-day group (87.56%) did not differ significantly from either the 0-day or the 14-day group ($p < 0.05$).

Table 4. Effect of sulfur or cyanide-utilizing bacteria (CUB) supplementation and fermentation time on the *In vitro* degradability of fermented total mixed rations (FTMR).

Treatment	Fermentation (day)	IVDMD, %DM			IVOMD, %DM		
		24 h	48 h	Mean	24 h	48 h	Mean
Control	0	53.78	62.93	58.36	87.40	86.48	87.23
	7	53.64	60.43	56.80	87.38	87.96	87.67
	14	52.52	58.37	55.45	90.03	87.27	89.05
Sulfur	0	54.12	61.75	57.94	86.85	86.44	86.64
	7	50.40	57.13	54.31	88.22	87.17	87.69
	14	49.18	54.92	53.15	89.77	85.64	87.70
CUB	0	53.57	62.51	57.05	87.05	87.22	87.13
	7	51.65	59.53	55.59	88.34	86.29	87.32
	14	51.30	61.18	56.24	87.72	87.12	87.42
SEM		1.53	1.70	0.69	0.54	0.52	0.42
Main effect							
Additives	Control	53.31	60.57^{a}	56.95	88.27	87.23	87.75
	Sulfur	51.23	57.93^{b}	54.58	88.28	86.41	87.35
	CUB	52.17	61.07^{a}	56.62	87.70	86.87	87.29
TIME	0	53.82^{a}	62.39^{a}	58.11^{a}	87.10^{b}	86.71	86.90^{b}
	7	51.89^{b}	59.03^{b}	55.46^{b}	87.98^{b}	87.14	87.56^{ab}
	14	51.00^{b}	58.15^{b}	54.57^{b}	89.17^{a}	86.68	87.92^{a}
Significance of main effects and interaction							
Additives		0.06	<0.01	0.13	0.34	0.18	0.19
TIME		<0.01	<0.01	<0.01	<0.01	0.49	<0.05
Additives x TIME		0.28	0.09	0.24	0.059	0.11	0.40

Note: Additives = FTMR with sulfur or CUB added, without fermentation, TIME = day of fermentation, CUB = cyanide-utilizing bacteria, DM = dry matter, SEM = standard error of the means, IVDMD = in vitro dry matter degradability, IVOMD = In vitro organic matter degradability, different superscripts in the same column mean the significant difference at $p < 0.01$.

3.3.2. Rumen pH and Ammonia-Nitrogen (NH_3-N)

Supplementation and fermentation significantly affected ruminal pH at 48 hours in Table 5. The lowest pH (6.64) was found in the CUB-supplemented group without fer-mentation, while the highest pH values were recorded in the control group fermented for 14 days (6.77), the sulfur-supplemented groups fermented for 7 and 14 days (6.78 and 6.81), and the CUB-supplemented groups fermented for 7 and 14 days (6.77 and 6.80, respectively) ($p < 0.05$).

NH_3-N concentrations at 24 hours were significantly higher in the sulfur-supplemented group without fermentation (16.65 mg/dL), while the lowest value (11.85 mg/dL) was recorded in the CUB group fermented for 14 days ($P < 0.01$). At 48 hours, NH_3-N was highest in the CUB group fermented for 14 days (22.43 mg/dL), and lowest in control (15.05 mg/dL) and sulfur-supplemented groups without fermentation (15.11 mg/dL) ($p < 0.01$). Sulfur additives resulted in the highest pH (6.82), while CUB shows the lowest (6.70). The control group had an intermediate pH value (6.80), which was not

significantly different from either of the other groups ($p < 0.05$). Regarding fermentation time, the 7 and 14 days fermented groups had the highest pH (6.81), whereas the lowest pH (6.77) was observed in the 0-day group ($p < 0.01$).

Table 5. Effect of sulfur or CUB supplementation and fermentation time on the ruminal fermentation of FTMR.

Treatment	Fermentation (day)	Ruminal pH		Ammonia nitrogen, mg/dL	
		24 h	48 h	24 h	48 h
Control	0	6.79	6.65^{bc}	15.88^{ab}	15.05^{e}
	7	6.81	6.68^{b}	14.49^{cde}	17.70^{c}
	14	6.81	6.77^{a}	14.93^{bcd}	16.68^{d}
Sulfur	0	6.79	6.69^{b}	16.65^{a}	15.11^{e}
	7	6.84	6.78^{a}	13.46^{f}	17.85^{c}
	14	6.84	6.81^{a}	14.32^{def}	19.50^{b}
CUB	0	6.75	6.64^{c}	15.41^{bc}	16.66^{d}
	7	6.80	6.77^{a}	13.77^{ef}	17.93^{c}
	14	6.80	6.80^{a}	11.85^{g}	22.43^{a}
SEM		0.01	0.01	0.25	0.24
Additives	Control	6.80^{ab}	6.70	15.10	16.48
	Sulfur	6.82^{a}	6.76	14.81	17.49
	CUB	6.70^{b}	6.74	13.68	19.01
TIME	0	6.77^{b}	6.66	15.98	15.61
	7	6.81^{a}	6.74	13.91	17.83
	14	6.81^{a}	6.79	13.70	19.54
Additives		<0.05	<0.01	<0.01	<0.01
TIME		<0.01	<0.01	<0.01	<0.01
Additives x TIME		0.61	<0.05	<0.01	<0.01

Additives = FTMR with sulfur or CUB added, without fermentation, TIME = day of fermentation, CUB = cyanide-utilizing bacteria, SEM = standard error of the means, different superscripts in the same column mean the significant difference at $p < 0.01$.

3.3.3. Total and individual VFA concentrations

In Table 6, neither additives nor fermentation time significantly affected TVFA concentrations at 24 or 48 h ($p > 0.05$), and no interaction effects were observed. For acetate (C2), no significant interaction effects between additives and fermentation time were found at either incubation period. However, fermentation time significantly influenced C2 concentration at 24 h ($p < 0.01$), with the highest value observed in the unfermented group (65.38 mmol/L; 64.10%), followed by lower values in the 7-day (60.53 mmol/L; 59.75%) and 14-day groups (60.56 mmol/L; 60.56%). No significant effects of fermentation time on C2 were observed at 48 h ($p > 0.05$). For propionate (C3), no significant interaction effects were detected at either incubation time. At 24 h, fermentation time significantly affected C3 ($p < 0.05$), with the highest value in the 7-day group (27.96%) and the lowest in the

unfermented group (26.63%). At 48 h, no significant differences were observed among fermentation times ($p > 0.05$). For butyrate (C4), a significant interaction between additives and fermentation time was found at 48 h ($p < 0.05$). The highest C4 concentration (12.6 mmol/L; 10.82%) was observed in the CUB group, fermented for 7 days, while the lowest values occurred in the control (9.12 mmol/L; 8.10%) and sulfur-supplemented group (8.15 mmol/L; 7.64%) without fermentation. At 24 h, fermentation time significantly influenced C4 ($p < 0.01$), with the highest values in the 7-day (12.28%) and 14-day (11.64%) groups, and the lowest in the unfermented group (9.29%).

Table 6. Effect of sulfur or CUB supplementation and fermentation time on volatile fatty acid profile of FTMR.

Treatment	Fermentation (day)	Total VFAs (mmol/L)		Acetate		Propionate		Butyrate	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control	0	94.50	132.54	65.38	65.98	25.63	26.01	9.12	8.15^{e}
	7	90.16	120.47	60.31	64.88	27.81	27.45	11.88	9.37^{cd}
	14	89.47	126.07	60.91	63.17	27.25	26.26	11.84	10.56^{ab}
Sulfur	0	87.36	124.19	63.84	64.96	27.17	27.39	8.98	7.64^{e}
	7	85.79	122.28	59.4	62.89	28.01	27.36	12.6	9.74^{bc}
	14	83.21	120.69	60.62	62.74	27.22	28.36	11.32	10.57^{ab}
CUB	0	88.60	131.15	63.09	63.65	27.11	27.5	9.79	8.84^{d}
	7	90.92	121.64	59.54	62.54	28.09	26.63	12.36	10.82^{a}
	14	87.66	127.82	60.16	63.7	28.05	25.77	11.78	10.52^{ab}
SEM		3.15	3.63	0.59	1.12	0.50	0.57	0.38	0.26
Main effect									
Additives	control	91.38	126.36	62.20	64.68	26.90	26.57	10.94	9.34
	Sulfur	85.45	122.39	61.29	63.53	27.46	27.70	10.97	9.32
	CUB	89.06	126.87	60.93	63.30	27.75	26.63	11.31	10.06
TIME	0	90.15	129.29	64.10^{a}	64.86	26.63^{b}	26.97	9.29^{b}	8.19
	7	88.95	121.46	59.75^{b}	63.44	27.96^{a}	27.15	12.28^{a}	9.98
	14	86.78	124.86	60.56^{b}	63.20	27.50^{ab}	26.80	11.64^{a}	10.55
Significance of main effects and interaction									
Additives		0.15	0.42	0.06	0.29	0.09	0.06	0.16	< 0.01
TIME		0.47	0.06	< 0.01	0.17	< 0.05	0.78	< 0.01	< 0.01
Additives x TIME		0.9	0.74	0.65	0.65	0.52	0.13	0.56	< 0.05

Note: Additives = FTMR with sulfur or CUB added, without fermentation, TIME = day of fermentation, CUB = cyanide-utilizing bacteria, VFAs = volatile fatty acids, different superscripts in the same column mean the significant difference at $p < 0.01$.

4. Discussion

Overall, the fermented total mixed ration maintained chemical composition values well within the typical nutritional ranges for ruminants. DM levels (27%–30%) align with ideal silage moisture content (30%–40%), supporting good preservation and intake [29]. OM remained high (~93%), indicating minimal ash and high digestible nutrient content. NDF ranged from 41%–47% DM, within the recommended intake-supporting range of 40–60% DM, and ADF values (25%–29% DM) were

below the threshold of 35% DM for adequate digestibility [30]. These compositional profiles confirm that, regardless of additives or fermentation time, the feed remains nutritionally balanced, ensuring sufficient fiber for rumination, digestible energy, and overall dietary suitability for ruminant animals.

The increase in CP content with extended fermentation can be attributed to microbial proliferation, particularly lactic acid bacteria and other fermentative microbes, which contribute microbial protein to the feed [31]. Fermentation also increases non-protein nitrogen (NPN) compounds, such as ammonia, which ruminants can use for microbial protein synthesis. Additionally, the acidic environment formed during fermentation inhibits proteolysis by undesirable microbes and enzymes, helping preserve protein. These mechanisms are consistent with findings by Su et al. [32], who reported CP increases of 14.28% and 25.53% when fermenting with S. cerevisiae and *Lactobacillus plantarum*, respectively.

The reduction in pH with longer fermentation durations is attributed to the microbial degradation of substrates, which releases organic acids and other acidic metabolites. The lowest pH values (3.77, 3.85, and 3.81) were recorded in the control 14-day group, sulfur-supplemented 14-day group, and CUB-supplemented 7-day group, respectively ($p < 0.01$). However, a prolonged drop in pH may inhibit cellulolytic and methanogenic microbial populations in the rumen, potentially contributing to reduced fiber digestion and lower gas production observed in extended fermentation treatments [33–35]. Microorganisms such as *Lactobacillus* and S. *cerevisiae* convert sugars to organic acids, ethanol, and CO_2 during fermentation. The latter dissolves into carbonic acid (H_2CO_3), further reducing pH. The chemical equilibrium involving increased hydrogen ion (H+) concentration also contributes to this reduction. These results corroborate findings by Yang et al. [36] and Song et al. [37], who reported lower pH in FTMR compared to unfermented TMR.

Initial HCN concentrations (~82–83 ppm DM) in the experimental feed were already below the commonly accepted acute toxicity threshold for ruminants (> 200 ppm fresh weight or > 600 ppm DM) [13]. Remarkably, after 14 days of fermentation, HCN levels dropped to as low as 4.12 ppm DM in the CUB-supplemented group, placing it well within the 'very low risk' category (< 200 ppm DM). This reduction can be attributed to fermentation processes that facilitate the enzymatic hydrolysis and volatilization of cyanogenic compounds from plant cells under acidic conditions [38]. The greater reduction observed in the CUB-supplemented group further suggests that microbial biodegradation contributed to enhanced detoxification efficiency. These findings align with previous studies, such as Chanangam [39], who reported a 31.11%–85.44% reduction in HCN content in cassava leaves fermented for 21 days, and Lambri et al. [12], who demonstrated that fermentation with Saccharomyces cerevisiae for 48 hours reduced HCN by up to 65.9%, compared to only a 33% reduction using a traditional 24-hour fermentation process.

Gas production kinetics and cumulative gas volume were highest in the control group at 0 day, likely due to cassava's high starch content providing abundant fermentable substrates. In contrast, the sulfur-supplemented group showed a steep decline in gas output, indicating an inhibitory impact on fermentation. Gas a was significantly reduced at day 0 (–4.14), suggesting immediate suppression of microbial activity. This aligns with Wu et al. [18], who reported that sulfate addition fosters sulfate-reducing bacteria (SRB) such as *Desulfovibrio* spp., redirecting hydrogen away from methanogens and reducing methane and overall gas production [40]. After 14 days of fermentation, the sulfur group exhibited only gas $b \approx 37.6$ mL and cumulative gas at 96 h of 34.95 mL, supporting the hypothesis that sulfur enriches sulfate-reducing bacterial (SRB) populations. These bacteria utilize hydrogen to reduce sulfate into hydrogen sulfide (H_2S), which at elevated concentrations is known to inhibit fibrolytic microbes and suppress fiber fermentation, leading to reduced gas production. Although Uniyal et

al. [41] reported that selected SRB isolates from goat rumen improved fiber digestibility, such effects may depend on the specific SRB strain and fermentation environment. In the present study, prolonged sulfur supplementation likely led to excessive H_2S accumulation, negatively affecting fiber-degrading microbial populations and overall fermentative capacity [42]. While reduced total gas production may compromise feed energy availability, it may concurrently lower methane emissions, a positive environmental outcome. As reviewed by Zhao and Zhao [43], enhancing SRB activity through sulfur supplementation can competitively inhibit methanogenesis by siphoning hydrogen away from methanogen-producing archaea [43]. Thus, there exists a clear trade-off: sulfur supplementation, especially with prolonged fermentation, effectively reduces HCN and methane but may also suppress fiber fermentation. Optimizing sulfur levels and fermentation time is therefore essential to balance cyanide detoxification, fermentative efficiency, and environmental sustainability in ruminant diets. The CUB-supplemented group demonstrated robust fermentation performance. At 0 days, it achieved high fermentation potential ($b \approx 91.9$ mL) and cumulative gas production (88.1 mL), comparable to the control, indicating that adding CUB does not hinder initial carbohydrate fermentation [44]. Moreover, CUB enhanced IVDMD by approximately 11% compared to the non-CUB group, suggesting improved feed utilization efficiency [45]. With continued fermentation (14 days), the CUB group maintained better fermentation kinetics and digestibility than the sulfur-treated group, despite a moderate decline in gas production ($b \approx 64.6$ mL; cumulative ≈ 59.9 mL). This decline likely reflects depletion of readily fermentable substrates during ensiling [46]. Extended fermentation durations of 7 and 14 days were associated with reduced degradability, contrary to expectations. Although FTMR generally improves microbial efficiency, prolonged fermentation may shift microbial populations unfavorably, reducing degradability [47].

Although the differences in rumen pH among treatments were statistically significant, the values remained safely within 5.5–7.5, indicating that supplementation had minimal impact on rumen acid-base balance [48]. Notably, the sulfur-treated group showed the lowest NH_3-N levels at both 24 and 48 hours, suggesting potential inhibition of proteolytic or deaminating microbial populations, as excess sulfur or hydrogen sulfide can suppress these microbes and reduce ammonia production [49]. In contrast, the highest NH_3-N was observed in the CUB-14-day group, reflecting active protein or non-protein nitrogen breakdown that corresponds with its enhanced gas kinetics and digestibility.

The study revealed an NH_3-N response in the CUB-14 treatment, with levels rising from moderate at 24 h to a peak of 22.43 mg/dL at 48 h, well within the optimal rumen range (13–50 mg/dL) required for maximal microbial protein synthesis [50]. This pattern reflects an initial phase of microbial uptake followed by intensified proteolysis and deamination, potentially driven by microbial turnover after prolonged fermentation. Importantly, all NH_3-N values ranged between 11.85 and 22.43 mg/dL, falling within physiological levels that support microbial growth without causing toxicity [51]. The elevated ammonia in the CUB-14 group aligns with its higher gas production and digestibility, suggesting that CUB enhances nitrogen turnover and feed degradation efficiency. Meanwhile, stable total VFA across treatments implies that these nitrogen fluctuations did not compromise overall ruminal fermentative energy production. The pronounced increase in NH_3-N at 48 h is also attributed to ongoing protein degradation and microbial metabolic activity under in vitro conditions, where the absence of absorptive mechanisms leads to ammonia accumulation over time, consistent with observations by Suntara et al. [52], who reported similar NH_3-N elevations on in vitro rumen fermentation system.

In our study, fermentation time significantly influenced VFA profiles. As fermentation increased from 0 to 14 days, acetate (C2) declined (64.1% → ~60.6%), while propionate (C3) increased (~26.6%

→ ~27.5%–28.0%), hinting at a metabolic shift toward gluconeogenic VFAs. This shift may be attributed to lactic acid accumulation during ensiling, which serves as a substrate for propionate-producing bacteria (via the acrylate pathway), redirecting fermentation from acetate toward propionate formation [53]. Additionally, butyrate (C4) rose from 9.29% to ~12%, suggesting enhanced activity of butyrate-producing bacteria that thrive on intermediate substrates during extended fermentation. Mechanistically, prolonged ensiling alters the rumen inoculum's substrate composition and redox balance, favoring microbes that convert lactate and succinate into propionate and butyrate. Such changes are well-documented in rumen fermentation studies, where lactate-derived pathways contribute to shifts in VFA proportions [54]. In conclusion, extended fermentation increases the proportion of propionate and butyrate at the expense of acetate, possibly driven by lactic acid metabolism and the selective growth of lactate-utilizing bacteria. This shift may enhance feed energy utilization and support improved rumen fermentation profiles, particularly in silage-based feeding systems.

5. Conclusions

This study demonstrated that both additive supplementation (CUB or sulfur) and fermentation time significantly influenced the quality and fermentation characteristics of FTMR. CUB supplementation was particularly effective in enhancing digestibility, nitrogen turnover, and fermentation efficiency while reducing HCN, making it a promising additive for safe and sustainable ruminant feeding. Future research should explore CUB's effects under farm-scale conditions, its interaction with different feed ingredients, and its long-term impact on animal performance, health, and environmental outcomes.

Author contributions

Chanon Suntara: Planning, conceptualization, study design, and funding acquisition, Literature review and protocol development, Statistical analysis and visualization, Data interpretation, Manuscript drafting, Manuscript review, editing, and final approval; Anusorn Cherdthong: Planning, conceptualization, study design, and funding acquisition, Literature review and protocol development, Manuscript review, editing, and final approval; Kannika Saisombut: Literature review and protocol development, Sample preparation, data collection, and laboratory work, Statistical analysis and visualization, Data interpretation, Manuscript drafting, Manuscript review, editing, and final approval; Suphakon Pramotchit: Literature review and protocol development, Sample preparation, data collection, and laboratory work, Statistical analysis and visualization, Data interpretation, Manuscript drafting, Manuscript review, editing, and final approval; Molthida Rungchaicharoenphai: Sample preparation, data collection, and laboratory work, Statistical analysis and visualization, Data interpretation, Manuscript review, editing, and final approval. All authors have read and agreed to the published version of the manuscript.

Use of AI tools declaration

In the preparation of this article, the authors utilized Artificial Intelligence (AI) tools to enhance the quality of the language and writing. The principal ideas and analysis presented in this work are

entirely those of the authors.

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Conflict of interest

The authors declare no conflicts of interest.

References

1. Somendrika D, Wickramasinghe I, Wansapala MAJ, et al. (2017) Nutritional composition of cassava cultivar "CARI-555". *Pak J Nutr* 16: 216–220. https://doi.org/10.3923/pjn.2017.216.220
2. Okechukwu RU, Dixon AGO (2008) Genetic gains from 30 years of cassava breeding in Nigeria for storage root yield and disease resistance in elite cassava genotypes. *J Crop Improv* 22: 181–208. https://doi.org/10.1080/15427520802212506
3. Bolarinwa I, Olaniyan SA, Olatunde S, et al. (2016) Effect of processing on amygdalin and cyanide contents of some Nigerian foods. *J Chem Pharm Res* 8: 106–113.
4. Burns AE, Bradbury JH, Cavagnaro TR, et al. (2012) Total cyanide content of cassava food products in Australia. *J Food Compos Anal* 25: 79–82. https://doi.org/10.1016/j.jfca.2011.06.005
5. Ameny MA (1990) Traditional post-harvest technology of cassava in Uganda. *Trop Sci* 17: 41–50.
6. Yooyen A (2019) Cyanide poisoning from cassava leaves in livestock. *NIAH Newslett* 18: 1–4.
7. Ospina MA, Tran T, Pizarro M, et al. (2024) Content and distribution of cyanogenic compounds in cassava roots and leaves in association with physiological age. *J Sci Food Agric* 104: 4851–4859. https://doi.org/10.1002/jsfa.13123
8. Uchechukwu-Agua AD, Caleb OJ, Opara UL (2015) Postharvest handling and storage of fresh cassava root and products: A review. *Food Bioprocess Technol* 8: 729–748. https://doi.org/10.1007/s11947-015-1478-z
9. Watson AG, MA S, TB N, et al. (2024) A simple solar crop drying and pasteurizing system appropriate for smallholder and subsistence farmers in tropical and subtropical regions. *Drying Technol* 42: 407–422. https://doi.org/10.1080/07373937.2023.2182316
10. Li M, Zhang L, Zhang Q, et al. (2020) Impacts of citric acid and malic acid on fermentation quality and bacterial community of cassava foliage silage. *Front Microbiol* 11: 595622. https://doi.org/10.3389/fmicb.2020.595622

11. Pongsub S, Suntara C, Khota W, et al. (2022) The chemical composition, fermentation end-product of silage, and aerobic stability of cassava pulp fermented with *Lactobacillus casei* TH14 and additives. *Vet Sci* 9: 617. https://doi.org/10.3390/vetsci9110617
12. Lambri M, Fumi MD, Roda A, et al. (2013) Improved processing methods to reduce the total cyanide content of cassava roots from Burundi. *Afr J Biotechnol* 12: 2685–2691. https://doi.org/10.5897/AJB2012.2899
13. Khota W, Kaewpila C, Kimprasit T, et al. (2023) The isolation of rumen *Enterococci* strains along with high potential utilizing cyanide. *Sci Rep* 13: 13176. https://doi.org/10.1038/s41598-023-40488-9
14. Sriwicha P, Piboonkunsamlit S, Phowang N, et al. (2024) The influence of two cyanide-utilizing bacteria species on kinetics of gas production and rumen digestibility using *in vitro* gas techniques. *Khon Kaen Agric J* 2: 243–244.
15. Lukbun S, Suntara C, Prachumchai R, et al. (2024) Exploring the *in vitro* effects of cassava diets and *Enterococcus* strains on rumen fermentation, gas production, and cyanide concentrations. *Animals* 14: 3269. https://doi.org/10.3390/ani14223269
16. Supapong C, Sommai S, Khonkhaeng B, et al. (2022) Effect of rhodanese enzyme addition on rumen fermentation, cyanide concentration, and feed utilization in beef cattle receiving various levels of fresh cassava root. *Fermentation* 8: 146. https://doi.org/10.3390/fermentation8040146
17. Sumadong P, Cherdthong A, So S, et al. (2021) Sulfur, fresh cassava root, and urea independently enhanced gas production, ruminal characteristics, and *in vitro* degradability. *BMC Vet Res* 17: 304. https://doi.org/10.1186/s12917-021-02999-3
18. Wu H, Li Y, Meng Q, et al. (2021) Effect of high sulfur diet on rumen fermentation, microflora, and epithelial barrier function in steers. *Animals* 11: 2545. https://doi.org/10.3390/ani11092545
19. Supapong C, Cherdthong A, Wanapat M, et al. (2019) Effects of sulfur levels in fermented total mixed ration containing fresh cassava root on feed utilization, rumen characteristics, microbial protein synthesis, and blood metabolites in Thai native beef cattle. *Animals* 9: 261. https://doi.org/10.3390/ani9050261
20. Bureenok S, Yuangklang C, Vasupen K, et al. (2012) The effects of additives in Napier grass silages on chemical composition, feed intake, nutrient digestibility, and rumen fermentation. *Asian-Australas J Anim Sci* 25: 1248–1254. https://doi.org/10.5713/ajas.2012.12081.
21. The Working Committee of Thai Feeding Standard for Ruminant (WTRS) (2010) *Nutrient requirements of beef cattle in Indochinese Peninsula*, 1st Ed., Khon Kaen: Klungnanavithaya Press.
22. AOAC (1990) *Official methods of analysis of the Association of the official Analytical Chemists*, Washington: Association of official Analytical Chemists. Available from: https://law.resource.org/pub/us/cfr/ibr/002/aoac.methods.1.1990.pdf.
23. Van Soest PJ, Robertson JB, Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *J Dairy Sci* 74: 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2
24. Sommart K, Parker DS, Rowlinson P, et al. (2000) Fermentation characteristics and microbial protein synthesis in an *in vitro* system using cassava, rice straw, and dried ruzi grass as substrates. *Asian-Australas J Anim Sci* 13: 1084–1093. https://doi.org/10.5713/ajas.2000.1084
25. Ørskov ER, McDonald I (1979) The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *J Agric Sci* 92: 499–503. https://doi.org/10.1017/S0021859600063048

26. Fawcett JK, Scott JE (1960) A rapid and precise method for the determination of urea. *J Clin Pathol* 13: 156–159. https://doi.org/10.1136/jcp.13.2.156
27. Samuel M, Sagathewan S, Thomas J, et al. (1997) An HPLC method for estimation of volatile fatty acids of ruminal fluid. *Indian J Anim Sci* 67: 805–807.
28. Sommart K, Jaikhan W, Wongtungthinthan S, et al. (2022) *Applied Animal Nutrition Laboratory: Laboratory manual*. Khon Kaen: Faculty of Agriculture, Khon Kaen University.
29. Yitbarek M, Tamir B (2013) Silage additives: Review. *Open J Appl Sci* 4. https://doi.org/10.4236/ojapps.2014.45026
30. Shi R, Dong S, Mao J, et al. (2023) Dietary neutral detergent fiber levels impacting dairy cows' feeding behavior, rumen fermentation, and production performance during peak-lactation. *Animals* 13: 2876. https://doi.org/10.3390/ani13182876
31. Yan Y, Zhao M, Sun P, et al. (2025) Effects of different additives on fermentation characteristics, nutrient composition, and microbial communities of *Leymus chinensis* silage. *BMC Microbiol* 25: 296. https://doi.org/10.1186/s12866-025-04023-2
32. Su W, Wang C, Hao L, et al. (2020) Solid-state fermentation of corn by-products mixture feed with *Saccharomyces cerevisiae, Lactobacillus plantarum,* and protease for improving protein quality and nutrient utilization. *Res Square.* https://doi.org/10.21203/rs.3.rs-21430/v1
33. Cerrato-Sánchez M, Calsamiglia S, Ferret A (2008) Effect of the magnitude of the decrease of rumen pH on rumen fermentation in a dual-flow continuous culture system. *J Anim Sci* 86: 378–383. https://doi.org/10.2527/jas.2007-0180
34. Terry RA, Tilley JMA, Outen GE (1969) Effect of pH on cellulose digestion under *in vitro* conditions. *J Sci Food Agric* 20: 317–320. https://doi.org/10.1002/jsfa.2740200514
35. Slyter LL (1986) Ability of pH-selected mixed ruminal microbial populations to digest fiber at various pHs. *Appl Environ Microbiol* 52: 390–391. https://doi.org/10.1128/aem.52.2.390-391.1986
36. Cao Y, Takahashi T, Horiguchi K-i, et al. (2010) Methane emissions from sheep fed fermented or non-fermented total mixed ration containing whole-crop rice and rice bran. *Anim Feed Sci Technol* 157: 72–78. https://doi.org/10.1016/j.anifeedsci.2010.02.004
37. Song J, Ma Y, Zhang H, et al. (2023) Fermented total mixed ration alters rumen fermentation parameters and microbiota in dairy cows. *Animals* 13: 1062. https://doi.org/10.3390/ani13061062
38. Jadav C, Makwana R, Parikh SS, et al. (2019) Hydrocyanic acid (HCN) estimation during different stages of growth in Gundrijowar (*Sorghum vulgare*) fodder crop. *Int J Curr Microbiol Appl Sci* 8: 1328–1333. https://doi.org/10.20546/ijcmas.2019.809.152
39. Chanangam C, and Narongwanichgarn, W. (2021) Toxicity of cyanide in cassava leaves for beef cattle health in Na Noi district, Nan province. Nan Province, Thailand: Nanoi District Livestock Office and National Institute of Animal Health, Department of Livestock (In Thai). Available from: https://pvlo-nan.dld.go.th/webnew/images/stories/doc_download/wijai/wijai2564.pdf.
40. Uniyal S, Chaudary L, Kala A, et al. (2020) Effect of sulphur supplementation on methane emission, energy partitioning and nutrient utilization in goats. *Anim Nutr Feed Technol* 20: 111. https://doi.org/10.5958/0974-181X.2020.00011.6
41. Uniyal S, Chaudhary LC, Kala A, et al. (2022) Effect of supplementing sulphate-reducing bacteria along with sulphur on growth performance, nutrient utilization and methane emission in goats. *Trop Anim Health Prod* 55: 3. https://doi.org/10.1007/s11250-022-03419-w

42. Uniyal S, Chaudary L, Kala A, et al. (2022) Isolation and characterization of sulphate reducing bacteria from goat rumen and its inclusion to improve *in vitro* feed fermentation. *Indian J Anim Sci* 92: 96–100. https://doi.org/10.56093/ijans.v92i1.120932
43. Zhao Y, Zhao G (2022) Decreasing ruminal methane production through enhancing the sulfate reduction pathway. *Anim Nutr* 9: 320–326. https://doi.org/10.1016/j.aninu.2022.01.006
44. Wang L (2025) Advances in nutritional manipulation of rumen fermentation. *Animals* 15: 833. https://doi.org/10.3390/ani15060833
45. Sombuddee N, Prachumchai R, Khota W, et al. (2023) Effect of cyanide-utilizing bacteria and sulfur on feed utilization, microbiomes, and cyanide degradation in cattle supplemented with fresh cassava root. *Sci Rep* 13: 18689. https://doi.org/10.1038/s41598-023-45993-5
46. Prachumchai R, Cherdthong A, Wanapat M (2021) Screening of cyanide-utilizing bacteria from rumen and *in vitro* evaluation of fresh cassava root utilization with pellet containing high sulfur diet. *Vet Sci* 8: 10. https://doi.org/10.3390/vetsci8010010
47. Paul O, Urmi S, Biswas A (2023) Effect of TMR and fermented TMR on ruminal *in vitro* digestion and gas production. *Adv Anim Vet Sci* 11: 586–594. https://doi.org/10.17582/journal.aavs/2023/11.4.586.594
48. Liu S, Xie B, Ji H, et al. (2024) Effects of dietary supplementation with alkaline mineral complex on *in vitro* ruminal fermentation and bacterial composition. *Front Vet Sci* 11. https://doi.org/10.3389/fvets.2024.1357738
49. Firkins JL, Yu Z, Morrison M (2007) Ruminal nitrogen metabolism: Perspectives for integration of microbiology and nutrition for dairy. *J Dairy Sci* 90: E1–E16. https://doi.org/10.3168/jds.2006-518
50. Niazifar M, Besharati M, Jabbar M, et al. (2024) Slow-release non-protein nitrogen sources in animal nutrition: A review. *Heliyon* 10: e33752. https://doi.org/10.1016/j.heliyon.2024.e33752
51. Martinez-Fernandez G, Jiao J, Padmanabha J, et al. (2020) Seasonal and nutrient supplement responses in rumen microbiota structure and metabolites of tropical rangeland cattle. *Microorganisms* 8: 1550. https://doi.org/10.3390/microorganisms8101550
52. Suntara C, Sombuddee N, Lukbun S, et al. (2023) *In vitro* evaluation of winged bean (*Psophocarpus tetragonolobus*) tubers as an alternative feed for ruminants. *Animals* 13: 677. https://doi.org/10.3390/ani13040677
53. Wang L, Zhang G, Li Y, et al. (2020) Effects of high forage/concentrate diet on volatile fatty acid production and the microorganisms involved in VFA production in cow rumen. *Animals* 10: https://doi.org/10.3390/ani10020223
54. Li MM, Ghimire S, Wenner BA, et al. (2022) Effects of acetate, propionate, and pH on volatile fatty acid thermodynamics in continuous cultures of ruminal contents. *J Dairy Sci* 105: 8879–8897. https://doi.org/10.3168/jds.2022-22084