Effects of sulfur and cyanide-utilizing bacteria in fermented total mixed ration containing fresh cassava root on rumen fermentation in Thai beef cattle

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ABSTRACT

This study investigated the effects of sulfur supplementation and cyanide-utilizing ruminal bacteria (CUB) on nutrient digestibility, rumen fermentation, and blood urea-nitrogen (BUN) in Thai native beef cattle fed fermented total mixed rations (FTMR) containing fresh cassava root. Two CUB strains, *Enterococcus gallinarum* KKU-BC10 and *Enterococcus faecium* KKU-BF7, were isolated from the rumen based on their hydrogen cyanide (HCN) degrading capacity. Four dietary treatments were prepared by formulating FTMR with 40% rice straw and 40% fresh cassava root (dry matter (DM) basis), supplemented with other concentrate ingredients, and differing by additive: (1) 1% sulfur, (2) 2% sulfur, (3) *E. gallinarum* KKU-BC10, or (4) *E. faecium* KKU-BF7. Each ration was fermented anaerobically for seven days before feeding. Four male Thai native beef cattle (2.5 years old; 222 ± 12.0 kg initial body weight) were allocated to the four treatments in a 4 × 4 Latin square design, with each animal receiving all four treatments across the four experimental periods. All FTMR treatments effectively reduced HCN to safe levels for ruminant feeding. Specifically, HCN content in the FTMR dropped from ~60–84 mg/kg DM before fermentation to ~41–52 mg/kg DM after 7 days, remaining well below the 100 mg/kg DM safety threshold for cattle. No significant differences were observed in dry matter intake, nutrient digestibility, or total volatile fatty acid concentrations ($p > 0.05$). However, fiber intake was enhanced by microbial inoculation. Cattle receiving *E. gallinarum* KKU-BC10 showed the highest neutral detergent fiber intake ($p < 0.05$). In contrast, *E. faecium* KKU-BF7 supplementation resulted in the highest acid detergent fiber intake ($p < 0.01$). Notably, cattle fed *E. faecium* KKU-BF7 also had significantly lower blood urea-nitrogen concentrations at 4 hours post feeding ($p = 0.04$), indicating improved nitrogen utilization. Rumen pH and ammonia–nitrogen levels remained within physiological ranges across all treatments. These findings confirm that sulfur and CUB supplementation in FTMR supports the safe use of fresh cassava root in ruminant diets. Moreover, *E. faecium* KKU-BF7 shows potential to enhance nitrogen efficiency beyond detoxification.

Keywords: detoxification strategy, energy source, microbial inoculation, ruminant nutrition, tropical forage systems

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a major tropical root crop valued for its high starch content and resilience in marginal soils. In ruminant feeding systems, cassava root is often processed into dried chips as an energy dense ingredient [1,2,3]. However, during the wet season, drying is impractical, leading to interest in fresh cassava root as a direct feed source. Its use is limited by hydrocyanic acid (HCN), a toxic compound that can impair animal health [4,5,6]. Reported HCN concentrations in fresh cassava typically range from 85 to 114 mg/kg DM [7,8], which can be hazardous depending on intake and detoxification capacity. Values above 100 mg/kg DM may pose subclinical risks, while levels exceeding 200 mg/kg body weight or 500 mg/kg DM are considered toxic [3,4,6]. Once ingested, HCN is rapidly released in the rumen and absorbed into the bloodstream, where it inhibits cytochrome oxidase in the mitochondrial electron transport chain, causing impaired oxidative phosphorylation and cellular hypoxia [4,9]. Effective detoxification strategies are therefore critical to safely harness cassava's nutritional potential [10,11].

Ruminants possess a natural defense mechanism against cyanide toxicity through the mitochondrial enzyme rhodanese, which converts cyanide into the less toxic thiocyanate [4,12]. This process requires sulfur as a co-substrate, highlighting the nutritional role of dietary sulfur in sustaining detoxification efficiency [16]. Sulfur supplementation has been shown to support this conversion, particularly when dietary sulfur amino acid levels are inadequate. Supapong et al. [16] reported that inclusion of 2% sulfur in FTMR containing 40% fresh cassava root (DM basis) reduced hydrogen cyanide concentrations from approximately 180–200 mg/kg DM to below 100 mg/kg DM, likely by enhancing sulfur availability for rhodanese activity. However, excessive sulfur supplementation can reduce feed palatability due to off odors and may cause toxicity symptoms such as polioencephalomalacia (PEM) if not properly managed [10,17,18]. These challenges emphasize the need for complementary strategies that ensure safe utilization of cassava without compromising intake or health, while remaining practical for smallholder farmers. Microbial detoxification has therefore emerged as a promising biological approach [9]. *Enterococcus gallinarum* KKU-BC10 and *E. faecium* KKU-BF7, isolated from the rumen contents of swamp buffalo and beef cattle, demonstrated rhodanese activity and HCN degrading ability under *in vitro* conditions [19]. Similarly, Lukbun et al. [11] reported that *E. gallinarum* KKU-BC10 enhanced cyanide degradation, fiber digestibility, and propionate production in high HCN cassava substrates. These findings suggest that cyanide-utilizing ruminal bacteria (CUB) could be effective inoculants for improving both safety and

fermentation quality of cassava based rations, although their persistence and activity *in vivo* remain to be confirmed.

Among detoxification platforms, FTMR offer several advantages for incorporating fresh cassava root into ruminant diets. This system stabilizes feed through lactic acid fermentation, enhances preservation, and lowers HCN concentrations during ensiling [9]. Moreover, FTMR ensures consistent nutrient intake and supports microbial protein synthesis under anaerobic conditions, providing an effective framework for evaluating both chemical and microbial detoxification methods [20,21,22]. Such integration could enhance feed safety and expand the usable range of cassava based rations under tropical conditions [6].

Although previous studies have reported the detoxification potential of both sulfur supplementation and microbial inoculation with cyanide-utilizing bacteria (CUB) in cassava based diets [11,16,23], no *in vivo* investigation has systematically compared these approaches within a unified feeding system to assess their effects on rumen function and nitrogen metabolism. Understanding how these detoxification strategies influence physiological outcomes such as ruminal fermentation dynamics and nitrogen utilization is important for refining dietary interventions in tropical cattle. Therefore, the present study evaluated the impact of supplementing FTMR containing fresh cassava root with either sulfur (1% or 2%) or CUB strains (*E. gallinarum* KKU-BC10 and *E. faecium* KKU-BF7) on hydrogen cyanide degradation, ruminal parameters, nutrient digestibility, and blood urea nitrogen in Thai native beef cattle. The hypothesis was that, beyond detoxification, microbial and chemical additives would exert distinct effects on rumen fermentation profiles and indicators of nitrogen metabolism.

MATERIALS AND METHODS

Ethical procedure

All procedures involving animals were approved by the Animal Ethics Committee of Khon Kaen University (Approval No. ACUC-KKU 32/67; dated 30 April 2024) and were conducted in accordance with institutional guidelines for the care and use of animals in research.

FTMR preparation

Four dietary treatments were prepared by formulating FTMR based on 40% rice straw and 40% fresh cassava root (DM basis), supplemented with soybean meal, palm kernel meal, rice bran, urea, molasses, salt, and a mineral premix. The treatments differed by additive as follows: 1% sulfur, 2% sulfur, *E. gallinarum* KKU-BC10, or *E. faecium* KKU-BF7 (Table 1). The inclusion levels of sulfur were selected based on previous reports indicating efficacy for cyanide detoxification without exceeding recommended safety limits for ruminants [8,10,16].

Fresh cassava roots (8 months old) were sourced from farms surrounding Khon Kaen University (16.4322°N, 102.8236°E). The roots were washed, left to drain overnight, then chopped into approximately 10 mm pieces before being mixed with the designated treatments. Sulfur was directly mixed into the TMR, while bacterial inoculants were sprayed evenly over the total mixed ration (TMR).

The CUB strains (*E. gallinarum* KKU-BC10 and *E. faecium* KKU-BF7) were originally isolated from the rumen of swamp buffalo and beef cattle, and selected based on their rhodanese activity, as reported by Khota et al. [19]. Bacterial cultures were prepared according to Khota et al. [24]: Each strain was incubated in de Man, Rogosa, and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) at 39 °C for 24 hours. The optical density (OD600) of the culture was adjusted to 1.0 using 8.5 g/L sterile NaCl, corresponding to approximately 10^8 colony forming units (CFU)/mL. The inoculum was applied at a rate of 1 mL/kg fresh TMR.

All FTMR treatments were ensiled in sealed plastic containers under anaerobic conditions and stored at ambient outdoor temperatures (25–32 °C) for 7 days. Such temperature variability may influence fermentation dynamics and the viability of added microbial strains. After fermentation, samples were analyzed for pH, chemical composition, and HCN concentration.

Experimental animals and treatments

Four male Thai native beef cattle, aged 2.5 years with an initial average body weight of 222 ± 12.00 kg, were used in a 4 × 4 Latin square design to evaluate the effects of four dietary treatments. The treatments consisted of FTMR containing fresh cassava root, each supplemented with one of the following additives: 1% sulfur, 2% sulfur, *E. gallinarum* KKU-BC10, or *E. faecium* KKU-BF7. Each animal received all four treatments across four consecutive 21 day experimental periods. The Latin square design was selected to control for individual animal variability and period effects, thereby enhancing the precision of treatment comparisons despite the limited sample size (n = 4). This experimental approach is commonly employed in ruminant nutrition studies, particularly for digestibility evaluations.

Each animal was housed in an individual pen and had free access to clean drinking water. The FTMR was offered ad libitum, divided into two daily feedings at 07:00 and 16:00. The experimental period lasted for four 21 day cycles, each comprising a 14 day dietary adaptation phase followed by a 7 day data collection phase. During the final 7 days of each period, cattle were moved to individual metabolism crates and fed FTMR at 90% of their average intake from the preceding adaptation period. This adjustment ensured complete feces collection for digestibility measurements. Daily feed intake was recorded by weighing the amount of feed offered and refusals, and body weight was measured at the beginning and end of each experimental period to monitor changes during the trial.

Sample collection and chemical analysis

During the last seven days of each experimental period, samples of the experimental feed, refusals, and feces were collected to determine nutrient digestibility and other parameters. Feces were collected using a total collection method while the animals were housed in metabolism crates. Each day, 5% of the total fresh feces were subsampled. One portion was used for daily DM analysis, while the remainder was stored at −20 °C and pooled by animal at the end of each period for chemical analysis.

At the conclusion of the 7 day collection period, pooled samples of feed offered, feed refused, and feces were thawed, thoroughly mixed, dried in a forced air oven at 60 °C for 72 hours, and ground to pass through a 1 mm screen using a Cyclotech Mill (Tecator, Hoganas, Sweden). These samples were analyzed for DM (ID 967.03), ash (ID 942.05), ether extract (EE; ID 954.02), crude protein (CP; ID 984.13), and acid detergent fiber (ADF; ID 973.18) following AOAC [25] procedures. The neutral detergent fiber (NDF) content was also determined using the method of Van Soest et al. [26], incorporating alpha amylase but excluding sodium sulfite.

After 7 days of ensiling, FTMR samples were analyzed for pH, chemical composition, and total HCN content. HCN concentrations in fresh cassava root and FTMR before and after fermentation were measured colorimetrically using a UV/VIS spectrometer (PG Instruments Ltd., London, UK), following the method of Khota et al. [24]. Briefly, 1 g of sample was homogenized with 9 mL distilled water and stored at –20 °C for 12 hours to promote cell lysis. After thawing with shaking at 120 rpm, the homogenate was centrifuged at 4200 rpm at 4 °C for 10 minutes. Then, 1 mL of the supernatant was transferred into a 15 mL conical tube (Eppendorf AG, Hamburg, Germany) and sequentially mixed with 0.4 mL of n-chlorosuccinimide oxidizing reagent, 0.4 mL of hydantoin pyridine reagent, and 8.2 mL distilled water. After mixing with a vortex and incubating at 25 °C for 1 minute, the absorbance was read at 403 nm using standardized potassium cyanide (KCN) as reference.

On day 21 of each period, 10 mL blood samples were collected from the jugular vein at 0 h (before feeding) and 4 h post feeding for the determination of blood urea-nitrogen (BUN). Blood samples were collected to evaluate systemic nitrogen metabolism and detect changes related to detoxification efficiency. Blood was drawn into EDTA containing tubes (12 mg/tube), centrifuged at 500 × g for 10 minutes at 4 °C, and plasma was analyzed using a commercial BUN assay kit (L-type Wako UN, Tokyo, Japan) following the method of Kohn et al. [21].

Simultaneously, approximately 45 mL of rumen fluid was collected via a stomach tube attached to a vacuum pump at 0 h (before morning feeding) and 4 h post feeding on day 21 of each period for the determination of rumen fermentation characteristics. These two time points were selected to represent baseline (pre feeding) and postprandial responses, which are commonly used in ruminant nutrition studies. Intermediate sampling was not performed; therefore, only absolute values at 0 h and 4 h were analyzed. Rumen pH and temperature were immediately measured using a portable pH/temperature meter (HI 8424, Hanna Instruments, Kallang, Singapore). The rumen fluid was strained through four layers of cheesecloth and divided into three subsamples. The first subsample (approximately 45 mL of rumen fluid) was preserved with 5 mL of 1 M H_2SO_4 in a plastic vial for ammonia–nitrogen (NH_3-N) and volatile fatty acid (VFA) analysis. Ammonia–nitrogen was determined spectrophotometrically using a UV/VIS spectrometer (Shimadzu UV-1800, Kyoto, Japan) [19]. Total and individual VFAs (acetate, propionate, and butyrate) were quantified using gas chromatography (5890A Series II, Hewlett Packard, Wilmington, DE, USA) equipped with a 180 cm × 4 mm glass column packed with 100 g/L SP-1200 and 10 g/L H_3PO_4 on 80/100 mesh Chromosorb WAW (Supelco, Bellefonte, PA, USA) [24]. The second subsample was used to determine hydrogen cyanide (HCN) concentration using the same procedure as described above for FTMR samples. The final subsample (1 mL) was diluted with 9 mL of a 10% formalin solution and used for protozoal enumeration. Ruminal protozoa were manually counted with a haemocytometer (Boeco, Hamburg, Germany) following standard procedures [8], and their abundance was considered a complementary index of microbial stability in response to dietary treatments.

Calculations and statistical analysis

All variables were tested for normality and homoscedasticity before GLM analysis. All data were analyzed using the General Linear Model (GLM) procedure of SAS version 9.0 (SAS Institute, Cary, NC, USA) according to a 4 × 4 Latin square design, using the following statistical model:

$$Y_{ijk} = \mu + M_i + A_j + P_k + \varepsilon_{ijk}$$

where: Yijk, observation from animal j, receiving diet i, in period k; μ, the overall mean, Mi, the fixed effect of dietary treatment (i = 1, 2, 3, 4); Aj, the effect of animal (j = 1, 2, 3, 4); Pk, the effect of period (k = 1, 2, 3, 4); and εijk the residual effect. All results are presented as least squares means with their associated standard errors (SEM). Differences among treatment means were evaluated using Tukey's multiple comparison test. Statistical differences among treatments are denoted by different superscript letters within each row ($p < 0.05$).

RESULTS

Chemical composition and HCN reduction

After 7 days of fermentation, the FTMR showed clear but small differences in chemical composition among treatments. The 1% sulfur group had the lowest dry matter (DM) content (26.52%), whereas the *E. gallinarum* KKU-BC10 treatment had the highest DM (29.41%). Crude protein content varied slightly, ranging from 10.23% to 10.74% of DM across treatments. The *E. gallinarum* KKU-BC10 diet also had the highest NDF concentration (41.03%), while the *E. faecium* KKU-BF7 diet showed the greatest ADF value (26.97%). All FTMRs reached low final pH (3.63 to 3.77), confirming successful fermentation. Hydrogen cyanide content decreased in all treatments after ensiling, falling from 58.42–84.47 mg/kg DM before fermentation to 40.95 to 51.56 mg/kg DM after 7 days.

Intake and digestibility

Dry matter intake (DMI) did not differ among treatments ($p > 0.05$) when expressed as kg/day, %BW, or $\%BW^{0.75}$ (Table 2). Numerically, cattle fed *E. gallinarum* KKU-BC10 had the highest DMI (4.69 kg/day), followed by *E. faecium* KKU-BF7 (4.51 kg/day), while the 1% sulfur group showed the lowest intake (4.12 kg/day). Organic matter and crude protein intakes were similar among treatments ($p > 0.05$). NDF intake differed ($p < 0.05$), with the highest value in the *E. gallinarum* KKU-BC10 group (1.92 kg/day), while the 1% sulfur and *E. faecium* KKU-BF7 groups had lower intakes (1.57 and 1.59 kg/day, respectively). ADF intake also differed ($p < 0.01$), with the highest intake in the *E. faecium* KKU-BF7 group (1.23 kg/day). Digestibility of DM, OM, CP, NDF, and ADF did not differ among treatments ($p > 0.05$).

Ruminal fermentations and blood urea nitrogen

Ruminal pH (6.81 to 7.13), ammonia–nitrogen (12.61 to 13.80 mg/dL), and protozoal counts (16.00 × 10^6 to 19.00 × 10^6 cells/mL) did not differ among treatments ($p > 0.05$; Table 3). Hydrogen cyanide degradation exceeded 83% in all treatments. Blood urea nitrogen (BUN) at 4 h post feeding differed among treatments ($p = 0.04$). The *E. faecium* KKU-BF7 group showed the lowest BUN concentration (6.50 mg/dL), whereas the control and other treatments had higher values.

Volatile fatty acid

Total volatile fatty acid (TVFA) concentration and the molar proportions of acetate, propionate, and butyrate did not differ among treatments at either 0 or 4 h post feeding ($p > 0.05$; Table 4). Across diets and sampling times, TVFA averaged about 107 mmol/L. Acetate accounted for roughly 62 mol/100 mol, with propionate around 22 mol/100 mol and butyrate 15–16 mol/100 mol. The acetate to propionate ratio remained stable at approximately 2.8 to 3.0 in all treatments.

DISCUSSION

The findings of this study underscore the potential of FTMR with sulfur or CUB as an effective strategy to reduce HCN concentrations in ruminant diets. The significant reduction in HCN content after a 7 day ensiling period reflects the combined action of chemical and microbial detoxification mechanisms. This result aligns with previous studies by Supapong et al. [23] and Sombuddee et al. [27], which highlighted the role of sulfur as a cofactor for rhodanese, facilitating the conversion of cyanide into the less toxic thiocyanate.

In lactic environments typical of ensiling, BC10 and BF7 likely contribute to cyanide detoxification through two complementary routes: (i) sulfurtransferase mediated conversion of cyanide (CN^-) to thiocyanate (SCN^-) using thiosulfate/mercaptopyruvate donors primarily via rhodanese (thiosulfate sulfurtransferase, TST) and the related 3-mercaptopyruvate sulfurtransferase (3-MST) and (ii) acidification assisted glycoside hydrolysis, in which LAB driven pH decline accelerates the breakdown of cassava cyanogenic glycosides (e.g., linamarin) and promotes subsequent CN^- handling by sulfurtransferases [4,12,28]. In the presence of supplemental sulfur, these enzymes have ready sulfur donors (e.g., thiosulfate), improving the stoichiometric conversion CN^- to SCN^-, a far less toxic anion that is readily absorbed and excreted [4,12]. This suggests that the detoxification process was not only chemical (sulfur mediated) but also biological, where microbial enzymes such as rhodanese may

have acted synergistically with low pH conditions to accelerate cyanide degradation. BC10 (*E. gallinarum*), a facultative heterofermentative LAB, is expected to drive rapid early acidification, stabilizing silage and maintaining conditions that favor sulfurtransferase activity and inhibit cyanohydrin reversion. BF7 (*E. faecium*), noted for probiotic robustness, likely sustains enzyme activity under low pH and anaerobiosis and may show higher survival across the ensiling feeding interface, supporting continued CN^- to SCN^- conversion post-ingestion [19,28]. Together, BC10's pH control and BF7's persistence plausibly increase net flux to thiocyanate under sulfur supplied conditions [4,12,19]. The incorporation of *E. gallinarum* KKU-BC10 and *E. faecium* KKU-BF7 likely enhanced cyanide degradation through their enzymatic activity, particularly via rhodanese production [4,12]. Microbial TST/3-MST activity likely pre processes CN^- in the silage and rumen, generating SCN^- that is absorbed and handled by the host, complementing hepatic rhodanese to complete detoxification. The availability of sulfur donors from the ration (supplemental sulfur) is rate limiting for this pathway; thus, CUB × sulfur interactions are biologically expected [4,12]. In ruminants, rhodanese is localized in both microbial and host tissues, and the presence of sulfur donors enhances the conversion of cyanide to thiocyanate, which is then excreted in urine. To mechanistically verify the pathway, follow up work should quantify SCN^- in rumen fluid, plasma, and urine, assay TST/3-MST activities in silage and rumen fractions, and (where feasible) screen for tst/mpst gene markers in BC10/BF7 populations across ensiling and feeding stages [4,12,19]. However, *in vivo* rhodanese activity was not directly measured in this study. As reported by Khota et al. [19], these strains are capable of functioning effectively under anaerobic conditions, which may explain their efficiency during silage fermentation. Low pH reduces cyanohydrin instability, limits re-liberation of CN^-, and preserves sulfur donor pools during ensiling, thereby maintaining substrate–enzyme alignment for microbial sulfurtransferases once the FTMR is fed [28]. Furthermore, the low pH values observed across all treatments support the success of lactic acid fermentation, contributing to feed preservation and providing a favorable environment for microbial detoxification [28]. Measuring microbial survival or activity post ensiling would strengthen the interpretation of these results. However, the study did not assess lactic acid content, microbial counts, or aerobic stability, which are critical indicators of silage quality; these should be prioritized in future investigations.

Although slight differences were observed in the chemical composition particularly in fiber fractions these did not result in significant changes in feed intake or nutrient digestibility. For example, the *E. gallinarum* KKU-BC10 group exhibited higher DM and NDF content, which may suggest improved fiber structure or fermentability. As proposed by Mertens [20], such characteristics can influence voluntary intake, though further investigation under extended feeding trials is warranted.

However, it should be noted that our assessment of detoxification focused primarily on the reduction of HCN concentrations in the feed before ingestion. Although ruminal HCN levels were monitored, we did not measure downstream detoxification products such as thiocyanate in rumen fluid or blood, which would provide a more complete understanding of cyanide metabolism. Nonetheless, the observed decline in feed HCN levels, along with stable fermentation characteristics and nutrient composition, supports the application of sulfur or CUB enhanced FTMR as a practical strategy to improve feed safety in tropical beef production systems [6]. Future *in vivo* research is warranted to evaluate the long term implications of these detoxification strategies on animal performance, nitrogen retention, and rumen microbial dynamics. Notably, performance indicators such as average daily gain or feed efficiency were not assessed in the present trial.

The results demonstrate that varying levels of sulfur and the inclusion of CUB in FTMR had no effect on DMI, whether expressed in absolute terms or relative to body weight. This aligns with previous findings by Drewnoski et al. [18], who reported that moderate sulfur supplementation does not adversely influence feed intake in ruminants. Although numerical differences were observed particularly with the *E. gallinarum* KKU-BC10 group exhibiting the highest DMI these variations did not reach statistical significance, indicating stable intake behavior in Thai native beef cattle across treatments. This may be attributed to the limited sample size, which constrains statistical power despite numerical trends. Future studies involving larger animal cohorts may help determine whether these trends hold biological relevance.

Organic matter and CP intake were similarly unaffected by the additives, suggesting that neither sulfur nor microbial supplementation impaired voluntary nutrient consumption. However, fiber intake showed distinct patterns among treatments. Notably, cattle fed *E. gallinarum* KKU-BC10 exhibited the highest NDF intake, while those receiving *E. faecium* KKU-BF7 had the greatest ADF intake. These outcomes may be attributable to strain specific effects on rumen microbial populations or differential influence on fiber palatability and fermentation [9]. Further investigation into rumen fibrolytic microbial populations may clarify these strain specific effects. As discussed by Mertens [20], fiber intake is governed not only by concentration but also by physical and fermentative characteristics, which can modulate satiety and gut motility.

The lack of significant digestibility changes suggests that the rumen microbiota were able to adapt functionally to the presence of sulfur and CUB without disruption of fibrolytic activity, reflecting the resilience of the rumen ecosystem. Despite these intake differences, nutrient digestibility including DM, OM, CP, NDF, and ADF remained statistically unaffected. This suggests that rumen microbial populations were able to adapt

effectively to the sulfur and CUB supplemented FTMR formulations. These results align with Mertens [20], who emphasized that digestibility depends more on effective fiber and particle size than intake volume alone.

This could reflect differences in nitrogen partitioning, where more nitrogen was captured for microbial protein synthesis rather than being degraded excessively in the rumen. Interestingly, the *E. faecium* KKU-BF7 group exhibited numerically lower CP digestibility than the other treatments. Although treatment effects on CP digestibility were not significant, numerical reductions in the BF7 group are noteworthy. This reduction may reflect differences in ruminal nitrogen utilization or microbial efficiency, which warrants further investigation. The adaptability of Thai native cattle and the resilience of their rumen ecosystem to such interventions support the use of FTMR incorporating fresh cassava root, detoxified via sulfur or microbial strategies [29]. For smallholder systems, both approaches offer accessible, cost effective methods to enhance the utility of local feed resources without impairing digestive performance.

Across all treatments, ruminal fermentation parameters remained within physiologically acceptable ranges, indicating that neither sulfur supplementation nor CUB disrupted rumen homeostasis. Rumen pH values were consistent with the optimal range required for cellulolytic microbial activity and fiber degradation [30]. This finding aligns with previous observations that the rumen possesses strong buffering capacity and regulatory mechanisms to maintain functional stability [31]. Such regulatory mechanisms include saliva secretion rich in bicarbonate and phosphate buffers, continuous rumen contractions that facilitate mixing and gas removal, and microbial cross feeding that stabilizes fermentation end products. Together, these processes maintain ruminal pH within the narrow range required for microbial activity and fiber degradation [31]. Moreover, no signs of sulfur toxicity (e.g., neurological symptoms of polioencephalomalacia) were observed at the 2% sulfur inclusion level, indicating this level was well tolerated in the short term. Neurological symptoms of PEM, including ataxia, blindness, and abnormal posture, were assessed by daily visual observation of animal behavior and health status. None of these clinical signs were observed in cattle across treatments.

The similarity in protozoal populations across treatments suggests that the inclusion of sulfur or CUB at the tested levels does not significantly alter the protozoal ecosystem. This stability is nutritionally relevant because protozoa contribute to fiber degradation and regulate nitrogen turnover through bacterial predation. Maintaining their populations indicates that both fiber fermentation and nitrogen recycling were preserved, supporting overall rumen fermentation efficiency [32,33]. The maintenance of optimal ruminal pH across treatments underscores the resilience of the rumen buffering system. This is consistent with the findings of Belanche et al. [34], who reported limited shifts in protozoal abundance under moderate dietary interventions.

Post ingestion, residual CN^- from cassava is expected to undergo immediate microbial conversion to SCN^- via sulfurtransferases from BF7 and, to a lesser extent, BC10, provided sulfur donors are present. This ruminal step complements the ensiling phase detoxification and aligns with the >83% degradation efficiency, consistent with a two stage (ensiling and rumen) sulfurtransferase cascade [4,9,12,19]. Cyanide degradation efficiency was consistently high, supporting the effectiveness of both chemical and microbial detoxification strategies [9]. This likely reflects rhodanese mediated conversion of HCN into thiocyanate, a less toxic compound readily excreted by the host [4,12]. It should be noted that the present experiment did not include performance parameters such as body weight gain, feed conversion ratio, or nitrogen balance. These omissions limit the extent to which the results can be extrapolated to practical feeding outcomes and highlight the need for additional studies that integrate both ruminal responses and animal performance indicators. It should be acknowledged that the present study utilized only four Thai native beef cattle within a 4 × 4 Latin square design. Although this design is statistically valid for controlling individual and period effects, the restricted number of animals inherently reduces statistical power and limits the generalizability of the results. Consequently, the outcomes should be interpreted as preliminary, recognizing that smaller treatment differences may not have been detectable under the current experimental conditions. It should be emphasized that the present study did not assess rumen microbiota composition or downstream detoxification products such as thiocyanate. These measurements would have provided stronger mechanistic evidence of how sulfur and cyanide-utilizing bacteria interact with rumen metabolism. Previous reports indicate that sulfur supplementation and microbial inoculation can modulate rhodanese activity, microbial adaptation, and cyanide detoxification pathways in ruminants [4,11,19,23]. Without such data, the interpretation of mechanisms remains limited and the current results should therefore be regarded as indicative rather than conclusive. These outcomes corroborate previous reports demonstrating the practical viability of sulfur based and microbial detoxification methods in cassava based rations [10,27]. Although the present results demonstrate that both sulfur and cyanide-utilizing bacteria can reduce hydrogen cyanide concentrations and maintain stable ruminal fermentation, the interpretation remains limited. Key production outcomes such as body weight gain, feed conversion efficiency, and nitrogen balance were not assessed, and microbial community shifts were not characterized. These limitations restrict the scope of the findings, which should be regarded as preliminary evidence requiring further validation under broader experimental conditions. However, *in vivo* measures of rhodanese activity or thiocyanate levels would help clarify the exact detoxification mechanism.

Because CN^- inhibits terminal oxidases, incomplete detoxification can depress microbial ATP yield. By diverting CN^- to SCN^-, BF7 may help preserve microbial energy status, supporting protein synthesis and ammonia capture, which is consistent with the lower BUN observed in *E. faecium* KKU-BF7 fed cattle [19,24,33]. However, as BUN is only a systemic surrogate of nitrogen utilization, this finding should be interpreted cautiously. Other indicators such as urinary nitrogen excretion, microbial protein synthesis, and isotopic nitrogen balance were not measured in the present study. Future work incorporating these parameters, alongside microbial profiling, is required to confirm whether reduced BUN truly reflects improved nitrogen utilization efficiency.

In ruminants, nitrogen not captured in microbial protein is converted to urea in the liver. This urea may be excreted or recycled into the gastrointestinal tract via urea nitrogen salvaging (UNS), where ureolytic microbes hydrolyze it into ammonia for further microbial protein synthesis [35]. When this recycling pathway is efficiently utilized, less urea accumulates in circulation, resulting in reduced BUN and nitrogen loss [21,35].

This finding is biologically relevant because lower BUN implies more efficient nitrogen capture by rumen microbes, reducing nitrogen waste and potential environmental losses. The observation that only *E. faecium* KKU-BF7 supplementation led to reduced BUN suggests a strain specific enhancement of nitrogen metabolism. Although not ureolytic itself, *E. faecium* may facilitate nitrogen efficiency indirectly by stabilizing the gut microbial community, supporting the growth of urease positive microbes, and optimizing fermentation balance [36]. Microbial sequencing or DGGE profiles would help validate this proposed community shift. Its established probiotic effects including modulation of immune responses and competitive exclusion of pathogens may create conditions conducive to improved nitrogen capture and microbial protein synthesis [36].

Taken together, these results suggest that *E. faecium* KKU-BF7 contributes not only to HCN detoxification but also to improved nitrogen economy through microbial and host mediated pathways [19,24,36]. This dual functionality detoxification and improved nitrogen efficiency could benefit cassava based rations in low protein systems. Further studies should investigate microbial shifts, protein synthesis rates, and animal performance over longer feeding durations to confirm these functional benefits and elucidate underlying mechanisms. Collectively, the findings support the potential of microbial detoxification strategies as a component of integrated feed safety protocols.

Volatile fatty acids are key end products of rumen microbial fermentation and serve as a primary energy source for ruminants, contributing approximately 40% to 70% of the total digestible energy in the diet [37]. In the present study, both the concentrations and molar proportions of VFAs were within expected physiological ranges, indicating that the rumen fermentation process remained stable across all treatment groups. This suggests that the

basal diet composition may have exerted a dominant effect, overshadowing treatment induced shifts [38]. The comparable VFA profiles among treatments support the similarity observed in digestibility coefficients, indicating that neither microbial inoculants nor sulfur additives negatively affected ruminal fermentation efficiency [39].

Despite intervention with sulfur and CUB, VFA concentrations and profiles remained largely stable across treatments. The major VFAs acetate, propionate, and butyrate are produced in varying ratios depending on dietary composition [40]. High fiber diets typically favor acetate production, while diets richer in starch promote propionate synthesis [38]. The VFA profiles observed in this study are consistent with diets containing fibrous ingredients such as cassava root and rice straw, suggesting that neither sulfur supplementation nor inoculation with CUB disrupted the fermentation balance.

Given that VFAs provide 40 to 70% of the host's metabolizable energy, stability of their proportions across treatments indicates that the energetic contribution from microbial fermentation was preserved regardless of detoxification strategy. The acetate to propionate (C2:C3) ratio is often used to evaluate energy utilization efficiency. Elevated ratios are generally associated with greater methane production and less efficient energy conversion, whereas a shift toward propionate reflects enhanced glucose availability for tissue metabolism [38,40]. The stability of this ratio across treatments implies that energy partitioning remained unaffected. In addition, the levels of butyrate detected likely support ruminal epithelial development and energy metabolism, further reflecting a well functioning microbial ecosystem. However, this remains an indirect inference, as microbial composition was not directly assessed.

Overall, these findings demonstrate that incorporating sulfur or CUB into FTMR did not impair the core pathways of microbial fermentation responsible for energy production. The consistent VFA profiles across treatments highlight the adaptive capacity of the rumen microbiota to dietary modifications, supporting the practical use of these additives in tropical beef cattle feeding systems [1,5]. Although microbial adaptation is inferred, this study did not quantify bacterial community composition. The similarity in VFA profiles aligns with the comparable digestibility coefficients among treatments, suggesting that microbial or sulfur interventions did not impair fermentation end products [11,16]. These results partially confirmed our hypothesis. Although all treatments reduced HCN as expected, only the CUB inoculants showed additional nutritional benefits. Specifically, *E. faecium* KKU-BF7 was associated with higher fiber intake, suggesting improved feed acceptability, while its lower BUN concentrations reflected more efficient nitrogen utilization. Thus, the benefits extended beyond detoxification to include improved feed use and potential enhancement of nitrogen metabolism.

While reduced BUN suggests a possible improvement in nitrogen capture, it cannot be taken as conclusive evidence of enhanced nitrogen utilization without supporting data on urinary nitrogen excretion, microbial protein synthesis, or isotopic nitrogen balance.

CONCLUSION

This study confirms that both sulfur supplementation and CUB effectively reduced hydrogen cyanide levels in FTMR containing fresh cassava root, without compromising feed intake, digestibility, or rumen fermentation in Thai native beef cattle. Notably, *E. faecium* KKU-BF7 improved nitrogen utilization, as indicated by reduced blood urea nitrogen, suggesting additional benefits beyond detoxification. These findings support the practical use of sulfur and CUB enhanced FTMR in tropical systems where cassava is abundant but underutilized due to HCN concerns. However, the small number of animals used in the design limits statistical power and the ability to generalize these results beyond the experimental conditions. Nevertheless, the limited sample size and the absence of performance indicators such as body weight gain, feed conversion efficiency, and nitrogen balance restrict the scope of interpretation. Furthermore, the lack of microbial community analysis and thiocyanate quantification represents an additional limitation, as these measurements would provide more direct insights into detoxification mechanisms and microbial contributions. The present findings should therefore be regarded as preliminary evidence, reinforcing the need for longer term in *vivo* studies to confirm the biological and practical significance under field conditions.

COMPETING INTEREST

No potential conflict of interest relevant to this article was reported

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467

468 **AVAILABILITY OF DATA AND MATERIAL**

469 Upon reasonable request, the datasets of this study can be available from the corresponding author.

470

471 **AUTHORS' CONTRIBUTIONS**

472 **Conceptualization:** Surakhai T, Suntara C, Srichompoo P, Phowang N, Khota W, Wanapat M,
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482

483 **ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

484 All procedures involving animals were approved by the Animal Ethics Committee of Khon Kaen
485 University (Approval No. ACUC-KKU 32/67; dated 30 April 2024) and were conducted in accordance with
486 institutional guidelines for the care and use of animals in research.

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601 **Table 1** Ingredients and chemical composition of fermented total mixed ration (FTMR) used in the
602 experiment.

Item	1% Sulfur	2% Sulfur	BC10	BF7	Fresh cassava root
Ingredients, % dry matter (DM)					
Rice straw	40	40	40	40	
Fresh cassava root	40	40	40	40	
Soybean meal	5	5	5	5	
Palm kernel meal	3	3	4	4	
Rice bran	4	3	4	4	
Urea	2	2	2	2	
Pure sulfur	1	2	-	-	
Mineral premix	1	1	1	1	
Molasses, liquid	3	3	3	3	
Salt	1	1	1	1	
Chemical composition					
Dry matter, %	26.52	27.39	29.41	28.52	35.20
Organic matter, %DM	90.92	91.20	92.21	91.28	93.93
Ash, %DM	9.08	8.80	7.79	8.72	6.07
Crude protein, %DM	10.74	10.23	10.35	10.42	2.23
Ether extract, %DM	1.47	2.67	1.34	1.82	1.21
Neutral detergent fiber, %DM	38.21	39.29	41.03	34.78	13.40
Acid detergent fiber, %DM	24.10	24.24	22.87	26.97	7.50
pH	3.71	3.77	3.66	3.63	-
HCN content (after 0 days of ensiling), mg/kg DM basis	82.65	78.42	84.19	84.47	190.78
HCN content (after 7 days of ensiling), mg/kg DM basis	51.56	49.95	45.07	41.45	-

BC10= *Enterococcus gallinarum* KKU-BC10; BF7= *Enterococcus faecium* KKU-BF7; HCN=Hydrogen cyanide concentration

603 **Table 2.** Effects of sulfur and cyanide-utilizing bacteria in fermented diets with fresh cassava root on
604 intake and digestibility in Thai beef cattle.

Item	1% Sulfur	2% Sulfur	BC10	BF7	SEM	*p*-Value
Dry matter (DM) intake						
Kg/d	4.12	4.45	4.69	4.51	0.14	0.12
%BW	1.63	1.87	1.87	1.89	0.09	0.19
$\%BW^{0.75}$	6.30	7.96	7.63	7.08	0.67	0.40
Nutrient intake, kg DM /d						
Organic matter	3.63	4.05	4.32	3.96	0.18	0.15
Crude protein	0.43	0.48	0.48	0.46	0.02	0.27
Neutral detergent fiber	1.57^{b}	1.75^{ab}	1.92^{a}	1.59^{b}	0.07	0.02
Acid detergent fiber	0.99^{b}	1.07^{b}	1.07^{b}	1.23^{a}	0.03	0.01
Digestibility coefficients						
Dry matter, %	68.04	68.75	64.51	70.89	2.35	0.65
Organic matter, %DM	71.76	72.54	70.97	73.52	1.47	0.86
Crude protein, %DM	68.37	68.20	65.71	59.29	2.50	0.12
Neutral detergent fiber, %DM	56.98	54.72	53.76	52.76	3.65	0.87
Acid detergent fiber, %DM	47.23	48.31	44.23	52.02	3.68	0.77

605 $^{a-b}$ Different superscript letters within a row indicate significant differences among treatments ($p < 0.05$).

606 BW = body weight; $BW^{0.75}$ = metabolic body weight; BC10= *Enterococcus gallinarum* KKU-BC10; BF7=
607 *Enterococcus faecium* KKU-BF7; SEM=standard error of mean.

608

609

610 **Table 3.** Effects of sulfur and cyanide-utilizing bacteria in fermented cassava based diets on rumen
611 fermentation, cyanide degradation, and blood urea in Thai beef cattle.

Item	1% Sulfur	2% Sulfur	BC10	BF7	SEM	*p*-Value
Ruminal pH						
0 h post feeding	6.83	7.02	6.92	7.13	0.24	0.95
4 h post feeding	6.88	6.94	6.85	6.81	0.09	0.83
Mean	6.85	6.98	6.88	6.97	0.12	0.99
Ammonia-nitrogen concentration, mg/dL						
0 h post feeding	8.59	11.21	11.63	11.49	0.75	0.27
4 h post feeding	16.62	15.83	14.85	16.11	1.29	0.39
Mean	12.61	13.52	13.24	13.80	0.79	0.64
Protozoa, $\times 10^6$ cell/mL						
0 h post feeding	14.50	12.33	12.50	14.00	1.72	0.77
4 h post feeding	23.00	19.67	19.50	24.00	3.34	0.84
Mean	18.75	16.00	16.00	19.00	0.83	0.27
Degradation efficiency of cyanide in the rumen (%)						
0 h post feeding	82.91	81.11	83.23	83.80	2.43	0.87
4 h post feeding	85.50	85.78	86.11	83.38	1.61	0.65
Mean	84.21	83.45	84.67	83.59	1.00	0.81
Blood urea-nitrogen concentration, mg/dL						
0 h post feeding	9.63	8.75	7.50	6.25	0.95	0.16
4 h post feeding	11.00[a]	10.70[a]	8.50[ab]	6.50[b]	0.26	0.04
Mean	10.32	9.73	8.00	6.38	1.01	0.07

612 [a-b] Different superscript letters within a row indicate significant differences among treatments ($p < 0.05$).

613 BC10= *Enterococcus gallinarum* KKU-BC10; BF7= *Enterococcus faecium* KKU-BF7; SEM=standard error of
614 mean.

615

616 **Table 4.** Effects of sulfur and cyanide-utilizing bacteria in fermented cassava based diets on volatile
617 fatty acids in the rumen of Thai beef cattle.

Item	1% Sulfur	2% Sulfur	BC10	BF7	SEM	*p*-Value
Total volatile fatty acid, mmol/L						
0 h post feeding	108.20	105.75	103.35	112.09	8.94	0.49
4 h post feeding	112.54	104.88	105.36	117.43	6.46	0.51
Mean	110.37	103.58	106.50	111.75	8.90	0.63
Volatile fatty acid profiles, %						
Acetic acid						
0 h post feeding	63.69	62.41	62.76	62.09	1.59	0.90
4 h post feeding	62.24	63.90	59.90	62.92	1.56	0.39
Mean	62.96	63.16	61.33	62.50	1.43	0.81
Propionic acid						
0 h post feeding	21.58	22.19	21.39	22.04	1.50	0.98
4 h post feeding	23.24	20.55	23.40	21.24	0.88	0.14
Mean	22.41	21.37	22.39	21.64	1.07	0.86
Butyric acid						
0 h post feeding	14.73	15.41	15.86	15.87	0.90	0.79
4 h post feeding	14.52	15.55	16.69	15.84	1.07	0.58
Mean	14.62	15.48	16.27	15.85	0.94	0.66
Acetic acid: propionic acid						
0 h post feeding	2.96	2.85	2.98	2.87	0.28	0.98
4 h post feeding	2.70	3.20	2.60	3.00	0.21	0.25
Mean	2.83	3.03	2.79	2.93	0.20	0.83

618 BC10= *Enterococcus gallinarum* KKU-BC10; BF7= *Enterococcus faecium* KKU-BF7; SEM=standard error of
619 mean.

620