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Improvement of the Nutritional Quality of *Psophocarpus tetragonolobus* Tubers by Fermentation with Ruminal Crabtree-Negative Yeasts on the In Vitro Digestibility and Fermentation in Rumen Fluid

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Abstract: The purpose of this study was to determine how ruminal Crabtree-negative yeast affects the nutritional characteristics of winged bean (*Psophocarpus tetragonolobus*) tubers (WBT), in vitro gas and digestibility, and rumen fermentation. The experiment was carried out in a randomized complete design with a 5 × 2 (+1) factorial arrangement. Factor A determined the WBT products (a_1 = dry WBT, a_2 = fermented WBT without yeast in media solution, a_3 = fermented WBT with *Pichia kudriavzevii* KKU20, a_4 = fermented WBT with *Candida tropicalis* KKU20, and a_5 = fermented WBT with *Saccharomyces cerevisiae*), whereas factor B determined the level of fermented WBT replacing cassava chips (b_1 = WBT at 50% and b_2 = 100% levels). The results of the experiment showed that the fermentation approach could increase the crude protein (CP) content of WBT by around 7% ($p < 0.01$). The WBT fermented with yeast lowered the number of aerobic bacteria during the fermentation process ($p < 0.01$). *P. kudriazevii* KKU20 yeast strain had a 17.3% higher final asymptotic gas volume (*Vf*) than the *C. tropicalis* KKU20. Crabtree-negative yeast had a higher in vitro dry matter digestibility (IVDMD) than Crabtree-positive yeast after 12 h of incubation ($p < 0.01$). Fermented WBT with yeast had a higher IVDMD after 24 h of incubation than fermented WBT without yeast in the media solution ($p < 0.05$). The fermented WBT with *C. tropicalis* KKU20 enhanced propionic acid (C3) concentrations when cassava chips were replaced for half of all of the diet (C3 ranged from 26.0 to 26.4 mol/100 mol; $p < 0.01$). Furthermore, Crabtree-negative yeast isolated from the rumen stimulates rumen bacteria more effectively than Crabtree-positive yeast ($p < 0.01$). According to our findings, nutritional enrichment with yeast might increase the in vitro gas production and digestibility of WBT. The study also demonstrated that Crabtree-negative yeast has

a promising lead in terms of improving rumen fermentation quality. However, further research is required before deciding on an effective approach for optimizing the potential of WBT as a feed source.

Keywords: tuberous plant**;** yeast from rumen; fermentation quality; in vitro gas production

1. Introduction

Cassava chips (*Manihot esculenta*) are tuberous plants that are widely farmed in Thailand's northeast. Furthermore, almost half of cassava chips are fed to cattle as a source of energy [1]. Many difficulties remain in the cassava field plant, causing production variances. One of the factors is the effect of climate change on agricultural production, which leads to inconsistency and volatility in prices [2]. In addition, there is considerable volatility in production [3]. Cassava chips are also commonly used in the food and energy industries [4]. For this reason, it makes growers' prospects for the plant even grimmer. The introduction of new tuberous plant species or other plants can assist in improving local feed variety and alleviating feed shortages in our regions.

The winged bean (*Psophocarpus tetragonolobus*) is a tropical legume that is only occasionally utilized in ruminant diets [5]. This plant is a valuable tropical legume vegetable with a high nutritional value [6], and it may be produced in several tropical regions, including Thailand [7]. The edible pods, seeds, leaves, blooms, and tuberous roots of the winged bean (WB) can be found in all of Thailand's provinces. However, there was very little information available on this plant's chemical composition, until Sriwichai et al. [8] revealed the tuber's chemical composition and showed that it may be used as an alternative animal feed. WBT provides nutritional advantages in feed formulas since it has a high nutritional value and can be used instead of cassava. WBT has a high energy content (3.82 Mcal/kg), which is comparable to cassava chips (4.04 Mcal/kg [9]), as well as a protein content of approximately 190 g/kg DM, 2.6–11.6 g/kg DM of ether extract, and 150–300 g/kg DM of neutral detergent fiber (NDF) [5]. This study also revealed a dual-purpose species with a massive tuber, but it is not widely used in animal feed. As a consequence, it was advocated that it be used as a cassava alternative for farmers to realize the benefits, which might eventually lead to large-scale cultivation for animal feed.

In Thailand, the tuber yield output of winged beans has been recorded at as high as 15.5 T/ha [5]. Although the nutritional content of WBT should make it a suitable alternative for animal feed, previous research has shown that WBT may be limited if fed directly to animals. WBT's odor and taste make it unappealing to animals [10]. As a result, there is concern that its usage on animals may be limited, and WBT quality will need to be improved. Previous studies have shown that there are several methods for avoiding these constraints using WBT, such as boiling [11] or pelleting [12] them before giving them to the animals. Furthermore, there are several potential approaches for boosting palatability while optimizing feed consumption.

Previous research has used yeast to improve feed quality, specifically yeast proliferation in a molasses and urea-containing medium solution followed by fermentation with feed raw materials [13–16], with the fermented product benefiting animals by increasing feed intake [17]. In addition, there is a considerable amount of data to support the approach in terms of improving nutritional value and optimizing rumen fermentation [18]. Employing cell growth as a protein source and improving rumen microbial activity, especially when yeast is incorporated or fermented with feed ingredients, improves the nutritional value of the feed [19]. However, previous studies have shown that not all yeast strains are equally capable of developing [20]. Under aerobic circumstances, respiration with oxygen as the ultimate electron acceptor is conceivable, but *S. cerevisiae* exhibits alcoholic fermentation before the quantity of sugar reaches a low level [21], and yeasts exhibiting this phenotype are referred to as "Crabtree-positive

yeasts." Under aerobic circumstances, "Crab-tree-negative" yeasts generate just biomass and carbon dioxide and do not make fermentative products [22]. In a solution medium with high glucose (more than 20–50 g/L), *S. cerevisiae* (Crabtree-positive yeast) generates sevenfold less biomass than Crabtree-negative yeast (*Kluveromyces marxianus*, Crabtree-negative yeast) [20].

As a result, while *S. cerevisiae* is a common yeast strain in animal feed, a recent study revealed that its biomass growth may be restricted. Prior to treating feedstuffs with *S. cerevisiae*, high sugar concentrations were employed to stimulate yeast growth. To explore the theory, glucose (which is utilized as a source of energy for yeast) was focused on at various concentrations. Some studies have shown that glucose concentrations of 20 g/L or more can inhibit the pyruvate dehydrogenase complex (PDH complex) in *S. cerevisiae*, resulting in less pyruvate entering the mitochondria and poorer biomass synthesis. Other yeast strains, such as Crabtree-negative yeast, can create more biomass than *S. cerevisiae* because pyruvate is still delivered to the mitochondria in a different manner [23]. As a result, the introduction of Crabtree-negative yeast is an exciting issue for increasing feed quality. Suntara et al. [24] isolated Crabtree-negative yeast from rumen fluid (*C. tropicalis* KKU20 and *P. kudriavzevii* KKU20) to increase the quality of rice straw after fermentation, feed digestion, and rumen fermentation quality. Furthermore, Crabtree-negative yeast fermentation with rice straw in dairy cow feed improved milk protein content [25].

The goal of this study was to investigate the effects of Crabtree-negative ruminal yeast and Crabtree-positive yeast in fermented WBT on fermentation quality, nutritional value, and microorganism composition, as well as evaluate the fermented WBT replacement of cassava chips using an in vitro gas production technique. We hypothesized that, following WBT fermentation with a high-potency yeast, it would be acceptable for use not only as animal feed but also as a novel feedstock for ruminants in the region at a time when feed costs are fluctuating.

2. Materials and Methods

2.1. Location, Winged Bean Tubers, and Yeast

This study was conducted at the Department of Animal Science, Faculty of Agriculture, Khon Kaen University (KKU), Khon Kaen (16°26′48.16″ N, 102°49′58.8″ E), Thailand. The winged bean was grown in an area of 1600 m^2 at the Agronomy Research Unit (Department of Agronomy, KKU), and the WBT was harvested at 43.5% DM by hand. The fresh WBT used in this in vitro experiment was chopped to a length of cut of 3–5 cm before being treated with fermentation methods. Ruminal yeasts included *C. tropicalis* KKU20 and *P. kudriavzevii* KKU20 with strain numbers CBS 94T (U45749) and CBS 5147T (MH545928), respectively, which were obtained from an experiment by Suntara et al. [23]. The yeast was isolated, screened, and identified from the rumen of Thai–Holstein Friesian dairy cattle until the product was in its dry form. In a De Deken [21] experiment, these two ruminal yeast strains were found to be expressed as Crabtree-negative yeasts. The Crabtree effect is determined by the ratio of fermented glucose to respiration glucose, which is positive if the ratio is greater than one and negative if the ratio is less than one. *C. tropicalis* and *P. kudriavzevii* are Crabtree-negative yeasts [20,21]. *On* the other hand, *S. cerevisiae* demonstrated yeast-like features in Crabtree-positive yeast derived from commercial yeast (Perfect Yeast Co., Ltd., Ubon Ratchathani, Thailand).

2.2. Yeast-Fermented Winged Bean Tubers

The product, yeast powder (1×10^{13} cell per g), was stimulated and expanded in a medium solution containing 250 g of molasses (Khon Kaen dairy cooperative Co., Ltd., Khon Kaen, Thailand) and 10 g urea per 1 L of water. The pH of the solution was then adjusted with formic acid (L.C. Industrial Co., Ltd., Nakhon Pathom, Thailand) to a final value of 3.5. To ensure adequate respiration and maximum yeast cell growth, the media solution was directly pumped into an electromagnetic air compressor (HAILEA ACO-318

oxygen pump, Sagar aquarium, Gujarat, India) that was flushed with oxygen for 60 h. The density of fully grown yeast populations in media solutions containing *S. cerevisiae*, *C. tropicalis* KKU20, and *P. kudriavzevii* KKU20 was 1.07×10^{10}, 1.15×10^{11}, and 0.53×10^{11} cells/mL, respectively.

The media solution was mixed with the WBT at a ratio of 0.2:1 (media solution: WBT) to provide WBT with a moisture content of approximately 700 g/kg (appropriate moisture for the fermentation process) as well as the required amount of yeast [26]. Fresh WBT was mixed with yeast and media solution in four different ways: fermented WBT without yeast in media solution (WBT-NO), fermented WBT with *P. kudriavzevii* KKU20 (WBT-P), fermented WBT with *C. tropicalis* KKU20 (WBT-C), and fermented WBT with *S. cerevisiae* (WBT-S) in order to further test the quality after fermentation. Once the WBT had been well-mixed with the yeast solution, 300 g of WBT yeast was manually packaged into a plastic bag (size 8 × 12 cm, E-Sann Pass Pack 1999 Co., Ltd., Khon Kaen, Thailand) with five replications for each ensilage. The sample was kept at room temperature and vacuum bagged (DZ-400 vacuum machine, Nakhoncenterpack Co., Ltd., Nakhon Ratchasima, Thailand). After 7 days, the fermentation products and nutritional composition of all fermented WBT samples were exposed in silos and analyzed [24].

2.3. Evaluation of Microorganism Count, Fermentation Product, and Chemical Composition

On the designated seventh day, five plastic bags per treatment were opened. The microbe numbers were determined by counting the individual colonies according to Kozaki et al. [27]. Ten grams of the fermented WBT were placed into a plastic bag containing 90 mL of sterilized distilled water and mixed thoroughly. Liquid containing a sample mixture (fermented WBT and distilled water) of 1 mL was serially diluted in 0.85% sodium chloride solution at 10^{-1}, 10^{-3}, and 10^{-5}. The 20 microliters of various solutions were then spread on agar plates. The lactic acid bacteria (LAB) were counted on Lactobacilli MRS agar (Sisco Research Laboratories Pvt. Ltd., Mumbai, India) in an anaerobic box at 30 °C for 48 h (Sugiyamagen Ltd., Tokyo, Japan). The presence of coliform was detected on blue light broth agar (Nissui-Seiyaku Co., Ltd., Nagoya, Japan). Aerobic microorganisms were grown on nutrition agar plates for 24 h at 30 °C under aerobic conditions, whereas yeast was grown on potato dextrose agar (Sisco Research Laboratories Pvt., Ltd., Mumbai, India). Yeasts are classed as microorganisms based on the presence of colonies and the form of their cells. The remaining liquid samples were split into two. The first component was centrifuged at 6000 rpm for 15 min to extract organic acid, and the liquid above the solid residue was filtered through a 0.45-micron syringe filter. Lactic acid and volatile fatty acid (VFA, acetic acid, propionic acid, and butyric acid) analyses were performed with gas chromatography (Model HP 6890, Hewlett-Packard Co., Ltd., New York, NY, USA) equipped with a flame ionization detector and a capillary column (Nukol fused silica capillary column 30 m × 0.25 mm × 0.25 μm, Supelco, Inc., Bellefonte, PA, USA). The temperature program functioned as follows: oven temperature was initially set at 90 °C to 180 °C. The heater temperatures of both the injector and detector were kept at 185 °C and 210, respectively [27]. According to Fawcett and Scott [28], ammonia nitrogen (NH_3-N) measurement was performed using a spectrophotometer (UV/VIS Spectrophotometer, PG Instruments Ltd., London, UK). In the second portion, the pH of the liquid samples was measured using a glass electrode pH meter after being kept at 4 °C for 24 h (Hanna HI-8424 Portable pH/ORP Meter, Woonsocket, RI, USA).

Fermented WBT samples were dried at 60 °C for 48 h in a forced-air drying oven until constant moisture was reached. The samples were ground by forcing a grinder machine through a 1 mm steel screen (Wiley mill, Arthur H. Thomas Co., Philadelphia, PA, USA) prior to chemical analysis. Fermented WBT was determined by dry matter (DM; ID 967.03), ash (ID 492.05), and ether extract (EE; ID 445.08). Crude protein (CP) was analyzed by determining CP percentage (CP; nitrogen content multiplied by 6.25 conversion factor) using a Leco FP-828 P combustion analyzer (Leco FP828 Nitrogen

analyzer, LECO Corporation, MI, USA). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) content were determined using a detergent analysis method [29].

2.4. Inoculum Preparation In Vitro Gas Technique

Rumen liquor was extracted from two rumen-fistulated dairy steers weighing 400 ± 15.0 kg body weight (BW). Following WTSR [30], the steers were fed rice straw ad libitum and supplemented with a concentrated diet (150 g/kg CP). The animal needed to be supplemented with a mineral lick and have continual access to clean water for at least 14 days. A volume of 1500 mL of rumen fluid was obtained from the animals via cannula prior to morning feeding. The rumen fluid inoculum was brought to the lab and filtered through four layers of cheesecloth in a warm (39 °C) chamber while being continuously gassed with CO_2. According to Menke [31], artificial saliva was made by mixing distilled water (1095 mL), micro-mineral (0.23 mL), macro-mineral (365 mL), and buffer solution (730 mL) with rumen fluid (660 mL) in an anaerobic condition (1.00: 3.32, filtered rumen fluid to artificial saliva).

2.5. Dietary Treatments and Design

The Khon Kaen Complete Feed (KCF) 2020 Program based on NRC [32] was used to formulate an experimental diet to meet the nutritional requirements for maintenance of a growing steer using rice straw (roughage source) and a concentrate diet. Dietary treatments were administered in a randomized complete design with a 5 × 2 (+1) factorial arrangement. Factor A was evaluated as a WBT product: a1 = dry WBT (WBT), a2 = fermented WBT without yeast in media solution (WBT-NO), a3 = fermented WBT with *P. kudriavzevii* KKU20 (WBT-P), a4 = fermented WBT with *C. tropicalis* KKU20 (WBT-C), a5 = fermented WBT with *S. cerevisiae* (WBT-S). The level of cassava substitute in the concentrate diet is represented by factor B, which includes b1 and b2, which represent cassava chips replaced by WBT at 50% and 100% of cassava chips, respectively (Figure 1). Factors A and B were arranged in a factorial form, and the control group was compared; the cassava chips were employed as a major ingredient (as +1), and the feed formula did not contain WBT.

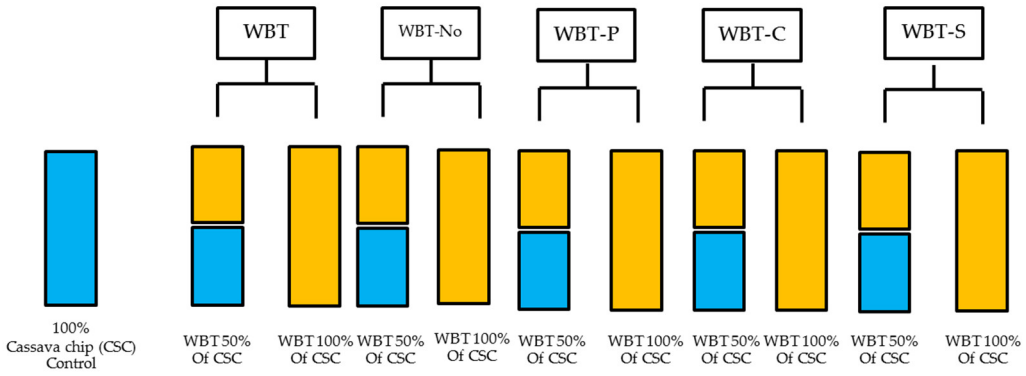

Figure 1. Schematic of the dietary treatments including: cassava chips 100% (control); dry WBT replacing cassava chips 50% (T1), 100% (T2); fermented WBT without yeast replacing cassava chips 50% (T3), 100% (T4); fermented WBT with *P. kudriavzevii* KKU20 replacing cassava chips 50% (T5), 100% (T6); fermented WBT with *C. tropicalis* KKU20 replacing cassava chips 50% (T7), 100% (T8); and fermented WBT with *S. cerevisiae* replacing cassava chips 50% (T9), 100% (T10).

The 50 mL bottles were weighed at 0.5 g of dietary treatment, and a total of 40 mL of rumen liquor medium was added to each treatment bottle using an 18 gauge, 1.5-inch needle. The experimental bottles were categorized into three groups: group 1 for gas

kinetics and production, group 2 for pH, ruminal NH_3-N, volatile fatty acids (VFA), and rumen microorganism count, and group 3 for nutrient degradability measurement. All experimental bottles were sealed with butyl rubber stoppers and aluminum caps before being placed in a hot-air oven at 39 °C for further testing. In group 1, a 20 mL glass aloe precision hypodermic syringe (U4520, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used. An 18-gauge injection needle was used to puncture the bottles. The pH, ruminal NH_3-N, volatile fatty acids (VFA), and rumen microorganism count were measured in the same bottle for group 2. According to Xia et al. [33], there are two times when rumen fermentation activity is at its peak, and the samples were obtained from three distinct duplicates of the bottle at 4 and 8 h of incubation time. Nutrient degradability was evaluated in group 3; samples were obtained from three bottle duplicates at 12 and 24 h after incubation.

2.6. In Vitro Gas Production and Fermentation Characteristics

The amount of gas generated was determined by monitoring and recording the volume of gas produced after incubation with a 100 mL glass syringe connected to the incubation bottle with a 1.5-inch needle [31]. In the same incubation bottle, gas output was measured using a glass syringe at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, 24, 48, 72, and 96 h after incubation. The amount of gas generated was calculated. The bottles were divided into three groups, the first of which was used to determine gas kinetics and production. The second set was used for ruminal parameter measurements at 4 and 8 h post-incubation, including pH (HANNA Instrument (HI) 8424 microcomputer, Hanna Instruments, Inc., Kallang, Singapore), NH_3-N (Kjeldahl methods; [34]), VFA [27], and ruminal microorganism direct counts (Boeckel & Co. GmbH & Co., Hamburg, Germany). The last set was used for the determination of in vitro NDF degradability (IVNDFD), in vitro ADF degradability (IVADFD) [35], and in vitro degradability (IVDMD) [36]. In an Ankom filter bag (ANKOM 200, ANKOM Technology, New York, NY, USA), the residual material was filtered and dried at 60 °C in an oven for 72 h and assessed for IVDMD. To evaluate IVNDFD, dry residues were mixed with a neutral detergent solution and alpha-amylase.

2.7. Statistical Analyses

The following model was used to evaluate data from fermented WBT, including chemical composition, fermentation products, and microbe counts, in a completely randomized design:

$$Yij = \mu + \tau i + \varepsilon ij \quad (1)$$

where Yij = observation, μ = overall mean, τi = effect of treatment (i = dry WBT, fermented WBT without yeast in media solution, fermented WBT with *P. kudriavzevii* KKU20, fermented WBT with *C. tropicalis* KKU20, and fermented WBT with *S. cerevisiae*), and εij = residual error.

An incubation series was performed as described by Schofield et al. [37] for the recording of gas produced, and the data concerning the produced gas were entered into the equation:

$$Vt = Vf \times (1 - \exp^{(-k(t-L))}) \quad (2)$$

where Vt is the volume of gas at time t, Vf is the final asymptotic gas volume corresponding to complete substrate digestion (mL/g DM), t is the incubation time (h), k is a rate constant (units time^{-1}), and L is a discrete lag term (h).

Gas kinetics, ruminal fermentation, and in vitro digestibility of fermented WBT-substituted cassava chips in concentrate diet were examined. Data were measured in a completely randomized design with a 5 × 2 (+1) (type of WBT × WBT proportion) factorial treatment by ANOVA using general linear model (GLM) procedures. The average

treatment value was calculated using the least-square means (LSMEANS) function of the SAS [38] software (version 6.2.9200, Cary, NC, USA), using statistical modeling as follows:

$$Yij = \mu + \alpha i + \beta j + \alpha\beta ij + \varepsilon ij \quad (3)$$

where Yij = observation, μ = overall mean, αi = type of WBT effect (i = WBT without fermentation, fermented WBT without yeast in media solution, fermented WBT with *P. kudriavzevii* KKU20, fermented WBT with *C. tropicalis* KKU20, and fermented WBT with *S. cerevisiae*), βj = level of fermented WBT replacing cassava chips (j = WBT at 50% and 100% levels), $\alpha\beta$ij = type of WBT × WBT proportion, and εij = error. When F-tests were significant, a single degree of freedom orthogonal contrast was used to determine the contrast between factors. Duncan's new multiple-range test (DMRT) was used to determine the difference mean of treatments at $p < 0.05$ [39].

3. Results

3.1. Chemical Contents of Fermented Winged Bean Tubers

All treatments had no effect on the composition of neutral detergent fiber (NDF) and acid detergent fiber (ADF) ($p > 0.05$), but it had an effect on organic matter (OM), ether extract (EE), and crude protein (CP) ($p < 0.01$) (Table 1). Dry WBT had a higher OM than the other groups (963.2 g/kg DM, $p < 0.01$). In terms of process, the OM content of FWBT was about 0.8% lower than that of UWBT ($p < 0.01$). WBT's CP content might be increased by roughly 7% throughout the fermentation process ($p < 0.01$). WBT fermented with yeast contained more OM, particularly when Crabtree-negative yeast was used ($p < 0.01$). When *C. tropicalis* KKU20 was used, the EE was significantly higher ($p < 0.01$), whereas the EE in WBT increased by 52.2% as a result of the fermentation process ($p < 0.01$). However, the yeast strain could not discriminate against the CP in the FWBT group ($p > 0.05$).

Table 1. Chemical contents of the winged bean tubers prior to ensiling and its silage at 30 days after fermentation.

Items	Treatments					Process		Additives		Yeast		SEM	*p-Value*
	WBT	WBT-NO	WBT-P	WBT-C	WBT-S	UWBT	FWBT	No	Yeast	C-	C+		
Dry matter, g/kg	435.2 ^{a}	296.7 ^{b}	258.6 ^{c}	266.3 ^{c}	262.5 ^{c}	435.2 ^{a}	271.0 ^{b}	296.7 ^{a}	262.4 ^{b}	262.43	262.45	5.36	$p < 0.01$
Nutrient content, g/kg DM													
Organic matter	963.2 ^{a}	954.5 ^{c}	960.3 ^{b}	955.5 ^{c}	952.8 ^{d}	963.2 ^{a}	955.8 ^{b}	954.5 ^{b}	956.2 ^{a}	957.9 ^{a}	952.8 ^{b}	0.33	$p < 0.01$
Ether extract	3.79 ^{c}	5.43 ^{b}	5.46 ^{b}	6.69 ^{a}	5.51 ^{b}	3.79 ^{b}	5.77 ^{a}	5.43	5.88	6.07	5.51	0.22	$p < 0.01$
Crude protein	199.1 ^{b}	208.1 ^{ab}	213.7 ^{a}	217.7 ^{a}	212.7 ^{a}	199.1 ^{b}	213.1 ^{a}	208.1	214.7	215.7	212.7	3.46	0.03
Neutral detergent fiber	180.3	180.7	173.1	176.5	181.6	180.3	178.0	180.7	177.1	174.8	181.6	2.35	0.19
Acid detergent fiber	65.2	63.6	56.9	62.4	63.2	65.2	61.6	63.6	60.9	59.7	63.2	2.48	0.30

WBT: dry winged bean tubers; WBT-NO: fermented winged bean tubers without yeast; WBT-P: fermented winged bean tubers with *Pichia kudriazevii* KKU20; WBT-C: fermented winged bean tubers with *Candida tropicalis* KKU20; WBT-S: fermented winged bean tubers with *Saccharomyces cerevisiae*; FWBT: fermented winged bean tubers; C-: fermented winged bean tubers with ruminal yeast *(Pichia kudriazevii* KKU20/*Candida tropicalis* KKU20); C+: fermented winged bean tubers with *Saccharomyces cerevisiae*; SEM: standard error of the mean; $^{a–d}$ means in the same row with different lowercase letters differ ($p < 0.05$, $p < 0.01$).

3.2. Fermentation Characteristics and Microbial Counts in Products

WBT-S had a substantially higher pH (5.56) than the other groups, but WBT-P had the lowest pH (4.28, $p < 0.01$) (Table 2). On day seven of the fermentation process, WBT-P had the lowest pH value, while WBT-S had the highest ($p < 0.01$). The pH of WBT was marginally higher with yeast than without yeast (4.78 vs. 4.62; $p < 0.01$). Furthermore, the pH of fermented winged bean tubers with Crabtree-negative yeast was greater than that

of fermented winged bean tubers with Crabtree-positive yeast (4.39 vs. 5.56; $p < 0.01$). WBT-S had larger amounts of NH_3-N than the other treatments, while WBT-P had the lowest levels ($p < 0.01$). NH_3-N was found to be higher in yeast-fermented WBT than in WBT-NO ($p < 0.01$). Crabtree-negative yeast reduced NH_3-N. In winged bean tubers, WBT-NO had the same lactic acid content as the yeast inoculated group ($p > 0.05$). Inoculation of *P. kudriazevii* KKU20 into WBT (WBT-P) increased lactic acid by approximately 33.7 and 30.0% when compared to *S. cerevisiae* (WBT-S) and *C. tropicalis* KKU20 (WBT-C), respectively ($p < 0.01$). In additives, when compared to WBT-NO, fermented WBT reduced acetic acid by about 18.5% ($p < 0.05$). Except for Crabtree-positive yeast ($p < 0.01$), propionic acid levels were less than 0.10 g/kg DM in all treatments, and butyric acid was not detected in this study. The lactic acid bacteria (LAB) in WBT develop quicker than those in the other groups. On the other hand, *P. kudriazevii* KKU20 raised the population of LAB more than other yeast strains ($p < 0.01$) and was equivalent to WBT-NO ($p > 0.05$). In the inoculated yeast group, the yeast population increased significantly ($p < 0.01$), from Log10 3.32 to Log10 5.15 cfu/g fresh matter, counteracting a decrease in the population of aerobic bacteria ($p < 0.01$).

Table 2. The effect of fermented winged bean tubers for 7 days with various yeast species on the alteration of fermentation characteristics and microbial populations.

Items	Treatments				Additives		Yeast		SEM	*p-Value*
	WBT-NO	WBT-P	WBT-C	WBT-S	No	Yeast	C-	C+		
pH	4.62 ^{b}	4.28 ^{d}	4.49 ^{c}	5.56 ^{a}	4.62 ^{b}	4.78 ^{a}	4.39 ^{b}	5.56 ^{a}	0.05	$p < 0.01$
Organic acids content (g/kg DM)										
Ammonia nitrogen	0.82 ^{c}	0.73 ^{c}	0.96 ^{b}	1.29 ^{a}	0.82 ^{b}	0.99 ^{a}	0.85 ^{b}	1.29 ^{a}	0.02	$p < 0.01$
Lactic acid	4.87 ^{ab}	5.63 ^{a}	4.33 ^{b}	4.21 ^{b}	4.87	4.73	4.98	4.21	0.23	0.038
Acetic acid	2.05	1.70	1.64	1.67	2.05 ^{a}	1.67 ^{b}	1.67	1.67	0.08	0.053
Propionic acid	0.08 ^{b}	0.08 ^{b}	ND	0.31 ^{a}	0.08 ^{b}	0.13 ^{a}	0.04 ^{b}	0.31 ^{a}	0.01	$p < 0.01$
Microbial count Log10 (cfu/g fresh matter)										
Lactic acid bacteria	8.67 ^{a}	8.72 ^{a}	8.23 ^{b}	8.33 ^{b}	8.67 ^{a}	8.43 ^{b}	8.48 ^{a}	8.33 ^{b}	0.03	$p < 0.01$
Yeast	3.32 ^{b}	5.59 ^{a}	4.95 ^{a}	4.91 ^{a}	3.32 ^{b}	5.15 ^{a}	5.27	4.91	0.19	$p < 0.01$
Aerobic bacteria	3.05 ^{a}	1.09 ^{b}	1.26 ^{b}	1.32 ^{b}	3.05 ^{a}	1.22 ^{b}	1.17	1.32	0.06	$p < 0.01$
Coliform	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

WBT-NO: fermented winged bean tubers without yeast; WBT-P: fermented winged bean tubers with *Pichia kudriazevii* KKU20; WBT-C: fermented winged bean tubers with *Candida tropicalis* KKU20; WBT-S: fermented winged bean tubers with *Saccharomyces cerevisiae*; C-: Crabtree-negative yeast; C+: Crabtree-positive yeast; SEM: standard error of the mean; $^{a–d}$ means in the same row with different lowercase letters differ ($p < 0.05$, $p < 0.01$), ND: not detected.

3.3. In Vitro Gas Production and the Model Parameters

The WBT had less influence on increasing the gas production pattern than the cassava chips. In the absence of a fermentation process, the WBT group had lower GP line curve values than the other groups ($p < 0.01$) (Figure 2). In contrast, when yeast was introduced into winged bean tubers, GP increased by 22.1% closer to cassava chip GP, and by 11.3% even after yeast-free fermentation ($p < 0.01$). Table 3 depicts the kinetics of gas models as a final asymptotic gas volume (*Vf*), a fractional rate of gas production (*k*), and a discrete lag term (*L*). Interactions exist between yeast species and the proportion of cassava chip substitutions. When cassava chips were totally substituted in the diet, WBT-P exhibited a substantially greater *Vf* of gas generation ($p < 0.01$) than the other groups. *Vf* was reduced to a bare minimum ($p < 0.01$) when 100% WBT was used in the diet. The statistical results of the comparison revealed that the fermentation process, specifically WBT fermentation with yeast, increased *Vf* by up to 49.7% ($p < 0.01$). Furthermore, when yeast groups were compared, Crabtree-positive yeast had a higher *Vf* value than Crabtree-negative yeast ($p < 0.01$). *P. kudriazevii* KKU20 had a 17.3% higher *Vf* than *C. tropicalis* KKU20 in the

Crabtree-negative yeast group. The fractional rate of gas production (*k*) in WBT-substituted cassava chips is the highest at both 50% and 100% ($p < 0.01$). Unimproved WBT had higher *k* values than the fermentation group, in contrast to *Vf*. The fermented WBT without yeast in the media solution had a higher *k* value than the inoculated yeast groups ($p < 0.01$). The usage of the cassava chips and WBT had no influence on the change in discrete L, nor on the interaction between yeast species and the proportion of cassava chip replacements ($p > 0.05$). Crabtree-positive yeast, on the other hand, had greater L than Crabtree-negative yeast ($p < 0.01$). In the Crabtree-negative yeast group, *P. kudriazevii* KKU20 had greater L than *C. tropicalis* KKU20 ($p < 0.05$).

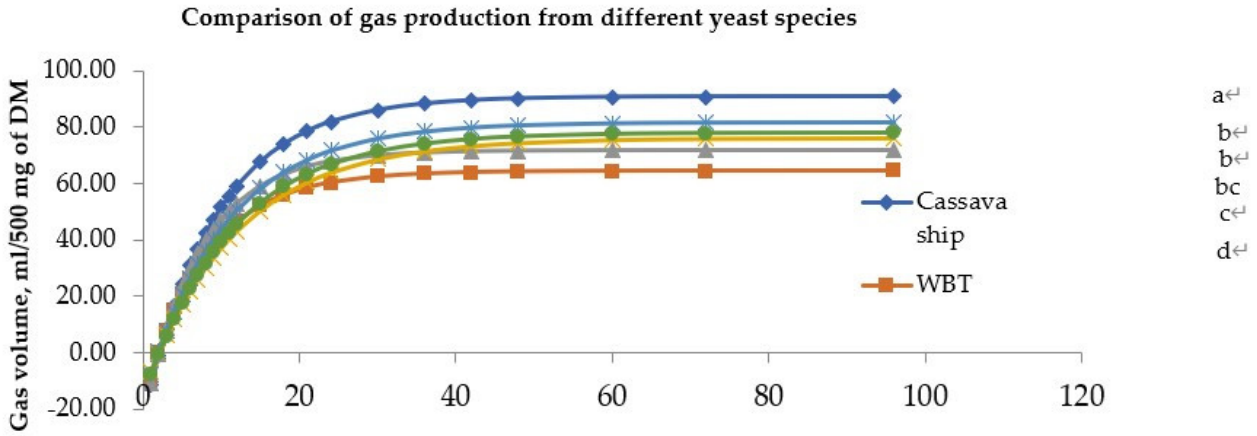

Figure 2. Cumulative gas production curves for fermented winged bean tubers over the incubation period (0 to 96 h) from the observed data fitted by gas production model. WBT: dry winged bean tuber; WBT−NO: fermented winged bean tubers without yeast; WBT−P: fermented winged bean tubers without *Pichia kudriazevii* KKU20; WBT−C: fermented winged bean tubers without *Candida tropicalis* KKU20; WBT−S: fermented winged bean tubers without *Saccharomyces cerevisiae.*

Table 3. The effect of fermented winged bean tubers with various yeast species and cassava substitution levels on the kinetics of gas production.

Yeast Species	WBT Level	Gas Kinetics [1]		
		Vf	***k***	***L***
Cassava chips	100	86.0 ^{cd}	0.110	2.09
WBT	50	61.5 ^{f}	0.134 ^{a}	2.00
	100	52.3 ^{g}	0.131 ^{a}	2.00
WBT-NO	50	88.0 ^{bcd}	0.124 ^{ab}	2.07
	100	55.9 ^{fg}	0.144 ^{a}	2.03
WBT-P	50	91.7 ^{abcd}	0.074 ^{c}	1.97
	100	96.4 ^{a}	0.096 ^{bc}	2.05
WBT-C	50	85.3 ^{c}	0.102b ^{c}	2.00
	100	75.0 ^{e}	0.089 ^{c}	1.85
WBT-S	50	95.6 ^{ab}	0.091 ^{c}	2.13
	100	93.4 ^{abc}	0.085 ^{c}	2.07
SEM		2.50	0.01	0.04
Cassava chips vs. Others		0.0214	0.96	0.07
Interaction		$p < 0.01$	$p < 0.01$	0.47
Yeast species	Comparison			
	WBT	56.9	0.133	2.00 ^{bc}
	WBT-NO	72.0	0.134	2.05 ^{ab}
	WBT-P	94.1	0.085	2.01 ^{b}
	WBT-C	80.2	0.096	1.93 ^{c}

WBT level	WBT-SC	94.5	0.088	2.10 [a]
	50	84.4	0.105	2.03
	100	74.6	0.109	2.00
Contrast 1	Unfermented	56.9 [b]	0.133 [a]	2.00
	Fermented	85.2 [a]	0.101 [b]	2.02
Contrast 2	No yeast	71.9 [b]	0.134 [a]	2.05
	With yeast	89.6 [a]	0.089 [b]	2.01
Contrast 3	Crabtree-negative	87.1 [b]	0.090	1.97 [b]
	Crabtree-positive	94.5 [a]	0.088	2.10 [a]
Contrast 4	*P. kudriazevii* KKU20	94.1 [a]	0.085	2.01 [a]
	C. tropicalis KKU20	80.2 [b]	0.096	1.93 [b]

WBT: dry winged bean tubers; WBT-NO: fermented winged bean tubers without yeast; WBT-P: fermented winged bean tubers with *Pichia kudriazevii* KKU20; WBT-C: fermented winged bean tubers with *Candida tropicalis* KKU20; WBT-S: fermented winged bean tubers with *Saccharomyces cerevisiae*; C-: Crabtree-negative yeast; C+: Crabtree-positive yeast; SEM: standard error of the mean; [a–g] means in the same row with different lowercase letters differ ($p < 0.05$, $p < 0.01$),
[1]Gas kinetics including: *Vf* = final asymptotic gas volume corresponding to complete substrate digestion (mL/500 mg DM), *k* = fractional rate of gas production (units time^{-1}), *L* = a discrete lag term (h).

3.4. In Vitro Degradability

The IVDMD, in vitro organic matter digestibility (IVOMD), and in vitro neutral detergent fiber digestibility (IVNDFD) of fermented winged bean tubers with various yeast species and cassava substitution levels are shown in Table 4. An interaction between yeast strains and cassava substitution levels was observed for the IVDMD and IVOMD at 12 h ($p < 0.01$) and only for the IVDMD at 24 h ($p < 0.05$). At 12 h of incubation, WBT-P replacing cassava chips at 50% had the greatest amounts of IVDMD and IVOMD, whereas WBT had the lowest degradability ($p < 0.01$). At 24 h, the degradability of DM in WBT remained low, while WBT-S was more effective than the other groups ($p < 0.01$). When cassava chips were 100% of the diet, IVNDFD was low at 12 h of incubation, but it reached a value that was comparable to the other groups at 24 h ($p < 0.01$). The statistics of the contrast indicated that improving the WBT process by fermentation enhanced the degradability of DM and OM by around 7.5% and 4.1%, respectively, at 12 h of incubation time ($p < 0.01$). Even at 24 h of incubation, the degradability of DM was still 2.25% higher than the unfermented group ($p < 0.05$). After 12 h of incubation, Crabtree-negative yeast had a greater IVDMD than Crabtree-positive yeast (593.5 vs. 546.4 g/kg DM; $p < 0.01$). Fermented WBT with yeast had a higher IVDMD after 24 h of incubation than unfermented WBT ($p < 0.05$).

Table 4. The effect of fermented winged bean tubers with various yeast species and cassava substitution levels on in vitro degradability.

Yeast Species	WBT Level	Degradability at 12 h (g/kg Dry Matter)			Degradability at 24 h (g/kg Dry Matter)		
		IVDMD	IVOMD	IVNDFD	IVDMD	IVOMD	IVNDFD
Cassava chips	100	541.5	589.2	469.9 [b]	712.8	767.3	609.8
WBT	50	543.1 [de]	566.1 [bc]	522.9 [a]	686.4 [d]	729.1	633.0
	100	528.5 [e]	563.5 [c]	523.0 [a]	706.1 [bc]	743.8	634.5
WBT-NO	50	548.5 [de]	571.6 [bc]	538.0 [a]	698.6 [cd]	744.0	640.8
	100	593.9 [abc]	592.2 [ab]	511.6 [a]	698.1 [cd]	748.9	628.8
WBT-P	50	617.9 [a]	604.2 [a]	531.7 [a]	714.5[abc]	767.6	614.9
	100	574.3 [abcd]	598.7 [a]	533.6 [a]	723.3[ab]	764.1	663.3
WBT-C	50	611.0 [ab]	586.9 [abc]	541.3 [a]	721.1[ab]	770.5	645.9
	100	570.5 [abcd]	587.6 [abc]	520.5 [a]	713.7[abc]	750.2	642.2
WBT-S	50	542.5 [de]	584.6 [abc]	523.5 [a]	725.3[a]	737.6	625.1

	100	550.3 ^{de}	580.8 ^{abc}	517.5 ^{a}	700.7^{cd}	749.7	640.0
SEM		12.5	9.36	15.1	7.1	13.5	19.8
Cassava chips vs. Others		0.07	0.58	$p < 0.01$	0.60	0.27	0.37
Interaction		$p < 0.01$	$p < 0.01$	0.74	$p < 0.05$	0.19	0.81
		Comparison					
Yeast species	WBT	535.8	564.8	523.0	696.2	737.5	633.8
	WBT-NO	571.2	581.9	524.8	698.3	736.4	634.8
	WBT-P	596.1	601.4	532.6	718.9	765.8	639.1
	WBT-C	590.8	587.3	530.9	717.4	760.4	644.0
	WBT-SC	546.4	582.7	520.5	713.0	743.7	632.5
WBT level	50	572.6	582.7	531.5	709.2	749.8	631.9
	100	563.5	584.6	521.2	708.4	751.3	641.8
Contrast 1	Unfermented	535.8 ^{b}	564.8 ^{b}	523.0	696.2 ^{b}	736.5	633.8
	Fermented	576.1 ^{a}	588.3 ^{a}	527.2	711.9 ^{a}	754.1	637.6
Contrast 2	No yeast	571.2	581.9	524.8	698.3 ^{b}	746.4	634.8
	With yeast	577.8	590.5	528.0	716.4 ^{a}	756.6	638.6
Contrast 3	Crabtree-negative	593.5 ^{a}	594.3	531.7	718.1	763.1	641.6
	Crabtree-positive	546.4 ^{b}	582.7	520.5	713.0	743.7	632.5
Contrast 4	*P. kudriazevii* KKU20	596.1	601.4	532.6	718.9	765.8	639.1
	C. tropicalis KKU20	590.8	587.3	530.9	717.4	760.4	644.0

WBT: dry winged bean tubers; WBT-NO: fermented winged bean tubers without yeast; WBT-P: fermented winged bean tubers with *Pichia kudriazevii* KKU20; WBT-C: fermented winged bean tubers with *Candida tropicalis* KKU20; WBT-S: fermented winged bean tubers with *Saccharomyces cerevisiae*; C-: Crabtree-negative yeast; C+: Crabtree-positive yeast; SEM: standard error of the mean; $^{a–e}$ means in the same row with different lowercase letters differ ($p < 0.05$, $p < 0.01$).

3.5. In Vitro Volatile Fatty Acid

On total volatile fatty acids (TVFA), the cassava chips (CVC) produced significant TVFA values, and the augmentation of WBT utilizing yeast in combination with a 50% CVC substitution yielded similar findings ($p > 0.05$; Figure 3). There are interactions between yeast species and the amount of cassava chip replacements in WBT fermented with yeast. WBT was improved by fermenting with the yeast strains *P. kudriazevii* KKU20, *C. tropicalis* KKU20, and *S. cerevisiae* and substituting 50% cassava chips in the diet, which led to a higher rise in TVFA than the other groups (TVFA ranged from 78.9 to 79.5 mmol/L; $p < 0.05$). TVFA was lowest when WBT was not enhanced by fermentation, as compared to WBT (TVFA ranged from 65.8 to 69.1 mmol/L; $p < 0.05$). The interaction of fermented winged bean tubers without yeast and cassava substitution levels resulted in the greatest ($p < 0.05$) concentrations of acetic acid (C2) and butyric acid (C4) (Table 5). The use of WBT as a 50% and 100% replacement of cassava in the diet resulted in a higher C2 concentration than other groups at 8 h of incubation time, including the use of WBT-NO as a 100% replacement, which caused this incident ($p < 0.01$). In the statistics of contrast, the mean of C2 showed that unfermented WBT caused C2 to be 8.04% greater than fermented WBT ($p < 0.01$). Furthermore, fermented WBT with yeast increased the concentration of C2 at 4 h of incubation ($p < 0.05$). However, there was no significant difference between the C2 concentration at 8 h and the mean value ($p > 0.05$). At 4 h of incubation, the use of WBT at 50% and WBT-NO at 50% and 100% replacement of cassava chips increased C4 concentrations more than the other treatments ($p < 0.01$). The mean value of C4 concentrations was still high when WBT-NO was replacing cassava chips at 50% in the diet ($p < 0.05$). The amount of C4 was certainly high when WBT was not fermented (11.2–12.7 mol/100mol) or fermented without yeast (10.9–13.5 mol/100mol) ($p < 0.01$).

The cassava chips (CVC) generated a significant amount of propionic acid, while yeast-enhanced WBT yielded comparable findings ($p > 0.05$). For the concentration of propionic acid (C3), an interaction between yeast strains and cassava substitution levels was identified ($p < 0.01$, Figure 4). Fermented WBT with *C. tropicalis* KKU20 increased C3 concentrations (C3 range from 26.0 to 26.4 mol/100 mol; $p < 0.01$), but when WBT was not

enhanced by the fermentation process, C3 declined (C3 range from 19.9 to 20.4 mol/100mol; $p < 0.01$).

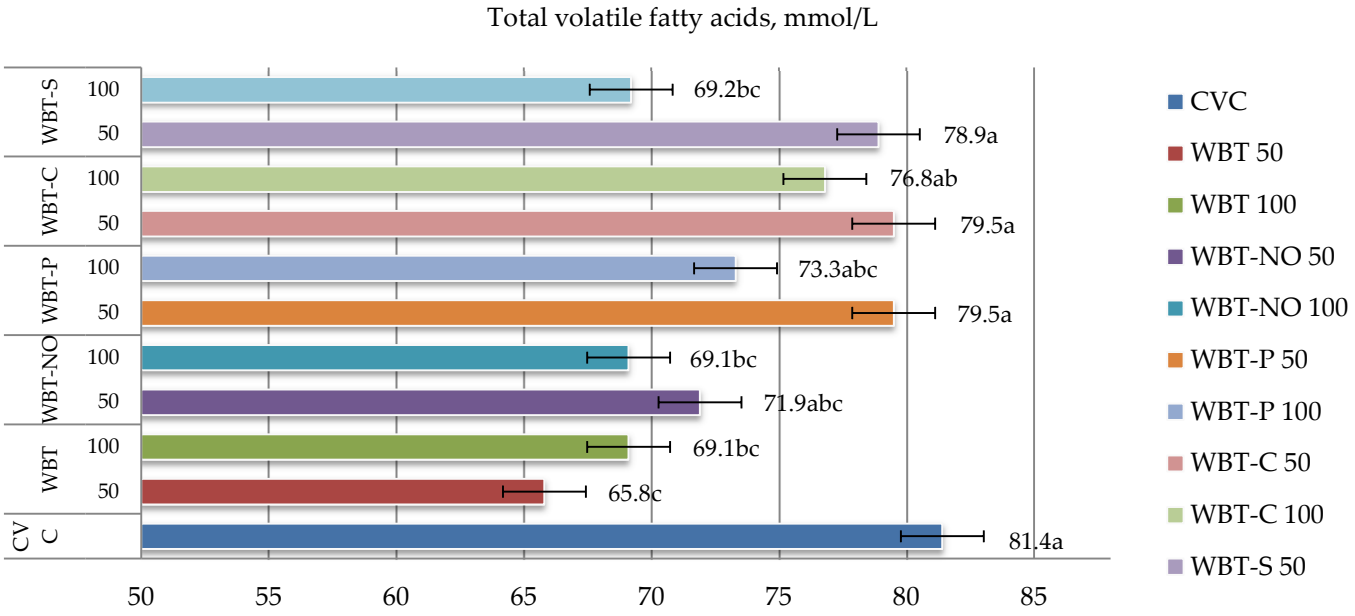

Figure 3. The effect of fermented winged bean tubers with various yeast species and cassava substitution levels on the average of total volatile fatty acids (between 4 h and 8 h). WBT: dry winged bean tubers; WBT-NO: fermented winged bean tubers without yeast; WBT-P: fermented winged bean tubers without *Pichia kudriazevii* KKU20; WBT-C: fermented winged bean tubers without *Candida tropicalis* KKU20; WBT-S: fermented winged bean tubers without *Saccharomyces cerevisiae*; CVC: cassava chips 100%. Interaction between yeast species × cassava substitution levels = $p < 0.01$; SEM = 1.88. $^{a–c}$ means in the same row with different lowercase letters differ ($p < 0.05$, $p < 0.01$).

Table 5. The effect of fermented winged bean tubers with various yeast species and cassava substitution levels on acetic acid and butyric acid.

Yeast Species	WBT Level	Acetic Acid, mol/100 mol			Butyric Acid, mol/100 mol		
		4 h	8 h	Mean	4 h	8 h	Mean
Cassava chips	100	63.2	65.2	64.2	10.3	8.3	9.3
WBT	50	66.5	70.8 ^{a}	68.6	13.3 ^{a}	9.7	11.5 ^{ab}
	100	65.6	71.6 ^{a}	68.6	12.1 ^{ab}	9.8	10.9 ^{abc}
WBT-NO	50	65.2	65.1 ^{b}	65.2	13.1 ^{a}	12.4	12.8 ^{a}
	100	62.2	70.7 ^{a}	66.4	13.8 ^{a}	9.3	11.6 ^{ab}
WBT-P	50	66.3	65.3 ^{b}	65.8	10.2 ^{bcd}	8.9	9.5 ^{bc}
	100	69.6	64.1 ^{b}	66.8	8.7 ^{d}	9.2	8.9 ^{c}
WBT-C	50	64.4	65.1 ^{b}	64.7	9.9 ^{cd}	8.5	9.2 ^{bc}
	100	65.1	64.9 ^{b}	65.0	8.5 ^{d}	8.8	8.6 ^{c}
WBT-S	50	66.0	64.8 ^{b}	65.4	11.1 ^{bc}	8.5	9.b ^{c}
	100	67.2	67.6 ^{ab}	67.4	8.d	9.9	9.3 ^{bc}
SEM		1.24	1.33	1.02	0.96	1.04	0.68
Cassava chips vs. Others		0.12	0.22	0.06	0.29	0.30	0.21
Interaction		0.82	$p < 0.01$	0.18	$p < 0.01$	0.69	$p < 0.05$
Comparison							
Process	WBT	66.0	71.2	68.6	12.7	9.8	11.2
	WBT-NO	63.7	67.9	65.8	13.5	10.9	12.2
	WBT-P	68.0	64.7	66.3	9.4	9.0	9.2
	WBT-C	64.7	65.0	64.9	9.2	8.6	8.9
	WBT-SC	66.6	66.2	66.4	9.9	9.2	9.6
WBT level	50	65.7	66.2	65.9	11.5	9.6	10.6
	100	65.9	67.8	66.9	10.4	9.4	9.9
Contrast 1	Unfermented	66.0	71.2 ^{a}	68.6 ^{a}	12.7 ^{a}	9.8	11.2 ^{a}
	Fermented	65.8	65.9 ^{b}	65.9 ^{b}	10.5 ^{b}	9.4	9.9 ^{b}
Contrast 2	No yeast	63.7 ^{b}	67.9	65.8	13.5 ^{a}	10.9 ^{a}	12.2 ^{a}
	With yeast	66.4 ^{a}	65.3	65.9	9.5b	8.9 ^{b}	9.2 ^{b}
Contrast 3	Crabtree-negative	66.4	64.8	65.6	9.3	8.8	9.1
	Crabtree-positive	66.6	66.2	66.4	9.9	9.2	9.6
Contrast 4	*P. kudriazevii* KKU20	68.0	64.7	66.3	9.4	9.0	9.2
	C. tropicalis KKU20	64.7	65.0	64.9	9.2	8.6	8.9

WBT: dry winged bean tubers; WBT-NO: fermented winged bean tubers without yeast; WBT-P: fermented winged bean tubers with *Pichia kudriazevii* KKU20; WBT-C: fermented winged bean tubers with *Candida tropicalis* KKU20; WBT-S: fermented winged bean tubers with *Saccharomyces cerevisiae*; C-: Crabtree-negative yeast; C+: Crabtree-positive yeast; SEM: standard error of the mean; $^{a–d}$ means in the same row with different lowercase letters differ ($p < 0.05$, $p < 0.01$).

3.6. pH, Ammonia-Nitrogen and Bacteria Populations in the Rumen

When fermented winged bean tubers were employed as a substrate, the yeast strains altered the pH, NH_3-N concentrations (Table 6), and bacterial population in the rumen (Figure 5) ($p < 0.01$). No alteration of rumen pH was found between the control group and the other groups ($p > 0.05$). However, fermented WBT had a lower pH than unfermented WBT after 4 h of incubation ($p < 0.01$). After 8 h of incubation, *P. kudriazevii* KKU20 maintains a slightly higher pH (7.03) in the rumen than *C. tropicalis* KKU20 (6.99) ($p < 0.05$). The level of NH_3-N in the rumen fluid was reported as an interaction among treatments ($p < 0.01$ at 4 h, $p < 0.05$ at 8 h, and $p < 0.05$ at the mean). Fermented WBT used at 100% in place of cassava chips resulted in increased NH_3-N at 4 h after incubation compared to the control group ($p < 0.01$). At 8 h after incubation, WBT-P and WBT-S had roughly 25% higher NH_3-N concentrations than the control group ($p < 0.01$). At the mean value, using only the cassava chips in the formulation resulted in the lowest NH_3-N value, which was close to that of 50% replacement of WBT or WBT-NO ($p < 0.01$). Furthermore, WBT fermentation is proven to enhance the concentration of NH_3-N at every time interval ($p <$

0.05). The mean number of rumen bacteria populations changed between conditions ($p < 0.01$). WBT fermentation resulted in a 3.5% increase in the rumen bacterial population compared to the unfermented group. WBT fermented with yeast increased rumen bacteria by 3.9% higher than WBT fermented without yeast. Furthermore, Crabtree-negative yeast isolated from the rumen is more efficient than Crabtree-positive yeast at stimulating rumen bacteria ($p < 0.01$).

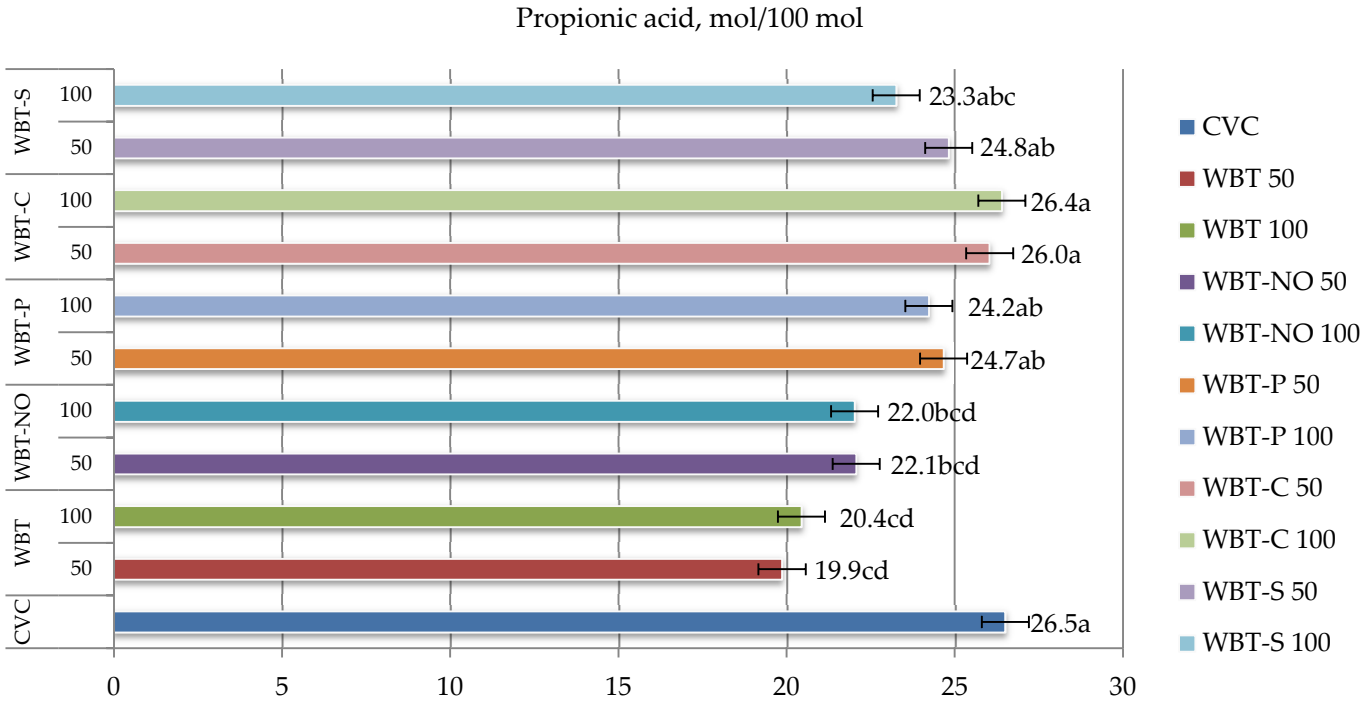

Figure 4. The effect of fermented winged bean tubers with various yeast species and cassava substitution levels on the average of propionic acids (between 4 h and 8 h). WBT: dry winged bean tubers; WBT-NO: fermented winged bean tubers without yeast; WBT-P: fermented winged bean tubers without *Pichia kudriazevii* KKU20; WBT-C: fermented winged bean tubers without *Candida tropicalis* KKU20; WBT-S: fermented winged bean tubers without *Saccharomyces cerevisiae*; CVC: cassava chips 100%. Interaction between yeast species × cassava substitution levels = $p < 0.01$; SEM = 0.99. $^{a–d}$ means in the same row with different lowercase letters differ ($p < 0.05$, $p < 0.01$).

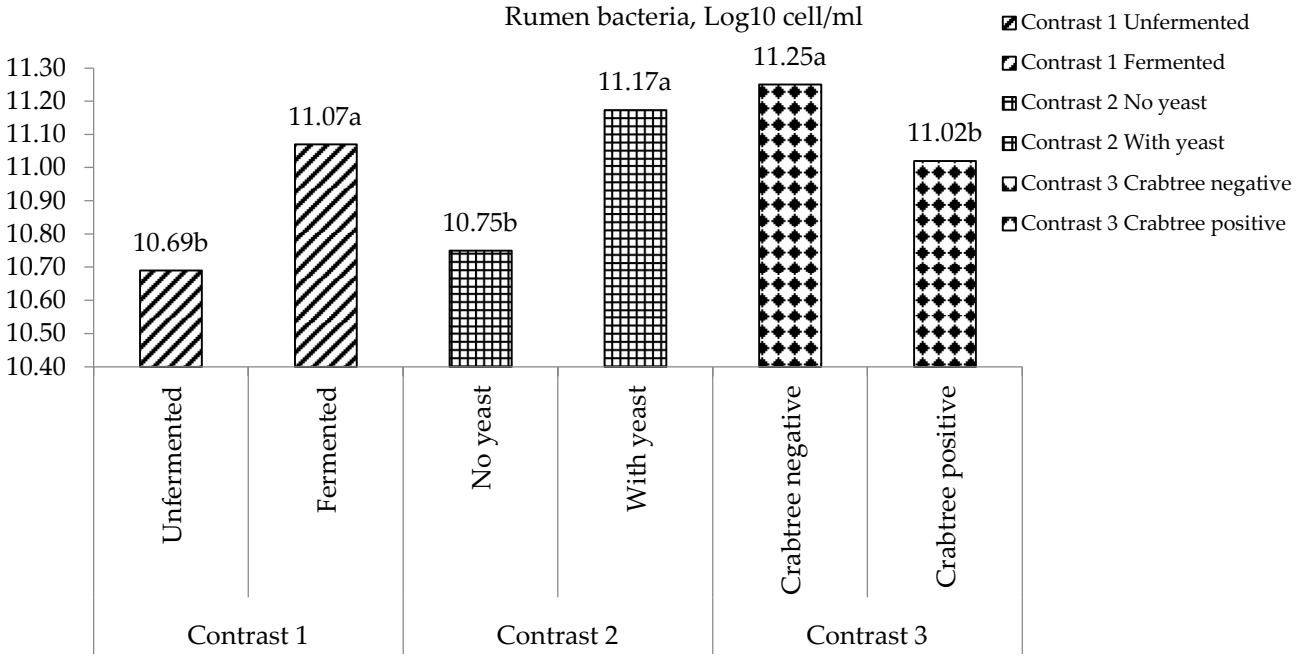

Figure 5. The effect of fermented winged bean tubers with various yeast species and cassava substitution levels on the bacteria population in the rumen. Unfermented: unfermented winged bean tubers; Fermented: fermented winged bean tubers; No yeast: fermented winged bean tubers with no yeast; With yeast: fermented winged bean tubers with yeast; Crabtree-negative: fermented winged bean tubers with ruminal yeast *(Pichia kudriazevii* KKU20/*Candida tropicalis* KKU20); Crabtree-positive: fermented winged bean tubers with *Saccharomyces cerevisiae.* Unfermented × Fermented = $p < 0.01$; No yeast × With yeast = $p < 0.01$; Crabtree-negative × Crabtree-positive = $p < 0.01$, SEM = 0.06. $^{a–b}$ means in the same row with different lowercase letters differ ($p < 0.05$, $p < 0.01$).

Table 6. The effect of fermented winged bean tubers with various yeast species and cassava substitution levels on pH and ammonia nitrogen in rumen.

Yeast Species	WBT Level	pH			Ammonia Nitrogen mg/dL		
		4 h	8 h	Mean	4 h	8 h	Mean
Control	100	7.12	7.00	7.06	14.71 ^{d}	17.95 ^{c}	16.33 ^{c}
WBT	50	7.11 ^{ab}	7.01	7.06	15.23 ^{d}	18.70 ^{bc}	16.96 ^{c}
	100	7.13 ^{a}	7.00	7.06	16.06 ^{cd}	18.85 ^{bc}	17.46 ^{b}
WBT-NO	50	7.09 ^{ab}	7.00	7.04	15.83 ^{d}	20.04 ^{abc}	16.86 ^{c}
	100	7.08 ^{b}	7.00	7.06	18.95 ^{abc}	21.29 ^{abc}	20.64 ^{ab}
WBT-P	50	7.10 ^{ab}	7.06	7.08	15.71 ^{d}	22.17 ^{ab}	20.81 ^{ab}
	100	7.07 ^{b}	7.01	7.04	19.22 ^{ab}	22.71 ^{a}	20.96 ^{ab}
WBT-C	50	7.11 ^{ab}	6.99	7.05	16.75 ^{bcd}	20.53 ^{abc}	18.64 ^{abc}
	100	7.08 ^{ab}	7.00	7.04	20.59 ^{a}	21.37 ^{abc}	20.98 ^{ab}
WBT-S	50	7.08 ^{ab}	6.99	7.04	17.05 ^{bcd}	20.52 ^{abc}	18.78 ^{abc}
	100	7.08 ^{ab}	7.02	7.05	20.68 ^{a}	22.47 ^{ab}	21.50 ^{a}
SEM		0.01	0.01	0.01	0.76	0.91	0.99
Control vs. Others		0.06	0.69	0.45	$p < 0.01$	0.022	0.016
Interaction		$p < 0.01$	0.70	0.11	$p < 0.01$	0.022	0.016
		Comparison					
Process	WBT	7.12	7.00	7.06	15.65	18.77	17.21
	WBT-NO	7.08	7.00	7.05	17.39	20.67	18.75
	WBT-P	7.08	7.03	7.06	17.46	22.44	20.89

	WBT-C	7.10	6.99	7.04	18.67	20.95	19.81
	WBT-SC	7.08	7.01	7.04	18.87	21.49	20.14
Level of WBT	50	7.10	7.01	7.05	16.11	20.39	18.41
	100	7.09	7.01	7.05	19.10	21.34	20.31
Contrast 1	Unfermented	7.12 [a]	7.00	7.06	15.65 [b]	18.77 [b]	17.21 [b]
	Fermented	7.08 [b]	7.01	7.05	18.10 [a]	21.39 [a]	19.90 [a]
Contrast 2	No yeast	7.08	7.00	7.05	17.39	20.67	18.75
	With yeast	7.09	7.01	7.05	18.33	21.63	20.28
Contrast 3	Crabtree-negative	7.09	7.01	7.05	18.07	21.70	20.35
	Crabtree-positive	7.08	7.01	7.04	18.87	21.49	20.14
Contrast 4	*P. kudriazevii* KKU20	7.08	7.03 [a]	7.06	17.46	22.44	20.89
	C.tropicalis KKU20	7.10	6.99 [b]	7.04	18.67	20.95	19.81

WBT: unfermented winged bean tubers; WBT-NO: fermented winged bean tubers without yeast; WBT-P: fermented winged bean tubers with *Pichia kudriazevii* KKU20; WBT-C: fermented winged bean tubers with *Candida tropicalis* KKU20; WBT-S: fermented winged bean tubers with *Saccharomyces cerevisiae*; C-: Crabtree-negative yeast; C+: Crabtree-positive yeast; SEM: standard error of the mean; [a–d] means in the same row with different lowercase letters differ ($p < 0.05$, $p < 0.01$).

4. Discussion

In this study, it was discovered that the fermentation process improved the chemical composition of WBT. Crabtree-negative yeast as an inoculant and urea as additives were used to improve the fermentation process. The increase in WBT CP was primarily due to urea-based fermentation, and the numerical supplementation of a feedstuff with urea has previously been studied. Kang et al. [40] used urea 10 g/L solution as one of the main ingredients to boost protein content in cassava top silage. This amount can dramatically enhance the protein content in an animal's feed from 220 to 325 g/kg. Wanapat et al. [41] found that treating rice straw with a 55 g/L urea solution increased protein from 25 g/kg to 78 g/kg. The protein content of WBT rose from 190 g/kg to 210 g/kg in this research, which was remarkably comparable to ours. The amount of CP is determined by the solution used to prepare the feedstuffs. As a result, while the protein level was higher, it was not as high as in the previous study. This is due to the modest amount of media solution that was employed to optimize the WBT moisture content. When there is enough carbon skeleton, yeast cells begin to use the N source from urea to build proteins [42,43]. In yeast cells, nitrogen is predominantly required to build different amino acids, followed by cell components such as proteins and nucleic acids [44,45]. The main source of the increased organic matter in yeast-fermented WBT is the transfer of inorganic nitrogen from urea into cell structures such as protein. Furthermore, due to their more efficient cell proliferation potential, Crabtree-negative yeasts can provide higher OM content than Crabtree-positive yeasts. Suntara et al. [25] found that the OM content of rice straw fermented by *P. kudriazevii* KKU20, a Crabtree-negative yeast, was greater than that of Crabtree-positive yeast (880 g/kg DM vs. 871 g/kg DM). Crabtree-negative yeast is well-known for its ability to multiply cells, particularly when the solution contains an abundance of glucose [21].

Wardrop et al. [46] investigated the influence of glucose levels ranging from 1 to 50 g/L on the biomass conversion of Crabtree-negative yeast, finding that Crabtree-negative yeast provided seven times more biomass than Crabtree-positive yeast. In the present study, 250 g/kg of sugarcane molasses was used as a carbon source, as sugarcane molasses contains 90 g/kg of glucose, according to Suntara et al. [23]; thus, glucose levels were around 22.5 g/L in this study and probably inhibited the activity of *S. cerevisiae* so that it could be compared with the Crabtree-negative yeast. Under aerobic conditions, respiration is possible with oxygen as the final electron acceptor, but *S. cerevisiae* inhibits the activity of the pyruvate dehydrogenase complex (PDH) enzymes and exhibits alcoholic fermentation until the sugar reaches a low level. The yeasts expressing this trait

are called "Crabtree-positive yeasts" [45]. In contrast, Crabtree-negative yeast can supply glucose to the mitochondria via PDH bypass, and because Crabtree-positive yeast lacks this channel, it distinguishes the two yeast species' proliferation [46]. The characteristics of WBT fermentation can be affected by differences in yeast strains. Crabtree-positive yeast increased the pH (5.56) of WBT after 7 days of fermentation in this experiment, which was a negative result for the fermented WBT product.

This phenomenon may be supported by the findings of McDonald et al. [47], who discovered that *S. cerevisiae* may increase the pH of silages by absorbing lactic acid, which may explain why lactic acid levels are decreasing. In this study, Crabtree-negative yeast, on the other hand, had no negative pH influence on WBT. The pH level that will most likely control undesirable microbes in fermentation products should be less than 4.0, but a pH range of 4.2–4.7 is typically regarded as suitable [48,49]. This experiment is inconclusive in terms of the statistical significance of the inconsistencies. The population of aerobic bacteria decreased when yeast was added to fermented WBT. In vitro investigations have revealed that yeast can lower the amount of oxygen used by 46–89% [50]. As a result, the initial yeast concentration of 10^{10}–10^{11} employed in WBT fermentation is a strong competitor for consuming residual oxygen in the WBT fermentation process, which may be the major reason for the reduction in aerobic bacteria count.

In each treatment, mathematical models describe gas production in response to feeding digestion over time [37]. The rich cassava treatment exhibited a high GP line curve, whereas WBT had not undergone a quality enhancement procedure, resulting in a low GP line curve (Figure 1). Variations in animal feed chemical composition have had a significant impact on gas production. These findings are consistent with the findings of Sommart et al. [51], who discovered that higher cassava levels in the diet affected GP. According to Makkar [52], protein fermentation produces less gas than carbohydrate fermentation. To compare the gas production capacity, 200 mg of casein and cellulose were weighed into the bottle; 23.4 mL and 80 mL of gas were produced, respectively, and it was clear that carbohydrates produced more gas. In our trial, WBT had a higher protein content (199 g/kg DM) than cassava chips (25 g/kg DM) in our trial, which might explain why the GP was low. However, using yeast to improve the quality of WBT could help increase GP and gas kinetics. The final asymptotic gas volume corresponding to complete substrate digestion (Vf) after yeast fermentation was used to clearly demonstrated the technique's advantage. Suntara et al. [24] discovered that adding yeast to feedstuffs enhanced the volume of gas produced from the insoluble fraction, the potential extent of gas production, and the gas volume at 96 h considerably. WBT fermented with yeast gave satisfactory results, particularly for Crabtree-negative yeast [51], and the gas output is related to feed digestibility (Table 4). Crabtree-negative yeast was found to be important in promoting IVDMD in the first 12 h of our research. According to Wang et al. [53], adding *C. tropicalis*, Crabtree-negative yeast, increased IVDMD when compared to Crabtree-positive yeast. Although the mechanism of Crabtree-negative yeast is unknown, there is evidence that the yeast can produce a cellulase enzyme [23]. However, yeast had no effect on the fibers in WBT in our study, and NDF digestibility was unrelated to Crabtree-negative yeast. This could be because WBT has less fiber than the roughage source used in previous studies. Therefore, neither enzyme release nor cellulase activity is significantly influenced. As a result, a variety of strategies for optimizing the utilization of yeast cultured with WBT to promote nutrient digestion are available. Various mechanisms have been proposed to explain yeast's beneficial effect on ruminal ecosystems [54,55]. According to Habeeb [50], yeast operates in the rumen as an oxygen scavenger, promoting microbial activity by completing respiration and sustaining anaerobic environments. This is congruent with the findings of Chaucheyras-Durand et al. [56], who discovered that employing yeast can enhance the ecological conditions for the development and activity of anaerobic microflora. These reasons were clear in this experiment, in which yeast was utilized and the rumen bacterial population was dominant (Figure 4). The results of this experiment also show that different yeast species

can stimulate different bacterial populations. Crabtree-negative yeast has a greater ability to improve rumen bacterial populations than Crabtree-positive yeast. Suntara et al. [24] observed that Crabtree-negative yeast had a 3.34% higher potential for bacterial population promotion than Crabtree-positive yeast. Therefore, Crabtree-negative yeast may improve feed digestion by raising rumen bacteria numbers. Crabtree-negative yeast also increases milk protein content, possibly due to a rise in microbial crude protein, according to a prior study [25].

The metabolism of rumen bacteria may produce a large amount of VFA, and VFA's principal function in ruminants is to provide energy [57]. Several prior investigations [18,58,59] confirmed yeast's supremacy in promoting ruminal bacteria, resulting in significant rumen VFA production. VFA levels can be enhanced by a variety of methods, including the use of yeast in feed formulations or the fermentation of feedstuffs. According to Ková et al. [60], using live yeast as a feed additive increased the level of VFA in Holstein cattle by up to 40%. Similarly, Elanthamil et al. [61] showed that adding live yeast to a diet comprising paddy straw as the primary roughage source increased rumen VFA by around 12.5%. The influence of yeast as a stimulus on a change in the bacterial population is thought to be the cause of the increase in VFA. The yeast strain, on the other hand, may influence the VFA proportions in the profile. Crabtree-negative yeast, such as C. tropicalis KKU20, showed a remarkable ability to cause an increase in C3 in our study (compared with WBT fermented without yeast and the control group). The most energetically efficient of the glucose-metabolized VFAs is propionate [62]. As a result, the significance of Crabtree-negative yeast in increasing the C3 ratio should be noted. The level of NH_3-N in the rumen indicates whether there is an immediate need for additional fermentable nitrogen in the diet [63]. As a result, rumen NH_3-N concentrations were determined in diets containing varying levels of fermented WBT. In our study, NH_3-N levels ranged from 16.3–21.5 mg/dL and were directly linked to the amount of fermented WBT consumed. Because urea is the main source of nitrogen in fermented WBT solutions, when it enters the diet, NH_3-N levels rise. (Table 6). Polyorach et al. [14] observed NH_3-N concentrations ranging from 18.9 to 26.6 mg/dL in a urea-containing solution utilized as a nitrogen source for yeast growth. When urea was used as a nitrogen source for yeast growth in the fermented cassava chip product, NH_3-N concentrations in the rumen ranged from 15.9–17.0 mg/dL [64,65]. Furthermore, Leng [66] discovered that NH_3–N concentrations of 15–30 mg/dL can support microbial growth. As a result of our research, the NH_3-N levels in the rumen remained normal, indicating that the amount of 10 g/L urea used to generate yeast is appropriate [67–69].

5. Conclusions

In conclusion, fermented WBT with yeast could benefit feed digestion and in vitro gas production more than with no yeast. The study also revealed that Crabtree-negative yeast has a promising future in terms of increasing rumen fermentation quality. The key process is the interaction of yeast with ruminal microorganisms, which increases the number of bacteria in the rumen and enhances fermentation. However, further evaluations of in vivo experiments involving yeast fermented with WBT are required.

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