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In vitro Ruminal Fermentation, Nutrient Digestibility, Methane Production of Cassave Leaf Waste as a Herbal Feed Additive

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ABSTRACT

Cassava leaf waste (*Manihot esculenta* Crantz) is an abundant agricultural by-product with potential as a natural herbal feed additive for improving ruminal fermentation and reducing methane production. This study aimed to evaluate the effects of cassava leaf waste supplementation on nutrient digestibility, ruminal fermentation, and methane production under in vitro conditions. The study used a Completely Randomized Design (CRD) with four treatments and four replications: P0 (basal diet without cassava leaf waste), P1 (basal diet + 5%), P2 (basal diet + 10%), and P3 (basal diet + 15%). The observed parameters included dry matter digestibility, organic matter digestibility, rumen fluid pH, volatile fatty acids, ammonia, total gas production, and methane production. Data were analyzed using ANOVA followed by Duncan's Multiple Range Test (DMRT) when significant differences were detected. The results showed that cassava leaf waste supplementation significantly affected nutrient digestibility, ruminal fermentation, and gas production ($P<0.05$). The P3 treatment showed the highest dry matter digestibility (62.79%), organic matter digestibility (75.40%), VFA concentration (148.42 mM), and the lowest total methane production (25.37%). It can be concluded that 15% cassava leaf waste improves nutrient utilization and ruminal fermentation while reducing methane production *in vitro*.

Keywords: *Cassava Leaf, Feed Additive, Methane Production, Nutrient Digestibility, Ruminal Fermentation*

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1. Introduction

The ruminant livestock sector plays a strategic role in the supply of national animal protein particularly meat and milk which is a vital component of efforts to achieve national food security. However, the ruminant production system faces two major challenges: feed conversion efficiency and environmental sustainability. One of the most prominent problems is the formation of methane gas as a byproduct of rumen fermentation, which not only contributes to greenhouse gas emissions but also results in the loss of feed energy that could otherwise be utilized by livestock [1,2]. Therefore, efforts to mitigate methane emissions from ruminant livestock are a critical issue that requires scientific investigation. A feed-based approach, particularly through the use of natural plant-derived additives, is considered an effective and sustainable strategy for modulating rumen fermentation. Plant-derived materials containing secondary metabolites, such as tannins and

flavonoids, are known to influence the activity of rumen microbes, particularly protozoa associated with methanogens [3,4]. The use of natural herbal additives is considered safer and more environmentally friendly than synthetic additives, and is in line with the concept of sustainable livestock production. Cassava (Manihot esculenta) leaf waste is an agricultural byproduct that is abundant in various regions of Indonesia, yet its utilization remains limited and suboptimal.

Until now, cassava leaves have more often been regarded as waste, even though they contain bioactive plant compounds that have the potential to be used as herbal additives in animal feed [5,6]. The use of cassava leaf waste as a herbal feed additive not only has the potential to reduce methane production by modulating rumen fermentation, but also adds value to local resources that are inexpensive and readily available [7,8,9]. Thus, this approach is expected to improve the efficiency of ruminant feed utilization while supporting national food security through a more efficient and sustainable livestock production system.

Although the potential of cassava leaves as a feed ingredient has been widely reported, studies specifically evaluating their role as herbal additives in influencing rumen fermentation and mitigating methane gas remain limited, particularly those using in vitro approaches that allow for a more controlled observation of fermentation mechanisms. Therefore, this study is important for providing a scientific basis for the utilization of cassava leaf waste as a herbal feed additive for ruminants.

This research is of high urgency because it offers a local-resource-based approach to improving the efficiency of the ruminant production system through methane mitigation. The research findings are expected to serve as a scientific basis for developing ruminant feeding strategies that are more sustainable, efficient, and environmentally friendly, as well as to contribute to supporting national food security.

Method

2.1. Materials

The ingredients provided are cassava leaf waste (*Manihot esculenta*), a basal diet for ruminants (elephant grass, rice bran, and coconut meal), fresh rumen fluid obtained from ruminant livestock, and a rumen fermentation buffer solution.

2.2. Tools

The tools used include a fermentation serum bottles, an incubator or waterbath, an analytical balance, a drying oven, a grinder, a shaker, a syringe or gas measuring device, a pH meter, a microscope, a hemocytometer, and standard laboratory glassware.

2.3. Research design

This study used a completely randomized design (CRD) with four treatment levels and four replications. The details of the treatments provided in this study are as follows:

P0 = basal diet without additives (control)
P1 = basal ration + 5% cassava leaf waste
P2 = basal ration + 10% cassava leaf waste
P3 = basal ration + 15% cassava leaf waste

The statistical model used, according to [7], is as follows:

$$(Yij) = 1 + 1 + \Sigma ij)$$

Where:

Yij = Variable being analyzed
μ = General average value
Ti = Effect of the first treatment
Σij = Experimental error of the treatment

2.4. Research procedure

2.4.1. Preparation and Characterization of Fresh Cassava Additives

Fresh cassava leaf waste was obtained from community farmlands and visually inspected to ensure there was no contamination from soil, mold, or other foreign materials. The cassava leaves were then washed under running water to remove any adhering dirt and drained until the surface water had subsided. Next, the material is dried in an oven at a temperature of ±60 °C until it reaches a constant weight to prevent damage to bioactive compounds caused by high temperatures [13]. The dried cassava leaves are ground using a grinder to achieve a relatively uniform particle size, then sieved to minimize particle size variation between samples. The

prepared material was stored in a sealed container under dry conditions until used as a herbal additive in in vitro rumen fermentation tests. This step aimed to ensure the homogeneity of the material and consistency of treatment throughout the study.

2.4.2. Formulation of Basal Rations and Preparation of Fermentation Substrates
The basal diet was formulated to resemble a standard ruminant diet consisting of forage and concentrate, as shown in Table 1. All ingredients in the basal diet were dried, ground, and weighed based on predetermined dry matter proportions. The basal diet was used uniformly across all treatments to ensure that any differences in fermentation results observed were due solely to the addition of herbal additives.

Table 1. Formulation of the Basal Diet for the Study [14,15]

Feed Ingredient	Proportion (%)
Elephant Grass	60
Rice bran	25
Coconut meal	15
Total	**100**

The fermentation substrate was prepared by weighing the basal diet according to the requirements of each experimental unit. Cassava leaf waste was then added as a herbal additive at levels of 5%, 10%, and 15% based on the dry matter content of the substrate, while the control treatment received no additive. All ingredients were mixed homogeneously before being used in the in vitro fermentation process.

2.5. Conducting In Vitro Rumen Fermentation
The prepared fermentation substrate was placed into fermentation flasks, and a mixture of rumen fluid and buffer was added under anaerobic conditions. The flasks were immediately capped with rubber stoppers and sealed to prevent gas leakage during incubation. The fermentation flasks were incubated at 39°C for a predetermined incubation period. During incubation, total gas production was monitored using either a pressure or volume measurement method, and a portion of the gas was collected for methane analysis. This step aimed to quantitatively evaluate the rumen fermentation response to the addition of herbal additives [16]. The in vitro study was conducted at the Nutrition Laboratory, Faculty of Animal Science, Universitas Andalas.

2.6. Stopping Fermentation and Taking Samples
After the incubation period ended, fermentation was stopped by cooling the fermentation bottles. Samples of the fermentation fluid were then taken for analysis of pH and ammonia (NH_3) concentration. The pH was measured immediately using a pH meter, while samples for NH_3 analysis were prepared according to laboratory procedures. The fermentation residue was filtered for subsequent use in determining the digestibility of dry matter and organic matter. In addition, a portion of the rumen fluid sample was used to count the protozoa population using a microscope and a hemocytometer. All observations were conducted consistently at the same time to minimize data variation.

2.7. Parameters
The parameters observed in this study included the rumen fermentation response to the addition of cassava (Manihot esculenta) leaf waste as a herbal feed additive for ruminants. The biological response of the substrate to rumen microbial activity was evaluated through in vitro testing, with the main parameters being dry matter digestibility (DMD) and organic matter digestibility (OMD) as indicators of nutrient utilization efficiency. Rumen fermentation characteristics were assessed by measuring total gas [17] and methane [18] production during the incubation period, rumen fluid pH, and ammonia (NH3) concentration [19] as indicators of fermentation activity and the efficiency of nitrogen utilization by rumen microbes. In addition, the rumen protozoa population [20] was examined to elucidate the biological mechanisms underlying changes in fermentation and methane production resulting from the addition of herbal additives.

2.8. Data analysis
The data obtained were analyzed using analysis of variance (ANOVA) based on a Completely Randomized Design (CRD) consisting of four treatments and three replications. The mathematical model used was as follows:

$$Yij = \mu + \alpha i + \varepsilon ij$$

Description:

$Yij = observation\ value\ of\ the\ i-th\ treatment\ and\ j-th\ replication$
$\mu = overall\ mean$
$\alpha i = effect\ of\ the\ i-th\ treatment$
$\varepsilon ij = experimental\ error\ associated\ with\ the\ i-th\ treatment\ and\ j-th\ replication$

Differences among treatment means were further tested using Duncan's Multiple Range Test (DMRT) when significant effects were detected.

3. Result and Discussion

Based on Table 1, the treatment involving a basal diet supplemented with cassava leaf waste had a statistically significant effect (P<0.05) on the digestibility of dry matter and organic matter. A follow-up analysis using Duncan's Multiple Range Test (DMRT) revealed that the best nutrient digestibility was observed in treatment P3, DMD at 62.79%, and OMD at 75.40%,

Table 1. Results of the Nutrient Digestibility Test

Treatment	Parameters (%)	
	DMD	OMD
P0	58.56[a]	73.74[a]
P1	59.99[a]	74.96[a]
P2	62.68[a]	74.31[b]
P3	62.79[b]	75.40[a]
SE	0.21	0.26

Note: Numbers in columns with superscripts differ significantly (P<0.05) SE: Standard Error

In addition to the fact that the same types of feed ingredients were used, the addition of cassava leaf waste to the basal diet had a significant effect on overall and total rumen microbial activity, although cumulative effects also played a role. Treatment P3 produced the best digestibility values compared to the other treatments, indicating that rumen microbial activity increased with each treatment, followed by an increase in activity [21]. An increase in microbial population also increased the digestible dry matter and organic matter. Rumen microbial activity is driven by the role of rumen microbes in the fermentation process [22]. Rumen microbial activity itself is influenced by the nutrients contained in the feed.

Table 2. Results of the Ruminal Fermentation Test

Treatment	Parameters		
	pH	VFA (mM)	NH3 (mg/100 ml)
P0	06.41[a]	131.11[b]	09.67[d]
P1	07.12[c]	136.95[a]	15.21[b]
P2	07.44[b]	143.58[b]	14.28[a]
P3	07.87[a]	148.42[c]	12.35[a]
SE	0.12	01.59	01.24

Note: Numbers in columns with superscripts differ significantly (P<0.050 SE: Standard Error

Table 2, shows that the treatment had a significant effect on rumen fluid pH, which ranged from 6.44 to 7.87. This means that feeding basal diet and cassava leaf waste can be beneficial. Feed conversion efficiency in ruminants is highly dependent on the rumen digestion process, which can lead to reduced feed efficiency. Normal rumen pH ranges from 6.0 to 7.0 to maintain rumen metabolism. A rumen pH below 6.0 can reduce fiber digestibility [23]. Rumen acidity can affect the number of microorganisms active in the fermentation process [24]. Rumen microbes function best at a pH of 6–7, breaking down amino acids into ammonia. An acidic rumen environment (low pH) can lead to a decrease in rumen microbial activity [25]. The addition of basal diet and cassava leaf waste is highly effective in increasing VFA levels in rumen fluid The higher the content of jackfruit and moringa leaves, the higher the total VFA in the rumen fluid, and vice versa. Administration of basal diet with cassava leaf waste increased total VFA by 148.42% under treatment D.

Cassava leaf are believed to contain tannin, saponins, which increase VFA concentrations [26]. Protozoa are predators of rumen bacteria, and a decrease in the protozoa population in the rumen leads to an increase in bacterial biomass, thereby enhancing the efficiency of microbial protein synthesis. In addition, saponins can bind ammonia in the rumen at high concentrations and release it again at low concentrations to ensure the availability of ammonia for microbial protein synthesis [27], thereby yielding more optimal results. Rumen growth ranges from 80–160 μm [28]. Volatile fatty acids are the end products of the biofermentation process in ruminants, providing 70–80% of their energy requirements [29].

A process that further catabolizes the hydrolytic digestion products of fermentation monomers the fermentation of carbohydrates into volatile fatty acids (VFAs) [30]. Furthermore, the inclusion of basal diet with cassava leaf waste as herbs feed in the diet had a significant effect on increasing total rumen NH3 to 12.35 mM in treatment P3 [31]. The NH3 concentration required for optimal feed digestion by rumen microbes is 5–20 mg/dl [32].

Table 3. Results of the Methane Production Test

Treatment	Parameters		
	Total Gas Production	CH4 (ml)	Total CH4
P0	97.65^{c}	43.60^{a}	30.96^{b}
P1	107.23^{b}	36.54^{b}	27.12^{a}
P2	121.80^{b}	39.86^{b}	26.31^{b}
P3	134.22^{a}	44.51^{a}	25.37^{b}
SE	0.35	1.50	01.41

Note: Numbers in columns with superscripts differ significantly (P<0.05) SE: Standard Error

The analysis results indicate that the addition of basal diet and cassava leaf waste had a significant effect (P<0.05) on total gas production. This is because Treatment P1, at 107.23 ml, was lower than Treatments P2 and P3, where gas production had entered the exponential phase of its growth cycle and the microbes had already adapted to their new environmental conditions; at the same time, there was still an abundance of substrate in the environment, allowing rumen microbes to produce gas optimally. These findings are consistent with the study by [32], which showed rapid growth from 24 to 48 hours because the rumen microbes had entered the exponential phase of their life cycle and had adapted to their new environmental conditions; at the same time, there was still an abundance of substrate in their environment, allowing the rumen microbes to produce gas optimally [33].

In addition, the total gas production in treatment P3, at 134.22 ml, was higher than in the other treatments; this increase is assumed to be due to the fact that rumen microbes need to adapt to the new feed, so it takes time for them to produce gas effectively. Feed fermentation in the rumen produces not only volatile fatty acids (VFAs) but also gases such as CO_2, CH_4, NO_2, and NO [34], as well as N_2 and 3.5% O_2 [35]. These gases are produced by the fermentation of organic matter contained in the feed consumed by livestock. The volume of gas produced depends on the nutritional content of the feed. The higher the carbohydrate content of the feed, the greater the amount of gas produced by its fermentation in the rumen. In addition, the tannin content in the feed inhibits the activity of rumen enzymes and microbes, thereby reducing total gas production [36]. Furthermore, reducing methane gas through the addition cassava leaf waste is one strategy for manipulating the fermentation process in the rumen, with the aim of increasing microbial protein synthesis and reducing methane gas in the feed [37].

Furthermore, the type of feed is one of the factors that influence methane (CH_4) production. Forage is a feed source that produces higher levels of CH_4 compared to low-fiber feeds. The different diets had a significant effect on CH_4 production in treatment P1 compared to treatments P0, P2, and P3. This is due to differences in gas production among the treatments, consistent with the findings [33], who stated that diets with higher crude fiber content result in greater CH_4 production. According to [36], feed components particularly fiber and protein contribute to gas production during the fermentation process. Which states that organic matter content, fiber component content, and microbial degradability influence methane production. These differences are due to the varying types of fiber in each treatment and the differing levels at which they were administered, resulting in differences in the degradation process into CH_4 gas. Fiber fermentation influences methane gas production, and that different fiber sources result in different total gas yields [30].

Conclusions

The results of treatment P3 showed a significant effect ($P<0.05$), optimally improving nutrient digestibility, increasing the NH_3 concentration in rumen fluid to 12.35 mg/100 ml, and reducing methane gas (total CH4) by 25.37%. The study results revealed that the *in vitro* feeding of a diet containing 15% cassava leaf waste improved nutrient digestibility, ruminal fermentation, and methane production. It was concluded that the P3 treatment diet can help optimize national food security.

Suggestion

It is recommended that cassava leaf waste 10-15% be utilized as a source of herbal feed additives for ruminants in feed formulation applications at companies that prioritize environmental conservation as part of their mission to address global warming (climate change), and that phytochemical compounds capable of effectively and efficiently reducing methane production be tested.

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