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In Vitro Evaluation of Feed Quality of Fermented *Tithonia diversifolia* with *Lactobacillus bulgaricus* and *Persea americana miller* Leaves as Forages for Goat

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ABSTRACT

Fermented *Tithonia diversifolia* and *Persea americana miller* or avocado leaves as local alternative forages for goats are potential as protein, mineral, and energy sources. Therefore, this study aimed to evaluate the effect of fermented *Tithonia diversifolia* (FTD) and avocado leaves (AL) combination on *in vitro* nutrient digestibility, rumen fluid characteristics, and methane production. This study consisted of 3 trials. Trial 1 evaluated FTD's nutrient content with *Lactobacillus bulgaricus* with different durations of fermentation arranged in a completely randomized design consisting of five treatments and four replications. The treatments were *T. diversifolia* without fermentation and fermentation of *T. diversifolia* for 2, 3, 4, and 5 days. Trial 2 was *in vitro* evaluation on different days of fermented *T. diversifolia* in a completely randomized design consisting of four treatments and four replications. Trial 3 was *in vitro* evaluation of FTD for 5 days and AL combination, which consisted of four combinations. FTDAL1 = 20% FTD+80% AL; FTDAL2 = 40% FTD+60% AL; FTDAL3 = 60% FTD+40% AL; and FTDAL4 = 80% FTD+20% AL. Experimental diets were incubated using Tilley and Terry method. Fermentation of *T. diversifolia* using *L. bulgaricus* significantly increased nutrient components ($p<0.01$), nutrient digestibility ($p<0.01$), and rumen fluid characteristics ($p<0.05$). *In vitro* evaluation of FTD and AL combination significantly increased nutrient digestibility, total volatile fatty acid, ammonia concentration, total gas production, and methane production ($p<0.05$), but insignificantly affected pH rumen fluid. It is concluded that the combination of 80% fermented *T. diversifolia* and 20% avocado leaves has the potential to increase dry matter, organic matter, crude protein, cellulose digestibility, and rumen fluid characteristics, but it is not optimum to decrease total gas and methane gas production.

Keywords: in vitro; Lactobacillus bulgaricus; Persea americana miller leaves; Tithonia diversifolia

INTRODUCTION

Feed is a critical aspect in determining animal output performance and forage is the primary feed for ruminants as nutrient and fiber source, which has a bulky characteristic. Forage availability requires a specific planting space. However, the application of specific planting space is restricted by land conversion into residential, industrial, and food crop land. Exploration of new and high-quality animal feed sources is critical for identifying alternatives. Accordingly, we can utilize *Tithonia diversifolia* and avocado leaves (*Persea americana miller*) as local alternative forages.

T. diversifolia leaves have nutritional composition of 88.7% dry matter and 23.56% crude protein (Osuga *et al.*, 2012). Ribeiro *et al*. (2016) and Mauricio *et al*. (2017) reported that *T. diversifolia* has greater amino acid and mineral contents. *T. diversifolia* is an annual weed having the potential as ruminant feed because of its high production, which is around 5.6-8.1 ton/ha/year in two pruning's (Purwani, 2011). If *T. diversifolia* is harvested 6 times/year it will produce 4.10-10.20 tons/ha/year in the form of dry matter or 24.00-46.80 tons/ha/year in a fresh form. However, the problem is that *T. diversifolia* has low palatability due to the content of phytic acid compounds that taste bitter to livestock. *T. diversifolia* contains high phytic acid, around 79.2 mg/100 g DM (Oluwasola & Dairo, 2016). The phytic acid contained in *T. diversifolia* can be reduced by fermentation using microorganisms that produce phytase enzymes, namely *Lactobacillus bulgaricus* (Pazla *et al.*, 2021c) by breakdown phosphorus-phytate bond. *L. bulgaricus* is optimum at pH 5.5-6.2, and the growth rate decreases in the early alkali media but can still grow at pH 8.1 (Malaka & Laga, 2005). Mohamed *et al.* (2011) reported that *L. bulgaricus* is the most effective bacteria for degrading phytic acid. Fermentation using this bacterium can decrease phytic acid by 77.0%, 69.2%, and 85.4% for soybean, green soybean, and red soybean, respectively. Sripo *et al.* (2016) also reported that *L. bulgaricus*

decreased phytic acid by 54.8% in fermented black glutinous rice.

Avocado leaves (*Persea americana miller*) are a waste of avocado plantation after branch pruning. Branch pruning needs to be done because it directly affects environmental factors and the sustainability of avocado farming. Another impact of pruning is it prevents nests from developing diseases and pests in leafy twigs and dense branches. Proper branch pruning will result in better production. The area of avocado plantations in Indonesia in 2018 reached 24.352 ha, with a tree population of 2.435.242 (Badan Pusat Statistik, 2018). Avocado production in Indonesia in 2020 reached 609.049 tons, while in West Sumatra, avocado production in 2020 reached 69.787 tons (Badan Pusat Statistik, 2020). The nutrient component of avocado is 11.70% dry matter, 93.23% organic matter, 11.60% crude protein, 27.60% crude fiber, and 3.12% ether extract (Marhaeniyanto *et al.*, 2019).

A combination of *T. diversifolia* and avocado leaves (*P. americana miller*) can complement the nutritional needs of goats. *T. diversifolia* has high protein and mineral contents, while avocado leaves have higher crude fiber, so they are complementary sources of good nutrition for goats. This study was conducted to evaluate the effect of the combination of fermented *T. diversifolia* with avocado leaves on digestibility and characteristics of rumen fluid *in vitro*.

MATERIALS AND METHODS

Sample Preparation and Experimental Diets

The study was conducted in the Ruminant Laboratory of Animal Science Faculty of Andalas University, Padang, West Sumatra. *T. diversifolia* was collected from Pandai Sikek, Tanah Datar Regency, West Sumatra. The area's geographical position is 0°17'39"S and 100°19'°51"E at an elevation of 750-1.000 m above sea level. The average rainfall is 1.750-2.000mm/year. Meanwhile, avocado leaves were collected from avocado plantations around the campus of Andalas University. The area's geographical position is 0°54'30"S and 100°27'48"E at an elevation of 250 m above sea level. The average rainfall is >4000 mm/year.

This study was conducted on an *in vitro* laboratory scale and did not use live farm animals. Rumen liquor was obtained from a slaughterhouse from four Kacang goats with an average BW ± 20 kg. The rumen liquor was filtered using nylon (of 100 µm sieve size) with 4 layers, and poured into a thermos container in a water bath with a temperature of 39 °C while flowing CO_2 gas.

Trial 1

Before fermentation, the samples of *T. diversifolia* were dried at 60 °C for 48 h in a forced-air oven and then milled through a 1 mm sieve. *T. diversifolia* mill was weighed 50 g each for 16 experimental units to be fermented by adding 80 mL of distilled water and putting in a plastic bag. Then the experimental preparations were sterilized in an autoclave at 121 °C for 30 min. All samples were cooled and placed in a laminar flow. *L. bulgaricus* was obtained from the Livestock Technology Laboratory, Faculty of Animal Husbandry, Bogor Agricultural University, Bogor, Indonesia. After that, 1.5 mL of *L. bulgaricus* brood stock was inoculated and homogenized. All samples were tightly covered with tape and plastic wrap and then stored at room temperature according to treatments. The flow diagram of *T. diversifolia* fermentation is presented in Figure 1.

In trial 1, a completely randomized design consisting of 5 treatments and 4 replications was used. The treatments were CT= control (without fermentation); FTD2= fermented *T. diversifolia* (FTD) for 2 days; FTD3= FTD for 3 days; FTD4= FTD for 4 days; and FTD5= FTD for 5 days. Phosphorus analysis was determined with the method of Bray I (Bray & Kurtz, 1945). A total of 2.5 g of sample was mixed with 25 mL of Bray and Kurt 1 extractant and shaken for 5 min. The solution was filtered until transpicuous. A total of 2 mL of clear extract was transferred into the solution. Then, the solution and

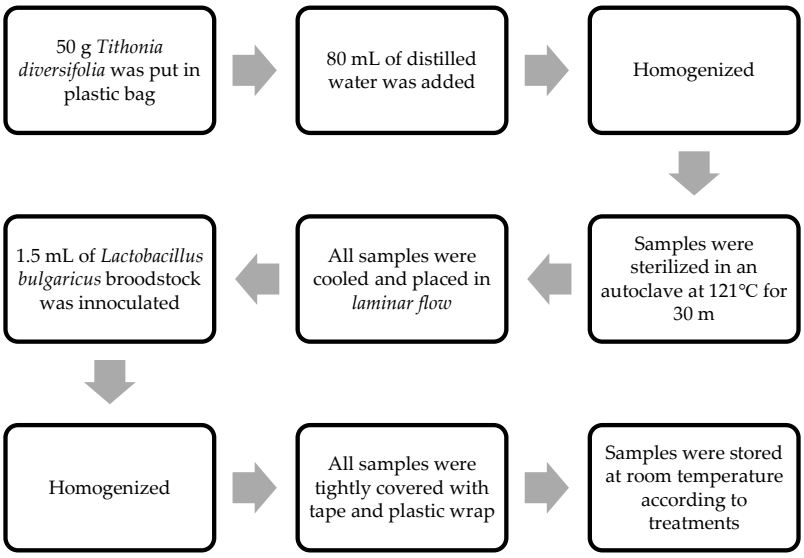

Figure 1. Flow diagram of *Tithonia diversifolia* fermentation

standard phosphate solution were mixed well with 10 mL phosphate reagent, and the mixture was left for 30 min. The absorbance was measured by a spectrophotometer at a wavelength of 889 nm.

Phytic acid was determined with a method suggested by Davies & Nightingale (1975). The principle of this method is that iron ions that form complexes with phytate cannot react with thiocyanate ions to form red-colored complexes. With amyl alcohol, the optical density of the solution measured with spectrophotometer at a wavelength of 465 nm is inversely proportional to phytate concentration. The higher the amount of phytic acid in the sample, the lower the absorbance.

Total phenol was determined with the method of Folin & Ciocalteau (1927). A 0.5 g sample was mixed with 1 mL 0.5% hexamethylenetetramine, 20 mL acetone, and 2 mL 25% hydrochloric acid (HCl). The mixture was kept in a refrigerator for 30 m and shaken with distilled water and ethyl acetate. The extract was separated into two solutions. The first part, the extract was mixed with 1ml aluminum chloride and 5% (volume/volume) methanol-acetic acid for further measurement. Meanwhile, the second part was mixed with 5% (volume//volume) methanol-acetic acid to produce a standard solution. After 30 m of incubation, the absorbances of both samples were measured with a spectrophotometer at a wavelength 425 nm (A). The total phenolic concentration was determined using this formula: (1.25xA) m, where m was mass of sample in grams.

Total tannin was measured using UV-visible (Shimadzu) spectrophotometer. The sample was mixed with 150 mL of distilled water and heated in a water bath for 30 min at 70 °C. The cooled extract was quantitatively transferred into a 250 mL volumetric flask, filtered, and used for the reaction. The extraction results were measured at a wavelength of 278.5 nm spectrophotometer using pure tannins as a standard. Nutrient components of all treatments were analyzed.

Proximate analysis (AOAC, 2005) and Van Soest (Goerning & Van Soest, 1970) were used to analyze the nutrient component of *T. diversifolia* and avocado leaves. A total 2.5 g sample was oven dried at 105 °C for 8 h to determine the dry matter. Ash content was measured by combustion at 600 °C for 4 h. Protein content was measured by following the Kjeldahl method, consisting of three steps: destruction, distillation, and titration. The gravimetric method was used to determine crude fiber by sequential digestion with H_2SO_4 and NaOH, followed by oven dried at 105 °C for 8 h and ignition in furnace at 600 °C for 4 h. Extractor Soxhlet method was used to determine extract ether. The sample was extracted with an organic solvent like hexane, and the remaining residue was dried and weighed. Neutral Detergent Fiber (NDF) was estimated by dissolving the sample with neutral detergent soluble (NDS), meanwhile, ADF (acid detergent fiber) was estimated by dissolving sample with acid detergent soluble (ADS). Cellulose was determined by soaking the residue of ADF with H_2SO_4, meanwhile ignition in furnace at 600 °C for 4 h of cellulose's residue will obtain lignin content. Hemicellulose was estimated from the subtraction of NDF content and ADF content. TDN was estimated with this following formula (Moran, 2005):

TDN= 5.31 + 0.412 CP% + 0.249 CF% + 1.444 EE% + 0.937 NFE%

where CP was crude protein; CF was crude fiber; EE was ether extract; NFE was nitrogen free extract.

Nutrient components of *T. diversifolia* and avocado leaves (% dry matter) are presented in Table 1.

Trial 2

In trial 2, sample preparation was the same as in trial 1. In trial 2, the experiment was arranged in a completely randomized design consisting of 4 treatments and 4 replications. The treatments were FTD2= fermented *T. diversifolia* (FTD) for 2 days; FTD3= FTD for 3 days; FTD4= FTD for 4 days; and FTD5= FTD for 5 days. Nutrient digestibility and rumen fluid characteristics of each treatment were determined using *in vitro* Tilley and Terry method (Tilley & Terry, 1963). Filtered rumen liquor was diluted with the buffer solution suggested by McDougall (1947). A 2.5 g sample was incubated in an Erlenmeyer flask with a rumen-buffer mixed solution (200 mL buffer and 50 mL filtered rumen liquor) anaerobically by flowing CO_2 gas into the flask then covering it with a rubber lid. Each Erlenmeyer flask was placed in a shaker water bath at a temperature of 39 °C for 48 h. Methane gas production was measured using the method of Fievez *et al*. (2005) by collecting gas at the volume of 100 µL used as a sample injected for methane estimation with gas chromatography for mass spectrophotometer Gas Chromatograph GC-2010 Plus, Shimadzu No. O205354. During incubation, methane gas production was measured for 12, 24, 36, and 48 h.

The incubation process was stopped by immersing the Erlenmeyer flask in the ice water, after which the pH was measured. pH was measured with an Eutech Instruments pH 700 device. Then, the supernatant and the residue were separated by using a centrifuge at 3000 rpm for 5 min at 4 °C. The supernatant was stored in bottles to determine total volatile fatty acid (VFA) and

Table 1. Nutrient components of *Tithonia diversifolia* and avocado leaves (% dry matter)

Nutrient component (%)	*Tithonia diversifolia*	Avocado leaves
Dry matter	98.06	94.24
Organic matter	83.18	95.38
Ash	16.82	4.90
Ether extract	6.33	6.23
Crude protein	26.04	11.19
Crude fiber	10.72	24.59
Nitrogen free extract	40.08	53.10
Neutral detergent fiber	35.83	40.00
Acid detergent fiber	28.14	37.14
Cellulose	16.39	17.90
Hemicellulose	7.69	2.86
Lignin	6.02	19.15
True digestible nutrient	65.41	74.79

ammonia (NH_3) contents. Total VFA was determined by steam distillation (Abdurachman & Askar, 2000) and NH_3 was determined by using Conway and O'Malley method (Conway & Malley, 1942). The residue was filtered using Whatman No. 41 filter paper and then dried in an oven at 60 °C for 24 h. The residue was analyzed to determine nutrient digestibility using proximate (AOAC, 2005) and van Soest analysis (Goering & Van Soest, 1970). The analysis procedure of residue followed the procedure used in Trial 1. Nutrient digestibility was determined by following these equations:

DM= {[DM samples - (DM residue - DM blanks)] / DM sample} x 100%
OM= {[OM samples - (OM residue - OM blanks)] / OM sample} x 100%
CP= {[CP samples - (CP residue - CP blanks)] / CP sample} x 100%
NDF={[NDF samples - (NDF residue - NDF blanks)] / NDF sample} x 100%
ADF={[ADF samples - (ADF residue - ADF blanks)] / ADF sample} x 100%
Cellulose= {[Cellulose samples - (Cellulose residue - Cellulose blanks)] / Cellulose sample} x 100%
Hemicellulose= {[HemiCl samples - (HemiCl residue - HemiCl blanks)] / HemiCl sample} x 100%

where DM is dry matter, OM is organic matter, CP is crude protein, NDF is neutral detergent fiber, ADF is acid detergent fiber, and HemiCl is hemicellulose.

Trial 3

Fermented *T. diversifolia* and avocado leaves were formulated into the ration with a completely randomized design consisting of 4 treatments and 4 replications. The treatments were FTDAL1= 20% fermented *T. diversifolia* (FTD) for 5 days + 80% avocado leaves (AL); FTDAL2= 40% FTD for 5 days + 60% AL; FTDAL3= 60% FTD for 5 days + 40% AL; and FTDAL4= 80% FTD for 5 days + 20% AL. Nutrient digestibility and rumen fluid characteristics were determined using *in vitro* Tilley and Terry method (Tilley & Terry, 1963) by following the procedure used in Trial 2. Proximate analysis (AOAC, 2005) and Van Soest analysis (Goering & Van Soest, 1970) were conducted by following the procedure used in Trial 2 to analyze the nutrient composition of the fermented *T. diversifolia* and avocado leaves combination, as shown in Table 7. The phytic acid content of fermented *T. diversifolia* by *L. bulgaricus* at the different durations of fermentation and total phenols of fermented *T. diversifolia* and avocado leaves are shown in Table 2 and Table 3.

Table 2. Phytic acid contents of fermented *Tithonia diversifolia* by *Lactobacillus bulgaricus* at different durations of fermentation

Treatments	Phytic acid (mg/100g)	Phytic acid degradation (%)
Control	9.93±0.58	-
FTD2	4.85±0.17	51.03±4.26
FTD3	3.99±0.18	59.74±3.10
FTD4	3.89±0.13	60.83±1.05
FTD5	3.48±0.34	64.81±4.58

Note: FTD2= fermented *Tithonia diversifolia* (FTD) for 2 days; FTD3= FTD for 3 days; FTD4= FTD for 4 days; and FTD5= FTD for 5 days.

***In Vitro* Experiment**

An *in vitro* technique was conducted using the Tilley and Terry method (Tilley & Terry, 1963) used in Trial 2 and Trial 3. Rumen liquor was obtained from a slaughterhouse from four Kacang goats with an average BW ± 20 kg. The rumen liquor was filtered using nylon (of 100 μm sieve size) with 4 layers and poured into a thermos container in a water bath with a temperature of 39 °C while flowing CO_2 gas. Filtered rumen liquor was diluted with the buffer solution suggested by McDougall (1947). A 2.5 g sample was incubated in an Erlenmeyer flask with a rumen-buffer mixed solution (200 mL buffer and 50 mL filtered rumen liquor) anaerobically by flowing CO_2 gas into the flask covering it with a rubber lid. Each Erlenmeyer flask was placed in a shaker water bath at a temperature of 39 °C for 48 h. Methane gas production was measured using the method of Fievez *et al.* (2005) by collecting 100 μL of gas produced and using it as a sample injected for methane estimation with gas chromatography for mass spectrophotometer Gas Chromatograph GC-2010 Plus, Shimadzu No O205354. During incubation, methane gas production was measured for 12, 24, 36, and 48 h.

The incubation process was stopped by immersing the Erlenmeyer flask in the ice water, after which the pH was measured. pH was measured with an Eutech Instruments pH 700 device. Then, the supernatant and the residue were separated by using a centrifuge machine at 3000 rpm for 5 min at 4 °C. The supernatant was stored in bottles to determine total volatile fatty acid (VFA) and ammonia (NH_3) contents. Total VFA was determined by steam distillation (Abdurachman & Askar, 2000), and NH_3 was determined using Conway and O'Malley (Conway & Malley, 1942). The residue was filtered using Whatman No. 41 filter paper and then dried in an oven at 60 °C for 24 h. The residue was analyzed to determine nutrient digestibility using proximate (AOAC, 2005) and van Soest analysis (Goering & Van Soest, 1970) by following the same procedure used in Trial 2.

Table 3. Total phenols and tannin levels of fermented *Tithonia diversifolia* at different durations of fermentation and avocado leaves

Treatments	Total phenols (mg GAE/100 g)	Tannin (%)
CT	12.21±0.45	15.36±0.51
FTD2	14.69±0.23	30.42±0.33
FTD3	14.00±0.11	28.68±0.16
FTD4	12.21±0.19	22.28±0.17
FTD5	12.01±0.18	9.17±0.20
Avocado leaves	74.52±0.67	30.50±0.73

Note: CT= control/no fermentation, FTD2= fermented *Tithonia diversifolia* (FTD) for 2 days; FTD3= FTD for 3 days; FTD4= FTD for 4 days; and FTD5= FTD for 5 days.

Variables Measured

In Trial 1, the nutrient content of fermented *T. diversofolia* was measured. Nutrient digestibility, pH, total VFA, ammonia concentration (NH_3), and methane production were measured in Trial 2 and Trial 3.

Statistical Analysis

This study used a completely randomized design. The data were statistically analyzed using analysis of variance (ANOVA) with the SPSS software version 21.0. The data group showed a significantly different effect ($p<0.05$) and a very significantly different effect ($p<0.01$). Further tests were carried out using Duncan's Multiple Range Test (DMRT). Variance analysis was performed using the following statistical model:

$$Y_{ij} = \mu + \gamma_j + \epsilon_{ij}$$

in which Y_{ij} is the j^{th} observation of the i^{th} treatment, μ is the population mean, γ_j is the fixed effect of treatment (j = 1, 2, ..., 4), ϵ_{ij} is the random error for the j treatment in the i replication.

RESULTS

Trial 1

Nutrient contents of *T. diversifolia* fermented for 2, 3, 4, and 5 days are presented in Table 4. The results showed that the increased duration of fermentation increased crude protein content and decreased the crude fiber content, and FTD5 had the highest crude protein (35.59%) content and the lowest crude fiber content (6.75%) ($p<0.05$). The protein content of fermented *T. diversifolia* was significantly ($p<0.05$) different from the control treatment. The crude protein content of fermented *T. diversifola* increased significantly from 26.04% to 35.59% ($p<0.05$). The crude fiber content of fermented *T. diversifolia* decreased significantly ($p<0.05$) with an increase in the duration of fermentation. The crude fiber content of fermented *T. diversifolia* decreased from 10.72 to 6.75% with the increased duration of fermentation.

There was an increase in the phosphorus content of fermented *T. diversifolia* compared to the control treatment from 11.51 to 33.23 ppm (Figure 2). Although the data group showed a nonsignificantly different effect ($p>0.05$), there was an increase in phosphorus content with the increasing duration of fermentation, with the highest phosphorus content was found in the FTD5 treatment (33.23 ppm).

Trial 2

Nutrient digestibility of fermented *Tithonia diversifolia*. The nutrient digestibility of fermented *T. diversifolia* at different durations of fermentation is presented in Table 5. Nutrient digestibility increased significantly in line with the increased duration of fermentation from two days to five days ($p<0.05$), and the highest digestibility was found in the fermented *T. diversifolia* for 5 days. The digestibility of dry matter, organic matter, crude protein, NDF, ADF, cellulose, and hemicellulose are 68.35%, 69.08%, 74.19%, 61.32%, 58.40%, 71.02%, and 79.80% respectively.

Rumen fermentation characteristics and methane production of fermented *Tithonia diversifolia*. Rumen fermentation characteristics of fermented *T. diversifolia* at different durations of fermentation are presented in Table 6. In this study, no significant effect, except total VFA and NH_3 concentrations, were observed in all treatments. *In vitro* ruminal pH of rumen ranged from 7.19 to 7.31 ($p>0.05$). NH_3 concentrations in this study ranged from 25.82 to 28.37 mg/100 mL ($p<0.05$), and the highest concentration was found in FTD5. Total VFA ranged from 102.50 to 117.25 mM ($p<0.05$), and the highest was found in the FTD5 treatment. No significant difference was observed in methane production in all treatments.

Table 4. Nutrient contents of fermented *Tithonia diversifolia* at different durations of fermentation

Nutrient content (%)	Treatments				
	CT	FTD2	FTD3	FTD4	FTD5
DM	98.06±1.32^{a}	97.93±0.76^{a}	97.11±0.50^{b}	94.70±1.24^{c}	92.84±0.41^{d}
OM	83.18±0.34^{a}	83.16±0.32^{a}	83.02±0.38^{ab}	82.90±0.12^{b}	81.01±0.17^{c}
CP	26.04±0.11^{a}	28.89±0.54^{b}	31.18±0.54^{c}	33.06±0.37^{d}	35.59±0.74^{e}
EE	6.33±0.94^{a}	5.94±0.34^{ab}	5.56±1.28^{b}	4.89±0.79^{c}	2.42±0.36^{d}
CF	10.72±0.66^{a}	10.19±0.38^{b}	9.18±0.39^{c}	8.58±1.11^{d}	6.75±0.44^{e}
NDF	35.83±0.45^{a}	34.88±1.12^{b}	33.91±0.86^{c}	32.58±1.06^{d}	30.75±0.48^{e}
ADF	28.14±0.10^{a}	27.88±0.19^{b}	26.83±0.29^{cd}	26.77±0.36^{d}	25.91±0.49^{e}
CEL	16.39±0.86^{a}	15.38±0.79^{b}	14.51±0.32^{cd}	14.02±0.80^{d}	12.87±0.75^{e}
HCEL	7.69±0.55^{a}	6.99±1.22^{ac}	7.08±1.12^{a}	5.81±0.93^{b}	4.84±0.78^{c}
NFE	40.08±0.97^{a}	38.15±0.86^{b}	37.10±1.68^{cd}	36.37±0.98^{d}	36.24±0.95^{d}
TDN	65.41±1.09^{a}	64.07±0.72^{b}	63.23±0.69^{c}	62.21±1.07^{d}	59.11±0.47^{e}
Ash	16.82±0.34^{e}	16.84±0.32^{de}	16.98±0.38^{cde}	17.10±0.12^{cb}	18.99±0.17^{a}

Note: DM= dry matter, OM= organic matter, CP= crude protein, EE= ether extract, CF= crude fiber, NDF= neutral detergent fiber, ADF= acid detergent fiber, CEL= cellulose, HCEL= hemicellulose, NFE= nitrogen free extract, TDN= true digestible nutrient, CT= control/no fermentation, FTD2= fermented Tithonia diversifolia (FTD) for 2 days, FTD3= FTD for 3 days, FTD4= FTD for 4 days, and FTD5= FTD for 5 days. Means in the same row with different superscripts differ significantly ($p<0.05$).

Trial 3

Nutrient digestibility of the combination of fermented *Tithonia diversifolia* and avocado leaves. Nutrient components of fermented *T. diversifolia* and avocado leaves combination are presented in Table 7, while the nutrient digestibility is presented in Table 8. The results showed that fermented *Tithonia diversifolia* and avogado leaves combination significantly affected the dry matter (69.14%), organic matter (67.14%), crude protein (74.70%), and cellulose (80.95%) ($p<0.05$) contents except for NDF (53.60%), ADF (47.94%), and hemicellulose (80.36%) digestibility ($p>0.05$).

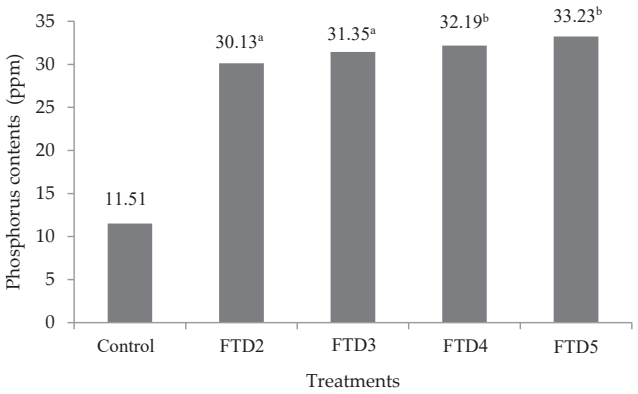

Figure 2. Phosphorus (P_2O_5) contents of fermented *Tithonia diversifolia* at different durations of fermentation. Note: FTD2= fermented *Tithonia diversifolia* (FTD) for 2 days; FTD3= FTD for 3 days; FTD4= FTD for 4 days; and FTD5= FTD for 5 days. Different superscripts in different bars mean significantly different ($p<0.05$).

Rumen fermentation characteristics and methane production of the combination of fermented *Tithonia diversifolia* and avocado leaves. Rumen fermentation characteristics and methane production of fermented *T. diversifolia* and avocado leaves combination are presented in Table 9. There was no significant effect of treatment on the pH of rumen fluid in this study ($p<0.05$), which ranged from 7.25 to 7.31. NH_3 concentrations ranged from 19.98 to 24.44 mg/100 mL and were significantly different ($p<0.05$) in each treatment. Increasing the percentage of fermented *T. diversifolia* and decreasing the percentage of avocado leaves in the ration had a significant effect on total VFA concentration ($p<0.05$), and the highest total VFA was observed in FTDAL4 (123.75 mM). In this study, treatments had a significant effects ($p<0.05$) on total gas and methane productions ranging from 106.25 to 139.08 mL/g DM and from 75.04 to 107.21 mL/g DM, respectively.

DISCUSSION

Trial 1

The method of forage fermentation can alleviate its nutritional value. Sharma *et al*. (2020) stated that various method technologies such as sprouting, fermentation, and milling enhance the nutritive quality, among which fermentation is the best. The crude protein content of fermented *T. diversifola* increased. This increase was caused by enzyme activity produced by *L. bulgaricus*, which produces protease enzyme with protease activity of 133.28 U/mL (Purkan *et al*., 2017). The activity of protease enzymes will increase with the increased duration

Table 5. Nutrient digestibility of fermented *Tithonia diversifolia* at different durations of fermentation

Nutrient digestibility (%)	Treatments				SE
	FTD2	FTD3	FTD4	FTD5	
DM	64.59±0.62^{d}	65.70±2.56^{c}	67.26±0.44^{b}	68.35±0.62^{a}	0.34
OM	64.07±1.60^{c}	65.53±2.41^{b}	67.76±0.78^{a}	69.08±0.81^{a}	0.39
CP	64.41±1.98^{d}	66.58±0.89^{c}	67.67±1.17^{bc}	74.19±2.37^{a}	0.43
NDF	55.71±0.73^{c}	56.51±1.68^{bc}	60.76±1.85^{a}	61.32±1.95^{a}	0.41
ADF	50.66±1.94^{d}	51.10±0.62^{cd}	56.77±1.27^{b}	58.40±1.99^{a}	0.39
Cellulose	65.65±2.61^{c}	66.16±1.54^{bc}	70.16±2.29^{a}	71.02±3.21^{a}	0.62
Hemicellulose	78.37±4.62	79.53±4.69	79.77±3.99	79.80±3.66	1.07

Note: DM= dry matter, OM= organic matter, CP= crude protein, NDF= neutral detergent fiber, ADF= acid detergent fiber, FTD2= fermented *Tithonia diversifolia* (FTD) for 2 days, FTD3= FTD for 3 days, FTD4= FTD for 4 days, and FTD5= FTD for 5 days. SE= Standard Error. Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 6. Rumen fermentation characteristics of fermented *Tithonia diversifolia* at different durations of fermentation

Item	Treatments				SE
	FTD2	FTD3	FTD4	FTD5	
VFA (mM)	102.50±2.89^{c}	113.00±5.72^{b}	116.50± 5.70^{a}	117.25±2.22^{a}	1.11
NH_3 (mg/100 mL)	25.82±1.17^{d}	26.67±0.94^{c}	27.37± 0.46^{b}	28.37±1.06^{a}	0.24
pH	7.23±0.09	7.24±0.06	7.19± 0.02	7.31±0.13	0.07
Total gas production (mL/g DM)	110.23±7.92^{c}	116.73±8.54^{b}	134.41±13.18^{a}	146.39±3.83^{a}	2.25
Methane production (mL/g DM)	74.50±3.32^{c}	79.58±2.94^{b}	87.25± 2.93^{a}	96.83±8.86^{a}	1.29

Note: VFA= volatile fatty acid, NH3= ammonia concentration, DM= dry matter, FTD2= fermented *Tithonia diversifolia* (FTD) for 2 days, FTD3= FTD for 3 days, FTD4= FTD for 4 days, and FTD5= FTD for 5 days. SE= Standard Error. Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 7. Nutrient components of combination of fermented *Tithonia diversifolia* for 5 days and avocado leaves (% dry matter)

Nutrient component (%)	Treatments			
	FTDAL1	FTDAL2	FTDAL3	FTDAL4
Dry matter	93.96	93.68	93.40	93.12
Organic matter	92.51	89.63	86.76	83.88
Ash	7.72	10.54	13.36	16.17
Ether extract	5.47	4.71	3.95	3.18
Crude protein	15.56	19.93	24.30	28.67
Crude fiber	21.02	17.45	13.89	10.32
Nitrogen free extract	49.73	46.36	42.99	39.62
Neutral detergent fiber	38.15	36.30	34.45	32.60
Acid detergent fiber	34.90	32.65	30.40	28.16
Cellulose	16.90	15.89	14.88	13.87
Hemicellulose	3.26	3.65	4.05	4.44
Lignin	16.22	13.30	10.38	7.45
True digestible nutrient	71.65	68.52	65.38	62.25

Note: FTDAL1= 20% fermented *Tithonia diversifolia* (FTD) for 5 days + 80% avocado leaves (AL); FTDAL2= 40% FTD for 5 days + 60% AL; FTDAL3= 60% FTD for 5 days + 40% AL; and FTDAL4= 80% FTD for 5 days + 20% AL.

Table 8. Nutrient digestibility of the combination of fermented *Tithonia diversifolia* for 5 days and avocado leaves

Nutrient digestibility (%)	Treatments				SE
	FTDAL1	FTDAL2	FTDAL3	FTDAL4	
DM	62.73± 2.78^{d}	65.59± 0.56^{c}	66.75± 0.89^{bc}	69.14± 2.48^{a}	0.48
OM	62.23± 2.59^{d}	63.91± 0.71^{c}	65.25± 0.72^{bc}	67.14± 2.65^{a}	0.48
CP	61.22± 5.46^{d}	65.98± 2.63^{c}	68.59± 0.72^{bc}	74.70± 3.19^{a}	0.84
NDF	49.63± 1.34	49.91± 1.42	51.21± 1.38	53.60± 3.73	0.55
ADF	46.46± 2.26	46.11± 2.69	47.41± 2.52	47.94± 5.73	0.90
Cellulose	64.71± 1.41^{d}	64.98± 7.63^{cd}	76.18± 3.92^{b}	80.95± 7.90^{a}	1.74
Hemicellulose	69.91±14.95	83.87±12.14	79.71±11.51	80.36±10.85	3.11

Note: DM= dry matter, OM FTDAL1= 20% fermented *Tithonia diversifolia* (FTD) for 5 days + 80% avocado leaves (AL); FTDAL2= 40% FTD for 5 days + 60% AL; FTDAL3= 60% FTD for 5 days + 40% AL; and FTDAL4= 80% FTD for 5 days + 20% AL. SE= Standard Error. Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 9. Rumen fermentation characteristics and methane productions of the combination of fermented *Tithonia diversifolia* for 5 days and avocado leaves

Item	Treatments				SE
	FTDAL1	FTDAL2	FTDAL3	FTDAL4	
VFA (mM)	101.25±13.15^{d}	110.00±12.25^{c}	122.50±2.89^{b}	123.75±12.50^{a}	2.76
NH_3 (mg/100 mL)	19.98± 1.96^{c}	22.74± 2.26^{b}	24.23±1.20^{a}	24.44± 1.92^{a}	0.47
pH	7.25± 0.06	7.31± 0.07	7.25±0.01	7.28± 0.06	0.01
Total gas production (mL/g DM)	118.13±20.01^{bc}	139.08±22.75^{a}	106.25±5.94^{c}	133.54±12.29^{a}	4.15
Methane production (mL/g DM)	84.00±13.70^{bc}	107.21±16.78^{a}	75.04±8.78^{c}	99.21±10.39^{a}	3.20

Note: VFA= volatile fatty acid, NH_3= ammonia concentration, DM= dry matter, FTDAL1= 20% fermented *Tithonia diversifolia* (FTD) for 5 days + 80% avocado leaves (AL); FTDAL2= 40% FTD for 5 days + 60% AL; FTDAL3= 60% FTD for 5 days + 40% AL; and FTDAL4= 80% FTD for 5 days + 20% AL. SE= Standard Error. Means in the same row with different superscripts differ significantly ($p<0.05$).

of fermentation. This result is in line with the result reported by Adeleke *et al*. (2017) that there was an increase in the crude protein content of fermented cassava peels compared to unfermented one. The protein content increased with an increase in the duration of fermentation. They stated that the increase of protein content of fermented cassava peels was caused by the high number of microorganisms that capable of secreting extracellular enzymes, such as amylases, linamarases, and cellulases during their breakdowns in the fermentation, the ability of the microorganisms to synthesize amino acids, the addition of crude proteins produced by bacterial isolate, and a combination of factors such as carbon dioxide, temperature, and other factors. The high protein content is required by ruminants and microbial living in the rumen (Putri *et al*., 2019). In addition, the crude fiber content of fermented *T. diversifolia* was significantly different ($p<0.05$) from the control treatment. Adeleke *et al*. (2017) also found that the crude fiber content of the fermented and unfermented cassava peel showed a general decrease with the increase in the duration of fermentation. They explained that the decrease was

caused by the ability to ferment microorganisms to degrade the crude fiber. *L. bulgaricus* will decompose or break the cell wall and indigestible coatings of these products physically and chemically (Sharma *et al.,* 2020). The decomposing of the cell walls was done under either anaerobic or aerobic conditions. *L. bulgaricus* will secrete an enzyme to break down the carbohydrate of the cell wall into cellulose, hemicellulose, and related polymers that are not digestible into simpler sugars and sugar derivatives (Hasan *et al.,* 2014). Fermentation can increase crude protein and decrease the crude fiber content, eventually increasing nutrient availability for ruminants. In line with Zhao *et al*. (2018) who reported that rice straw silage with addition of *L. plantarum* enhance its fermentation quality, nutritive characteristics, and *in vitro* digestibility.

Phosphorus contents of fermented *T. diversifolia* with *L. bulgaricus* for 2 to 5 days are presented in Figure 2. Hendarto *et al*. (2019) stated that *L. bulgaricus* produces phosphoglycerate enzyme that is able to escalate phosphorus content so that phosphorus is available for ruminants. Phosphorus is utilized for ATP synthesis in the body of microbial rumen. This mineral also plays a role as a buffer originating from the fermentative process in the rumen. The lack of phosphorus appeared to suppress the breakdown of protein in the rumen, causing a decrease in ruminal ammonia concentration and branched-chain fatty acid. This decrease will suppress the growth of microbial rumen (Jain & Mudgal, 2021).

Adding phosphorus to feed can increase the total bacterial population in the rumen (Zain *et al.,* 2010; Ningrat *et al.,* 2019; Pazla *et al.,* 2020). Rumen microbes require phosphorus for growth which will degrade the feed to produce final products in the form of volatile fatty acid (VFA) and ammonia (NH_3).

Trial 2

Nutrient digestibility of fermented *Tithonia diversifolia*. The high nutrient content of fermented *T. diversifolia for* five days (Table 4) contributed to the increased nutrient digestibility. In addition, the longer duration of fermentation will increase nutrient digestibility. *L. bulgaricus* can degrade a more complex structures with a longer duration of fermentation. Fermentation is a process of physically, chemically, and biologically degrading a hard structure so that materials from complex structures become simpler and digestibility is more efficient. A previous study by Sun *et al*. (2019) reported that corn stover silage with lactic acid bacteria improved *in vitro* digestibility of nutrients.

The low crude fiber content of FTD5 contributed to the high nutrient digestibility in FTD5. From Table 4 we can see that the longer the fermentation duration will decrease the feed's crude fiber content due to the increased digestibility of the feed. Feed ingredients with low crude fiber will be easier to digest because microbial rumen may easily enter the cell walls and ferment the feed (Jamarun *et al.,* 2017; Yanti *et al.,* 2021). On the contrary, high crude fiber content indicates the cell walls are thicker and resistant to fiber-digesting microbes. It can result in a decrease in the digestibility of the feed ingredients. Nutrient digestibility in this study is higher than that reported by Pazla *et al*. (2021a), who compared nutrient digestibility of fermented *T. diversifolia* with *L. plantarum* and *A. ficuum.* This difference may be caused by different feed ingredients in each study.

Rumen fluid characteristics and methane production of fermented *Tithonia diversifolia*. Ruminal pH is a crucial parameter in maintaining the efficiency of fermentation in rumen. Ruminants have well-developed systems for keeping ruminal pH within a normal range of 5.5–7.0 (Puniya *et al.,* 2015). Although not in the normal range, *in vitro* ruminal fermentation maintained a high nutrient digestibility. It means that this range still provides a suitable condition for fermentation, microbial rumen growth, and nutrient degradation in the rumen.

High NH_3 concentration in the rumen indicated that the protein content of the ration has high rumen degradable protein fraction and dry matter digestibility. FTD5 treatment also had high protein content, which contributed to the increase in NH_3 concentration. The rumen microbes can utilize the high NH3 concentration in the rumen as a nitrogen source for microbial protein synthesis (Uddin *et al.,* 2015; Yang *et al.,* 2016). In line with Chen *et al*. (2017), who reported that the ruminal NH_3 concentration increased linearly with the addition of *L. acidophilus.* The role of rumen microbes in converting protein to NH_3 results in increased NH_3 production (Polyorach *et al.,* 2016). As a result, NH_3 was used to provide carbon sources for microbial synthesis, which may be used as an amino acid for the host (Tan *et al.,* 2012). Therefore, adding *L. acidophilus* could affect microbial protein synthesis and cellulose degradation rates. The study by Sadarman *et al*. (2020) contradicts this study, which reporting that NH_3 observed an insignificant difference in the ensiled soy sauce-by product with lactic acid bacteria compared to ensiled soy sauce-by product with tannin. It indicated that protein solubility of treatments has similar degradation. Additionally, tannins could suppress protein degradation during ensiling, particularly in a high-protein silage such as soy sauce-by product.

Total VFA is the product of microbial rumen activity in degrading the energy sources of feed (Belanche *et al.,* 2012), which play as major energy source for ruminants. According to Putri *et al.* (2019), the optimum range of total VFA is 67.67 to 162.17 mM. High digestibility of organic matter in FTD5 caused a high total VFA concentration. VFA concentration is linked with the availability of fermentable carbohydrates in the rumen. Carbohydrates are the most influential compounds in determining the digestibility of organic matter because carbohydrates as energy producers (VFA) are the largest component in feed (Pazla *et al.,* 2021b).

There was no significant difference in total gas and methane gas production observed in all treatments, although a longer duration of fermentation increased total gas and methane gas production. This result was caused by the decreased total phenol and tannin (Table 3) contained in each treatment with the longer fermentation duration, so they cannot interfere with the growth and activity of protozoa. The CO_2 and CH_4

gases produced are the results of microbial activity in the rumen fluid digesting the food substances of the feed (Pazla *et al.,* 2021b). Feeds containing plant secondary metabolites such as saponins or phenols, especially tannins, can be used as a tropical nutrition strategy to reduce total gas and methane (CH_4) emissions from ruminants. In line with the report of Philippeau *et al.* (2017), who found a significant effect of *L. plantarum* on methane emission. Methane emission decreased in cows fed a low-starch diet supplemented with a combination of *Propionibacterium P63* and *L. plantarum*. The use of *L. plantarum* can alter the pattern of ruminal fermentation. Besides that, they also reported that *Lactobacillus spp.* can produce antimicrobial peptides such as bacteriocins that affect methane emissions.

Trial 3

Nutrient digestibility of the combination of fermented *Tithonia diversifolia* and avocado leaves. Nutrient digestibility increased with the increasing percentage of fermented *T. diversifolia* and decreasing percentage of avocado leaves and the highest nutrient digestibility was observed in FTDAL4. Low digestibility in FTDAL1, FTDAL2, and FTDAL3 was caused by a high ratio of avocado leaves in each treatment. High lignin content in avocado leaves became a barrier for the microbial rumen to degrade nutrients, eventually decreasing nutrient digestibility. Besides that, high nutrient content in fermented *T. diversifolia* contributed to the high digestibility in line with Pazla *et al.* (2022). According to Pazla *et al.* (2021d), lignin negatively correlates with ADF digestibility. High lignin content will decrease nutrient digestibility because rumen microbes cannot degrade lignin.

Fermented *T. diversifolia* and avocado leaves can complement each other's nutrient contents which eventually increases *in vitro* digestibility of nutrients. A significant increase in crude protein digestibility may be due to the high protein content of *T. diversifolia* and avocado leaves. Digested protein will be utilized by microbial rumen to synthesize microbial protein so that it increases the population of rumen microbes. In this study, increasing nutrient digestibility was expected that the population and activity of rumen microbes increase, eventually increasing nutrient digestibility. This is in line with Zain *et al.* (2020) who stated that nutrient digestibility is related to the high protein content of feed and its digestibility. Protein degradation produces $N\text{-}NH_3$, which the rumen microorganisms need to synthesize microbial proteins.

Avocado leaves have potential as a forage source for ruminant because they have adequate nutrient, 11.19% crude protein content and 24.59% crude fiber content (Table 1). This protein content of avocado leaves complemented fermented *T. diversifolia* and contributes to the increased nutrient digestibility by assisting the synthesis of microbial proteins. In line with de Evan *et al*. (2020) and Gbaguidi & Saricicek (2021), who stated that avocado by-product, which is released in large amounts, can also be used as a source of roughage in ruminant feeding. It was also discovered that adding avocado by-product to the ration of dairy goats can increase the quality of milk's fatty acid profile while having no deleterious impact on milk yield (Velarde *et al.,* 2018).

Rumen fluid characteristics and methane production of the combination of fermented *Tithonia diversifolia* and avocado leaves. *In vitro* study of feed formulation containing avocado leaves had a pH of rumen fluid 7.40 (Jayanegara *et al.,* 2011). pH value of rumen fluid in this study was higher than that of the recent study of Pazla *et al*. (2021a) who stated that *T. diversifolia* was fermented with *L. plantarum* for 5 days, had a 6.83 pH of rumen fluid. The high pH value of rumen fluid in the combination of fermented *T. diversifolia* by *L. bulgaricus* with avocado leaves was due to the high NH_3 production. NH_3 is alkaline, so the pH of the rumen fluid will be high. The high NH_3 production was due to the high protein degradation from *T. diversifolia* in the rumen. Rumen pH will remain normal because of the balance of VFA (acidic) and NH_3 (alkaline).

The increasing percentage of fermented *T. diversifolia* and the decreasing component of avocado leaves in the experimental ration have a significant effect on total VFA concentration and the highest total VFA was observed in FTDAL4. The high value of total VFA indicated that ration contains high fermentable energy sources, especially fermented *T. diversifolia.* Fermented feed is more digestible because degrading bacteria play a role in loosening the complex bond so that nutrient is more available for ruminants. This study is in line with the study of Pazla *et al*. (2021a), who showed a difference in total VFA production between fermented *T. diversifolia* with *L. plantarum* for three and five days. They reported it *in vitro* total VFA production is higher in fermented *T. diversifolia* with *L. plantarum* for five days compared to that fermented with three days. The duration of fermentation affects *in vitro* total VFA production. The fiber content of the treatment also contributed to total VFA production. Makmur *et al*. (2020) stated that fermentation pattern is related to treatments' decreased dietary fiber content. The total VFA in FTD5 increased due to its low crude fiber and lignin content. The duration of fermentation significantly affects the lignin content (Pazla *et al.,* 2021d), so microbial rumen was not inhibited from doing degrading activity.

The adequate concentration of NH_3 to support the growth of rumen microbes ranges of 4 to 12 mM or equivalent to 5.6 to 16.8 mg/100 mL (Sutardi, 1980). The highest concentration of NH_3 in this study was due to the high protein degradation in fermented *T. diversifolia.* Significant increase in NH_3 production may be due to the high protein content of *T. diversifolia* and avocado leaves. The concentration of NH_3 is largely determined by protein content in the feed given to the livestock. In line with Putri *et al*. (2021), who stated that the content of protein in the feed would affect the concentration of NH_3 in the rumen, and NH_3 concentration is also determined by the degree of degradability, feeding retention time in the rumen, and the acidity of the rumen pH.

Total gas and methane production was affected by different tannin contents in each treatment. Tannin in *T. diversofilia* and avocado leaves is 15.36% DM and

30.50% DM, respectively (Table 3). These tannins can decrease methane emission through the defaunation of protozoa, which is the hosts for methanogenic bacteria and inhibit the growth of microbes that produce hydrogen gas (Bhatta *et al.,* 2009; Cieslak *et al.,* 2012). Tannin depresses the population of protozoa by interfering permeability of the cell wall of the protozoa, which in turn will reduce the population of methanogenic bacteria as reported by Patra & Saxena (2010). Adding more than 2% of the total ration of tannin can consistently reduce rumen methane (CH_4) production but will also reduce dry matter digestibility of the feed. Reducing methane (CH_4) emissions can make livestock energy efficient. This condition is because the release of methane gas represents the amount of energy lost in the range of 2%-14%, depending on the type of feed given (Johnson & Johnson, 1995; Moss *et al.,* 2000).

CONCLUSION

This study declares that fermented *T. diversifolia* increases nutrient content and digestibility. Increasing fermented *T. diversifolia* and decreasing avocado leaves in diet formulation increase nutrient digestibility (dry matter, organic matter, crude protein, and cellulose), rumen fluid characteristics (total volatile fatty acid and ammonia concentration) but do not optimize the decrease in total gas and methane gas productions. The optimal ratio of fermented *T. diversifolia* and avocado leaves is 80%:20% and needs further *in vivo* research to evaluate the effect of this feed formulation on goat productivity.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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