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Evaluation of Rumen Degradable Protein Values from Various Tropical Foliages Using in Vitro and in Situ Methods

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ABSTRACT

There are no recommended methods for obtaining rumen degradable protein (RDP) values of tropical foliage to meet ruminant feed quality standards. Different methods have produced different RDP values, prompting this study to compare and adjust values of RDP in tropical foliage obtained using both *in situ* and *in vitro* methods. Nine types of tropical foliages (*Gliricidia sepium, Leucaena leucocephala, Calliandra calothyrsus, Indigofera zollingeriana, Moringa oleifera, Calopogonium mucunoides, Arachis hypogaea, Sesbania grandiflora,* and *Arachis pintoi*) were analyzed for their chemical composition (ash, crude protein (CP), crude fiber (CF), ether extract (EE), neutral detergent fiber (NDF) and acid detergent fiber (ADF) using proximate analysis, Van Soest's fiber fraction, and Cornell protein fraction. The samples were then evaluated for their degradation characteristics, including dry matter degradation (DMD), organic matter degradation (OMD), and RDP in rumen using 1) conventional or first-stage *in vitro* rumen fermentation technique, 2) modification of the first-stage *in vitro* method, and 3) *in situ* method. The evaluation was performed in three consecutive runs using a completely randomized design for chemical analysis and a randomized complete block design for degradation study. The result shows that *I. zollingeriana, M. oleifera, L. leucocephala,* and *G. sepium* are among the best tropical foliage for ruminants due to their higher CP, DMD, OMD, and RDP values resulting from lower CF, NDF and ADF values. *C. calothyrsus*, with its high CP and low fiber, resulted in DMD, OMD and RDP values but its high tannin content in the foliage may be a limiting factor. Conventional and *in situ* produced similar degradation characteristics, significantly higher than *in vitro* modification. It is recommended to cultivate *I. zollingeriana, M. oleifera, L. leucocephala,* and *G. sepium* for ruminant protein supplements, and it is suggested to use standard *in vitro* to obtain a precise RDP value of tropical foliages.

Key words: Tropical Foliage, RDP, Method, Ruminant, Protein Supplement.

INTRODUCTION

Protein is a crucial factor that determines the productivity of ruminants, which is utilized by both for host ruminant and the microbial rumen (Zain et al. 2020; Sari et al. 2022). Ruminant require high protein and energy to optimize their growth and production (Pazla et al. 2018a; Zain et al. 2019; Arief and Pazla 2023). One major constraint that hinders ruminant productivity in tropical regions is the lack of forage quality, which influences ruminant performances (Pazla et al. 2018b; Indah et al. 2020; Suyitman et al. 2020; Despal et al. 2021a). Generally, farmers use a source of fiber feed as a component of a complete ration for ruminants that contains low quality (Zain et al. 2010; Zain et al. 2014; Zain et al. 2015; Pazla et al. 2021a; Elihasridas et al.

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2023). Dairy farmers have increased concentrate utilization, which might be applied to a certain degree, but it increases the feed cost tremendously. The increasing concentrate used in dairy ration also reduces milk fat and milk fatty acids quality, lowering farmer income due to lower total solid in milk (Lestari et al. 2015; Despal et al. 2021b; Despal et al. 2021c; Anzhany et al. 2022). Besides that, high secondary metabolites also depress the utilization of feed for ruminants (Pazla et al. 2021b; Pazla et al. 2023a; Pazla et al. 2023b). Therefore, the utilization of tropical foliage as a protein supplement for ruminants is suggested. The tropical foliage is rich in protein with high degradability and digestibility. Among the available tropical foliage species are *Gliricidia sepium, Leucaena leucocephala, Calliandra calothyrsus, Indigofera zollingeriana, Moringa oleifera, Calopogonium mucunoides*, *Arachis hypogaea*, *Sesbania grandiflora,* and *Arachis pintoi*. The foliages vary in nutrient content and degradability in the rumen (Rahmat et al. 2021; Zain et al. 2023).

Another obstacle to achieving high ruminant performance in tropical regions is the lack of accuracy in ration formulation due to limited feeds and nutrient requirement information available. Many tropical feedstuffs databases have been developed, but they are mainly based on chemical information and digestibility (Despal et al. 2020; Indah et al. 2020; Agustiyani et al. 2021; Despal et al. 2021a; Zahera et al. 2022). Including rumen-degradable protein (RDP) information in the ration formulation improves ruminant performance accuracy since it provides sufficient information on the fulfillment of protein requirements for both microbes and hosts animal (Putri et al. 2019; Rosmalia et al. 2021; Rosmalia et al. 2022a). Putri et al. (2021) reported that RDP-RUP-based rations increase *in vitro* nutrient digestibility, rumen fermentation characteristics, and microbial protein synthesis. The Indonesia National Standard for ruminant feeds requires such information in ruminant feed trades in Indonesia.

Satter and Slyter (1974) noted the importance of ammonia requirement for rumen microbes in the 1970s, and the value of a protein fed to ruminants is heavily influenced by its extent of degradation in the rumen, known as Rumen Degradable Protein (RDP) (Santos et al. 1998). Despite this, there has been little intensive discussion on the method for quantifying the RDP percentage (Schwab 2017). The RDP values can be obtained using *in vivo*, *in vitro*, or *in situ* methods (Orskov and Mc Donald 1979; Silva et al. 2020), with results varying depending on the method used. *In vivo* method produces the most accurate value, but it is also the most costly, labor-intensive, and requires many samples (Putri et al. 2019). Therefore, the *in vitro* and the *in situ* methods have been developed to overcome these problems. Each method has its own advantages and disadvantages, and the results produced may differ. However, limited information is available on the equality of RDP values obtained using *in vitro* and *in situ* methods. Thus, the purpose of this study is to evaluate the quality of tropical foliage feed and compare the RDP values obtained using *in vitro* and *in situ* methods.

MATERIALS AND METHODS

Ethics Approval of Research

The surgery protocol for handling and caring for experimental animals is based on the IPB University Animal Ethics Committee with protocol number: 047/KEH/SKE/XI/2021.

Sample Preparations

Nine legumes were collected from various cities in Indonesia, including Padang, Bogor, and Bandung. The plants included of *Gliricidia sepium, Leucaena leucocephala, Calliandra calothyrsus, Indigofera zollingeriana, Moringa oleifera, Calopogonium mucunoides*, *Arachis pintoi*, *Sesbania grandiflora*, and *Arachis hypogaea*. Approximately 4kg of each fresh sample were subjected to oven-drying at 60^{o}C for 72hrs until a constant weight was obtained, in order to determine their dry matter content. The dried samples were then milled to pass through a 1mm screen for subsequent laboratory analysis, and were stored in an airtight container for further chemical and degradation analysis.

Chemical Analysis

The nutrient content in the tropical foliage was analyzed using proximate and van Soest's analysis to determine DM, ash, CP, EE, CF, NDF, and ADF. In addition, NIRS analyses were also conducted using NIRSFlex 500 from Buchi (made in Switzerland) with reference to the local database. Chemical analyses were performed following the guidelines of AOAC (2005). The DM and OM contents of the samples were determined using an oven at 105°C and a furnace at 550°C, respectively. Micro-Kjeldahl and Soxhlet extraction apparatus were employed to measure the contents of CP and EE, respectively. The ANKOM A200 fiber technology (made in the USA) and the procedures outlined by Van Soest et al. (1991) were used to determine NDF and ADF by sequential digestion with H_2SO_4 and NaOH solutions.

Degradation Determination of Tropical Foliage

Conventional *In Vitro* Method

The first-stage in vitro rumen fermentation technique of the ground samples was evaluated using the protocol of Tilley and Terry (1963). Specifically, 0.5gm of the sample was placed in a fermentation glass tube with 40mL of McDougall's buffer, followed by the addition of approximately 10mL of rumen fluid obtained from a slaughterhouse. To ensure anaerobic conditions, all tubes were immediately sealed with ventilated rubber stoppers after being continuously flushed with CO_2 for 30sec. Each sample was represented by two fermentation tubes in each run, with the in vitro incubation being carried out in three separate runs (replicates) over several weeks. Following the first stage of incubation, the tubes were centrifuged for ten minutes at 4,000rpm. The DM, OM, and CP contents of the residues were analyzed, and the DMD, OMD, and RDP values were calculated using the DM, OM, and CP residues, respectively.

Modification of *In Vitro* Method

A modified version of the conventional method was utilized by increasing the number of samples per tube to mimic local ruminant rumen conditions. One gram of the sample was placed into a 100mL fermentation tube containing 40mL of buffer and 10mL of sheep rumen liquor, which was used in place of the conventional cattle rumen liquor. Termination of fermentation in the modified method was achieved by adding two drops of saturated $HgCl_2$, while in the conventional method employed a temperature- reducing technique (freezing). The remaining steps of the procedure were similar to the standard method.

***In situ* Method**

A Frisian Holstein bull with a ruminal fistula and a body weight of approximately 500 kg was utilized in this study. The cattle were fed twice a day in the morning and evening with composition diets of 60% napier grass and 40% concentrate mix on a dry matter basis. A nylon bag measuring 10 × 20cm was self-produced using Abutai material, which had been previously studied (Despal et al. 2022a) and showed similar characteristics to ANKOM. Five grams of foliage samples were placed in the nylon bag and incubated in the rumen for 6, 9, 12, 24, 48, and 72hrs. The nylon bag with 0hrs was filled with the samples that were not incubated in the rumen. After incubation, the nylon bag was cleaned to remove any feed particle, digesta, rumen fluid, or microbes that might have stuck to it. The nylon bag was than dried in an oven at 60˚C for 48hrs and weighed using the digital scale laboratory type OHAUS PA214C (made in the USA). The residual samples in the nylon bag were collected to determine the dry matter (DM), organic matter (OM), and crude protein (CP), according to AOAC (2005) standards. The ruminal degradabilities of DM, OM, and CP or the kinetic parameters were estimated using an equation developed by Orskov and McDonald (1979). The CP calculation used to determine RDP was similar to the method used by Rosmalia et al. (2021).

Parameter Measured

Parameter measured in this study were nutrient composition (DM, ash, CP, CF, EE, NDF and ADF), degradation (DMD, OMD and RDP), and comparison of RDP using different methods.

Statistical Analysis

This study used a completely randomized design for nutrient content and a randomized block design for degradability studies. The data were analyzed using Analysis of Variance (ANOVA). Significantly differences among the treatments were further tested using the Tukey test. All the procedures were conducted using SPSS 20 statistical package software.

RESULTS

Nutrient Composition and Degradation of Tropical Foliages

Figure 1 and Table 1 shows that there were no significant differences (P>0.05) in the dry matter and ash content of the tropical foliages. However, significant differences (P<0.05) were observed in the crude protein, extract ether (Table 1), crude fiber, NDF, and ADF

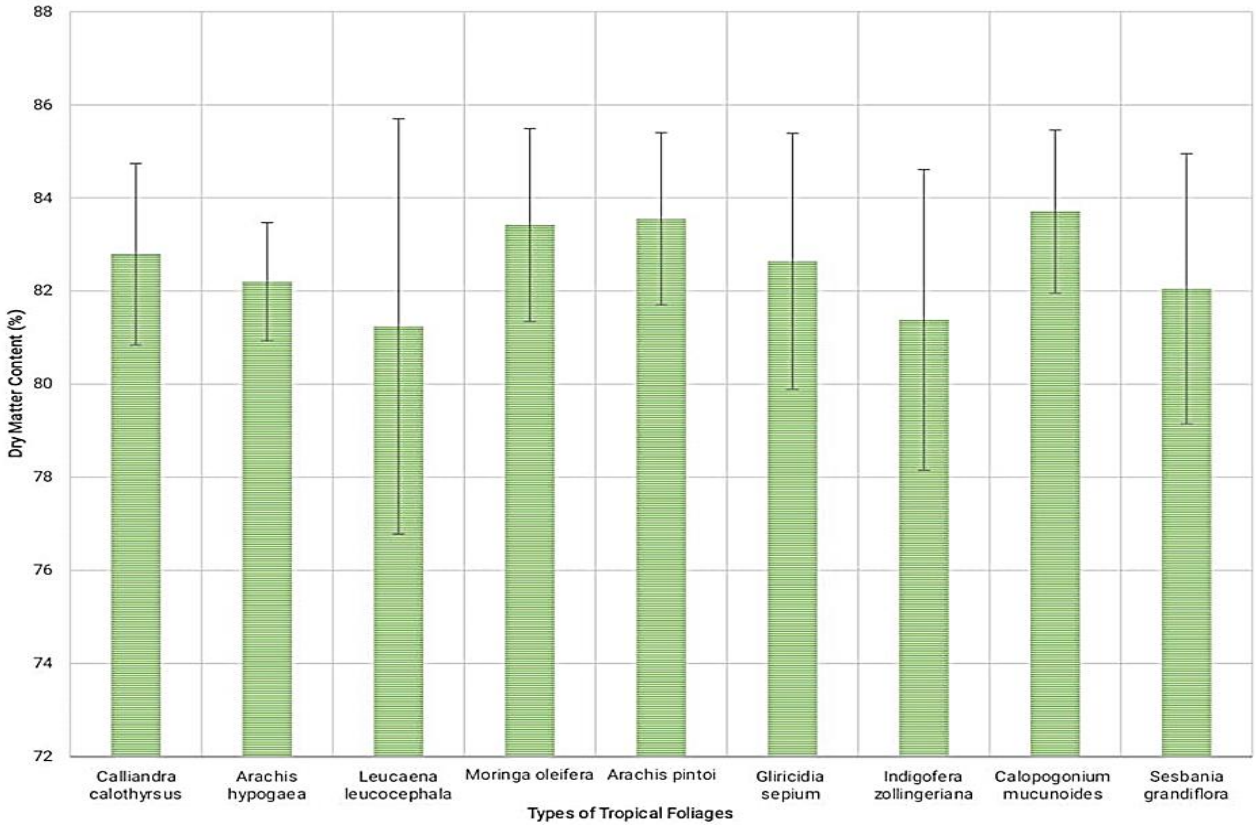

Fig. 1: Dry matter content of tropial foliages.

contents (Table 2). Among the tropical foliages, *M. oleifera* had the highest crude protein (23.18%), *C. mucunoides* had the highest crude fiber (19.60%), and *S. grandiflora* had the highest extract ether (3.88%). NDF and ADF were high in *A. hypogea* and *C. mucunoides* (38.98% and 34.78%). In contrast, the lowest NDF and ADF value were found in *M. oleifera* (25.96% and 9.21%).

Table 3 shows that the dry matter degradation (DMD), and organic matter degradation (OMD) of tropical foliages were significantly (P<0.05) different. *I. zollingeriana* had the highest DMD and OMD values among the tropical foliages, while *C. calothyrsus* had the lowest values. The rumen degradable protein (RDP) of tropical foliages in the study did not differ significantly (P>0.05). The highest RDP values was observed in *I. zollingeriana* (69.76%), while the lowest was found in *C. mucunoides*.

Comparison of Rumen Degradable Protein (RDP) Using Different Methods

A comparison of the degradation of dry matter, organic matter and protein (RDP) from different methods is shown in Table 4. The table shows that the degradation values obtained from different methods significantly differed (P<0.05). The M3 and M2 produced insignificantly different values of DMD and OMD compared to M1. However, the M3 was significantly higher than M2.

The RDP values produced using M1 and M3 were similar but different from the M2. Therefore, an adjustment is needed for the M2 data to achieve similar results with the M1 and M3 methods. Adjustment values of DMD, OMD and RDP obtained using the M2 method toward M1 and M3 can be done using the formula shown in Fig. 2a, 2b and 2c, respectively.

DISCUSSION

Nutrient Composition and Degradation of Tropical Foliages

The Fig. 1 and Table 1 indicates that DM and ash contents of the foliage were not significantly (P>0.05) different among the species. However, other parameters such as CP, CF, EE, NDF, and ADF varied among the foliage. *I. zollingeriana, M. oleifera, G. sepium, L. leucocephala. and C. calothyrsus* contained CP of more than 20% and can be categorised as a protein source (Zain et al. 2019). The extract ether content in the

Table 1: The ash, crude protein, and ether extract contents of tropical foliage

Tropical foliage	Ash (% DM)	CP (% DM)	EE (% DM)
Calliandra calothyrsus	8.71±7.68	20.46±3.64abc	2.22±0.23ab
Arachis hypogaea	9.13±1.21	16.32±3.13a	2.07±1.78ab
Leucaena leucocephala	7.13±0.83	21.28±3.20bc	2.45±0.67ab
Moringa oleifera	9.55±1.28	23.18±4.14c	2.80±1.65ab
Arachis pintoi	10.0±2.88	17.12±3.08ab	1.34±0.19a
Gliricidia sepium	8.17±1.16	21.34±2.92bc	2.80±0.34ab
Indigofera zollingeriana	7.58±0.83	22.98±3.29c	2.37±0.11ab
Calopogonium mucunoides	7.85±1.57	16.73±2.87a	2.09±0.07ab
Sesbania grandiflora	7.56±1.78	18.37±4.03ab	3.88±0.18b

Values (mean±SD) bearing different alphabets in a column differ significantly (P<0.05). CP: crude protein; CF: crude fiber; EE: ether extract.

Table 2: The crude fiber, neutral detergent fiber, and acid detergent fiber contents of tropical foliage

Tropical foliage	CF (% DM)	NDF (% DM)	ADF (% DM)
Calliandra calothyrsus	12.30±6.00a	33.30±8.89ab	20.02±8.93ab
Arachis hypogaea	16.41±1.17ab	38.98±17.63b	29.78±6.29c
Leucaena leucocephala	13.11±3.80a	31.60±1.54ab	20.23±6.27ab
Moringa oleifera	12.73±2.91a	25.96±10.78a	9.21±5.76a
Arachis pintoi	17.74±1.16ab	34.66±2.37ab	25.98±12.6cb
Gliricidia sepium	13.55±5.55ab	31.95±1.05ab	18.96±6.06ab
Indigofera zollingeriana	12.87±2.07a	32.57±2.07ab	22.45±2.32b
Calopogonium mucunoides	19.60±3.28b	36.73±2.80ab	34.78±5.59c
Sesbania grandiflora	14.07±4.96ab	31.24±4.87ab	18.68±5.62ab

Values (mean±SD) bearing different alphabets in a column differ significantly (P<0.05). CF: crude fiber; NDF: neutral detergent fiber; ADF: aciddetergent fiber.

Table 3: Degradation of dry matter, organic matter and protein of tropical foliage

Tropical foliage	DMD (%)	OMD (%)	RDP (% CP)
Calliandra calothyrsus	48.36±4.75a	50.36±2.99a	55.09±4.88
Arachis hypogaea	60.30±1.84bc	61.60±1.54bc	56.82±6.96
Leucaena leucocephala	62.40±2.54bcd	61.82±2.54bc	57.06±14.26
Moringa oleifera	68.48±8.72cd	69.12±8.06cd	68.76±13.76
Arachis pintoi	63.26±3.71bcd	63.09±5.60bc	54.07±12.69
Gliricidia sepium	65.38±5.37bcd	65.71±4.48bcd	67.99±14.26
Indigofera zollingeriana	72.47±7.76d	73.71±7.36d	69.76±14.46
Calopogonium mucunoides	54.50±10.45ab	55.73±9.78ab	53.30±13.20
Sesbania grandiflora	64.06±4.57bcd	64.06±4.36bcd	54.72±16.96

Values (mean±SD) bearing different alphabets in a column differ significantly (P<0.05). DMD: dry matter digestibility; OMD: organic matter digestibility; RDP: rumen degradable protein.

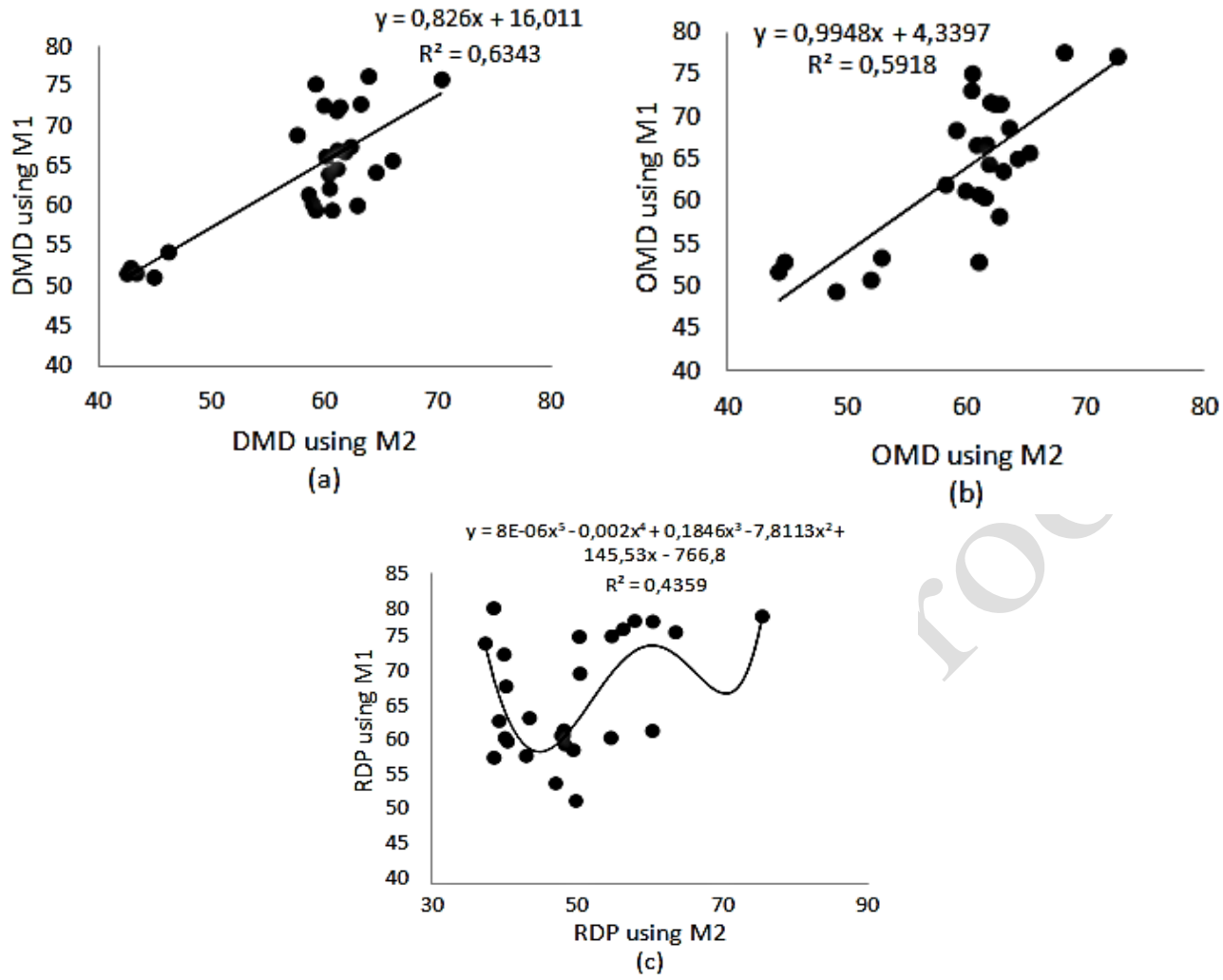

Fig. 2: Adjustment of M2 degradation to M1.

Table 4: The DMD, OMD, and RDP of tropical foliage using different methods

Methods	DMD (%)	OMD (%)	RDP (% CP)
In vitro (M1)	64.15±7.71ab	64.00±8.26ab	66.92±8.54b
Modified *in vitro* (M2)	58.12±7.34a	59.74±6.38a	49.31±9.27a
In situ (M3)	69.22±13.38b	69.10±13.28b	74.78±13.53b

Values (mean±SD) bearing different alphabets in a column differ significantly (P<0.05). DMD: dry matter digestibility; OMD: organic matter digestibility; RDP: rumen degradable protein.

S. grandiflora was significantly higher than *A. Pintoi*. The high EE content in *S. grandiflora* can increase ration energy since EE produces more energy than other nutrients (Riestanti et al. 2021). A feed can be classified as a fiber source if its CF content is more than 18% (Hasanah et al. 2017). In this case, only *C. mucunoides* fits the category. Although the CF content in the foliage studied was less than 18%, it was still higher than the average CF in concentrate (Despal et al. 2017; Riestanti et al. 2021; Rosmalia et al. 2021), therefore, it help maintain sufficient milk fat production. In contrast to CP content, CF content in the *I. zollingeriana, M. oleifera, G. sepium, L. leucocephala, and C. calothyrsus* were lower than others. The CF content in *C. mucunoides* was significantly higher than *L. leucocephala, M. oleifera* and *I. zollingeriana*. The high CF content in the foliage is valuable for dairy ration if it consists of unbounded hemicellulose and cellulose. *M. oleifera, C. calothyrsus, G. sepium, S. grandiflora, L. leucocephala*, and *I. zollingeriana* had a high difference between NDF and ADF (hemicellulose), which represents the high hemicellulose content, which is easier to degrade in the rumen (Despal et al. 2021c). The opposite was found for *C. mucunoides*, which contain more resistant fiber fraction and is related to a lower degradation in the rumen.

Based on the nutrient content, it is evident that *I. zollingeriana, M. oleifera, G. sepium, L. leucocephala* and *C. calothyrsus* are promising protein supplement for ruminants in the tropical areas, particularly for dairy cows and goats that require sufficient fiber to support milk fat synthesis (Anzhany and Toharmat 2022; Pazla et al. 2022; Arief et al. 2023b; Pazla et al. 2023a). Additionally, foliage is more affordable for traditional dairy farmers than concentrate feed (Zain et al. 2019). High protein and low CF in the *I. zollingeriana, M. oleifera, G. sepium, L. leucocephala and C. calothyrsus* are predicted to have higher DM and OM degradability. CP and EE were positively correlated with DMD and OMD, while the CF and ADF were negatively correlated (Despal 2010; Indah et al. 2020).

Table 3 indicates that *I. zollingeriana* had a significantly higher DMD value compared to *A. hypogaea, C. mucunoides,* and *C. calothyrsus*. A similar trend was observed for the OMD parameter. However, there was no significant difference in the RDP value between the foliages, which could be attributed to the high variation within the species or replication. Almost all of the foliages exhibited DMD values of more than 60%, except for *C. calothyrsus* and *C. mucunoides*, which have been reported to show low degradation (Ahn et al. 1989; Tiemann et al. 2010; Rahmat et al. 2021). Ahn et al. (1989) found that after 48hrs of incubation in the rumen, degradation of *Acacia, Albizia,* and *Calliandra* was under 60%.

The OMD of the tropical foliage followed a similar pattern as the DMD. Low CP and high CF were thought to cause the low DMD and OMD in *C. Mucunoides*. Indah et al. (2020) have reported a negative correlation between CF and digestibility, while Despal et al. (2022b) have reported a positive correlation between CP and degradation. Low DMD and OMD in *C. calothyrsus* were not caused by the low CP and high CF, but rather by high anti-nutrients, especially tannin (Tiemann et al. 2010; Pazla et al. 2021c). Although tannin can improve microbial protein synthesis and suppress methanogenesis, it also alters ruminal fermentation by binding protein and making it unavailable to the rumen microbes (Ahn et al. 1989). Tropical foliage rich in tannins has been shown to suppress protozoa in the rumen. Adequate tannin concentration in the ration can improves nutrient and fiber fraction digestibility by increasing the fibrolytic microbial population. However, high concentrations of tannin, as in *C. calothyrsus* may decrease fiber digestion by complexing with lignocelluloseand preventing microbial digestion or by inhibiting cellulolytic microorganisms (Zain et al. 2019). Ahn et al. (1989) reported that the tannin content in *C. calothyrsus* was higher than in other tropical legumes.

Table 3 indicates that the RDP values of the different foliage were not significantly different, likely due to significant variation observed between the replicates. However, the RDP values of *I. zollingeriana, M. oleifera,* and *G. sepium* were over 60%, indicating extensive degradation of the protein in the rumen. The high CP content in *I. zollingeriana, M. oleifera,* and *G. sepium* resulted in high ammonia concentration in the rumen, leading to the need for combining these with less degradable CP feed to achieve the ideal RDP to RUP ratio of 60:40 in dairy rations (Rosmalia et al. 2022b). Conversely, low RDP *L. leucocephala* should be combined with more degradable feed CP to achieve the ideal RDP to RUP ratio. Furthermore, protection of protein was recommended for high RDP protein feed sources (Schwab 2017).

Comparison of Rumen Degradable Protein (RDP) Using Different Methods

The determination coefficient for DMD and OMD was relatively high, but the RDP value was slightly lower. It is evident that the M2 method produced a different result from M1 and M3, and the adjustment failed to yield more precise data. The differing results bet ween M2 and other methods may have resulted from the use of rumen inoculant sourced from sheep in M2, while M1 and M3 used inoculant sourced from cattle. The rumen contains a diverse range of microorganisms, including bacteria, protozoa, fungi, archaea, and viruses (Loor 2016), with different ruminant species containing varying proportions of microorganisms that play a critical role in nutrient breakdown and utilization through the degradation and fermentation process in the rumen (Czerkawski 1986; Jamarun et al. 2017a; 2017b). The difference in rumen microbe composition between the two ruminant species may also resulted from the use of different feeds used. In tropical regions, cattle tend to be fed more concentrates than sheep, particularly dairy cattle that consume up to 50% concentrate, resulting in a lower solid-to-liquid ratio of rumen content.

Method M1 used a 0.5:50:10 of a sample (gm): rumen fluid (mL): buffer (mL) ratio, which resulted in a lower solid-to-liquid ratio in the fermentation compared to M2, which used a 0.5: 40: 10 ratios. The M3 method, being an *in situ* method, used the actual solid-to-liquid ratio of cattle rumen. In situ (M3) experiments were carried out in the actual rumen after fistulation of the cattle to allow the insertion of the nylon bag.

Conclusion

It is concluded that *I. zollingeriana, M. oleifera, L. leucocephala,* and *G. sepium* were among the best tropical foliage for ruminants for their higher CP, DMD, OMD, and RDP resulted in lower CF, NDF, and ADF values. Conventional *in vitro* and *in situ* methods produced similar degradation characteristics, significantly higher than method 2. The farmer is advised to cultivate *I. zollingeriana, M. oleifera, L. leucocephala,* and *G. sepium* for ruminant protein supplements. HighCP with high RDP proportion in *I. zollingeriana, M. oleifera, L. leucocephala,* and *G. sepium* was suggested to be combined with less RDP feed in the dairy ration or using protected protein.

Author's Contribution

MZ, D and UHT designed the concept, searched for funding, supervised the field and laboratory works, and drafted and reviewed the paper. RP, EMP, YIY, RZ, AR and RAA collected and prepared samples and conducted laboratory work. RZ, AM, EMP and LAS supervised the laboratory work. RZ, EMP, MM, SDP, RP and IMA conducted data analysis and drafting.

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Competing Interests

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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