

Effect of *Azolla filiculoides* Meal Inclusion in the Napier Silage Total Mixed Ration on the *In vitro* Cumulative Gas Production and Digestibility

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ABSTRACT

This study was carried out to determine the nutritional value and digestibility of total mixed ration (TMR) Napier silage with different *Azolla filiculoides* meal inclusion percentages. Samples of *Azolla* were cultivated in the tank with the media from 1.0 g/L dilution of sheep manure. Inclusion of 0% (control), 6% (T1), 10% (T2), 16% (T3), and 23% (T4) *A. filiculoides* meal was used to replace the proportion of Napier silage and soybean meal according to treatments with four replicates. All treatments were analyzed to determine the nutritional composition, and *in vitro* gas production was recorded for 96 h. In contrast, *in vitro* dry matter digestibility (IVDMD), *in vitro* organic matter digestibility (IVOMD), and metabolizable energy (ME) of each TMR mixture were determined using the published equation. As a result, only T4 had shown a significant difference ($p < 0.05$) in crude protein (CP) and ether extract (EE) compared to other treatments. Values of dry matter (DM), CP, and ash of the TMRs were not affected on T1, T2, T3, and control. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were increased significantly at T3 and T4 compared to other treatments even though higher ($p < 0.05$) acid detergent lignin (ADL) as

replacement of 5.0% Napier silage and 1.0% soybean meal had shown a competitive value in their nutritional compared to the common TMR for ruminants. Therefore, a fermentation process was suggested to degrade indigestible components of *A. filiculoides* to enhance the potential of this species as an alternative feed source for a ruminant.

Keywords: *Azolla filiculoides*, digestibility, *in vitro* gas production, ruminant, total mixed rations

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INTRODUCTION

Agriculture has become one of the fundamental industries in Malaysia. These industries had contributed lucrative employment and concurrently supplied the domestic food requirements for the population. This local industry establishment will ensure food security for domestic consumption and reduces dependency on imported livestock product. The livestock industry has contributed around 12.4% of total agricultural gross domestic product (GDP) in 2013, whereby the ruminant sub-sector had only contributed 12.1% from it (Shanmugavelu, 2014). Malaysian National Agro-food Policy 2011–2020 (NAP) had emphasized the demand and production of meat which is expected to be increased. From 2010 to 2020, an increment of local demand for meat is estimated from 1.4 million metric tons (MT) to 1.8 million MT with a growth of 2.4% per annum, while meat production is forecast to increase from 1.6 million MT to 2.1 million MT with a growth of 2.7% per annum in the same period. The demand increase is also expected for other livestock products such as milk and eggs. However, the ruminant sector, which consists of beef and dairy cattle, dairy, buffaloes, sheep, and goats, is still small-scale (Rosali, 2015). Positive progress has been observed in recent years, but it can still not meet the local demand. Thus, Malaysia imports most of the needed beef, mutton, and dairy product from abroad, especially India, Australia, and New Zealand, to cater to the shortage. In 2014, the level of self-sufficiency (SSL) for beef,

mutton, and milk were 24.84%, 13.10%, and 12.93%, respectively. The lag in this ruminant sector is normally associated with several factors such as the lack of land resources, high feed price, cheaper import substitutes, poor private sector involvement, disease prevention and control, and lack of quality breeds, expertise, and workforce (National Agro-food Policy 2011–2020). The insufficient local protein source for the domestic market and high dependency on imported meats are associated with the issues regarding Malaysia's ruminant industry, especially in feeds and production systems. Eventually, research and development of any abundance material or local by-product had been emphasized to ensure our ruminant industry could be viable and sustainable for our domestic consumption.

Components of nutrient requirement were based on animal species, and stages had been highlighted in the research in developing new feed for livestock. The fiber source that farmers had used was from local agriculture by-products such as oil palm frond (OPF), corn stalk, and bagasse, while fish meal, copra cake, and soybean meal were used as a protein source in the feed. Palm kernel cake (PKC) or palm oil sludge (POS) was also used as an alternative source of protein and energy for the animal (Kum & Zahari, 2011; Seephueak et al., 2011). POS is a by-product from the palm oil mill effluent (POME) filtration that consists of approximately 9.6%–16.0% CP (Devendra et al., 1983). However, due to some changes in the livestock production systems towards semi-intensive and fully intensive systems,

agriculture and industrial waste were highly demanded and became pricey in the market. Indeed, the availability of these products was on a seasonal basis, and the supply was unable to be sustained due to higher prices was offered by the exporter to support a huge industry such as construction, papers, and cosmetics (Akbari & Resalati, 2012, Kumar et al., 2020; Sahota, 2014). Eventually, farmers had chosen Napier grass and soybean meal as the main source of fiber and protein, respectively. Although Napier grass has become one of the renewable fiber sources, it requires areas and workforce to ensure an adequate amount of quality fodder could be produced. In addition, shortages of labor and the inability to manage the cutting interval at 6 to 8 weeks had decreased the fodder nutritional quality (Zailan et al., 2016b). Meanwhile, due to the runaway prices, farmers and feed producers had to reduce or replace the soybean meal in their feed formulation with other alternative ingredients, such as palm kernel cake, even though its availability in the market is relatively limited and its price is unstable. Therefore, an effort was made to discover an alternative source of fiber and protein that is practical and affordable for farmers to produce.

Meanwhile, most animal farms will have a drainage system that drains farm waste to the main canal. All drainage was predominant by several aquatic plant species such as *Eichhornia crassipes*, *Pistia stratiotes*, and *Azolla filiculoides*. Those species had been found necessary as bioremediation agents and bio-fertilizers,

which have an important role in ecology conservation (Escoto et al., 2019). However, the uncontrolled population of the floating aquatic plants has been reported to harm aquatic ecology. Therefore, previous researchers have realized the potential of these plants as a source of additional fiber and protein for livestock. Hence, studies related utilization of an aquatic plant as an animal feed were conducted many years before. However, *A. filiculoides* species was found to be more suitable than other aquatic plant species due to its growth potential and nutrient content (Kamaruddin et al., 2019). In an optimal environment, this species can achieve a doubling time of 2–7 days and produce up to 2.9 g/m² day⁻¹ with a crude protein (CP) content of 22.48% kg⁻¹ DM, crude fiber (CF), 14.70% kg⁻¹ DM, neutral detergent fiber (NDF) 37.6% kg⁻¹ DM, and acid detergent lignin (ADL) 8.03% kg⁻¹ DM (Kollah et al., 2016). This species was also able to be cultivated in the livestock manure liquid. The bio-phytoremediation role was proved to absorb up to 2.6 tons N/ha year⁻¹ and 0.43 tons P/ha year⁻¹ from the ‘farm waste treatment collector pond’ before being drained into the main drainage system (Costa et al., 1999). In this environment, 1.5 g/m² day⁻¹ can be harvested every 14 days with nutrient composition of CP 21.3 %kg⁻¹ DM, CF 16.4 %kg⁻¹ DM, NDF 37.6 %kg⁻¹ DM, ADF 27.64 %kg⁻¹ DM and ADL 8.03 %kg⁻¹ DM (Mohammad Fitri Rimi et al., 2021). Therefore, farmers will be able to maximize the use of existing resources in the farm to reduce the production cost. The objective of this study was to investigate the effect of

different levels of *A. filiculoides* meal as a fiber and protein source in a ruminant diet through *in vitro* gas production and feed degradability trials.

METHODS AND MATERIALS

Research Area

The study was conducted at Livestock Science Research Center MARDI headquarters, Serdang, Selangor (2°59'23"N 101°42'08"E) and MARDI's Livestock Centre of Excellence, Kluang, Johor (1°56'58"N 103°21'54"E). The cultivation location of *Azolla filiculoides* was conducted at the MARDI Serdang pasture study plot (2°59'23"N 101°41'43"E). At the same time, the mixing activity of total mixed ration and laboratory analysis was carried out at the MARDI feed bio-process incubator, Serdang (2°59'01"N 101°42'06"E). Meanwhile, rumen fluid was collected from cannulated animal husbandry of Kluang MARDI Research Station (1°57'27"N 103°21'35"E), and digestion studies were conducted at digestibility laboratory at Kluang MARDI Research Station (1°56'56"N 103°21'56"E).

Cultivation and Preparation of *Azolla filiculoides* Meal

Cultivation of *A. filiculoides* was conducted in the five units' canvas pools with 2.5m x 2.5m. All pools are placed in the opened area and directly exposed to sunlight. After filling the water approximately 1.0 m deep, all pools were left for 24 h. Sheep

manure was measured into 6.3 kg for each pool and was soaked until it spread evenly. Subsequently, approximately 100 grams of fresh *A. filiculoides* were spread into each pool. After 14 days of cultivation, harvesting was carried out using sieve containers to remove the water from the plant. Next, all harvested plants were dried using a forced air oven at 60 °C for 72 h and ground into a 1 mm size *Azolla* meal. Finally, the grounded sample was packed into a dry and clean container for storage in the 2 °C chillers.

Samples had been analyzed to determine the composition of total dry matter (DM), organic matter (OM), crude protein (CP), crude fiber (CF), ether extract (EE), and ash (IM) as guided in the Association of Official Analytical Chemists (AOAC) (2005) and the component of fiber (NDF, ADF, and ADL) was measured using fiber cap and fiber tech distillation machine. According to Van Soest et al. (1991), those measurement principles were made. The same procedures were used to determine the nutrient composition and fiber components of other materials used in this treatment.

Preparation of Napier Silage and Total Mixed Ration

The harvested Napier grass was wilted for 24 h and chopped into 2–4 cm before being ensilaged in the plastic drum for 21 days. All drums were prepared for the adaptation process of the cannulated animal, and it had been stored under the shaded area. The formulation was calculated based on the nutritional value of each material by setting the homogeneity on CP and ether

extract (EE) composition. The rations met the nutritional requirements for maintenance cattle (National Research Council [NRC], 2001). Then, all ingredients were mixed for 10 minutes using a 100 kg industrial horizontal mixer machine. Five total mixed rations (TMR) were formulated based on

the percentage of the inclusion of *Azolla filiculoides* meal which is 0% (control), 6% (T1), 10% (T2), 17% (T3), and 23% (T4). Sample from each TMR was taken for the proximate and fiber analysis. The actual nutritional composition for each treatment is shown in Table 1.

Table 1

Ingredients of the total mixed rations (TMR) with the different inclusion percentages of Azolla filiculoides meal

Indices	TMR				
	Control	T1	T2	T3	T4
Ingredients					
Molasses (%)	3.0	3.0	3.0	3.0	3.0
Palm oil (%)	3.3	3.3	3.3	3.3	3.3
Salt (%)	1.0	1.0	1.0	1.0	1.0
Mineral and vitamin (%)	0.7	0.7	0.7	0.7	0.7
Limestone (%)	1.0	1.0	1.0	1.0	1.0
Maize (%)	13.5	13.5	13.5	13.5	13.5
Napier silage (%)	60.0	55.0	52.0	47.0	41.0
Soybean meal (%)	17.5	16.5	15.5	14.5	13.5
<i>Azolla filiculoides</i> meal (%)	0.0	6.0	10.0	16.0	23.0

Note. Control = 0% *Azolla* meal; T1 = 6% *Azolla* meal; T2 = 10% *Azolla* meal; T3 = 16% *Azolla* meal; T4 = 23% *Azolla* meal

Chemical Analysis

Samples from each TMR were analyzed for DM, CP, EE, CF, and ash according to the Association of Official Agricultural Chemists (AOAC) (1975). In addition, the leaf samples were analyzed for NDF, ADF, ADL, and cellulose according to Van Soest et al. (1991).

Donor Animal’s Inocula

The adaptation process on three (3) cannulated bulls was carried out for 14 days. The cannulated bulls were fed with Napier silage and a total mixed ration containing 23% *Azolla* meal throughout the adaptation period. Napier silage and total mixed ration were given two times a day at a ratio of 55:45 according to the rate of 3% of the individual bodyweight and material DM.

Instruments to collect the rumen liquid were prepared a day before because it is recommended to make a collection in the morning before feeding the animals. Thermos (filled with hot water), polyvinyl chloride (PVC) perforated strainer pole, and carbon dioxide (CO₂) tank had been prepared a day before the water tub had been set up at 39 °C and the buffer solution was ready for the rumen liquid. Meanwhile, the bull was restrained while the rumen liquid collecting equipment was inserted into the rumen. Rumen liquid from the cannulated bulls was pooled in the flask. At the same time, it has been flushed using the CO₂ to maintain an anaerobic environment for the rumen microorganism until it is poured into the prepared buffer solution.

Gas Production Assay

According to Theodorou et al. (1994), the gas production assay was carried out. Approximately 30 mL buffer media was filled with 200 mg samples in the syringe. An arrangement of the syringe was according to randomized complete block design (RCBD) in the water tub. Anaerobic buffer solution, which is contained micro and macro elements reducing agent and a reduction indicator of resazurin, was added to the bottles containing 10 mL of ruminal fluid. Negative controls (blank) containing buffered rumen fluid but no substrates were also included in triplicate to correct gas produced from small particles present in the ruminal fluid. Cumulative gas production (mL/g DM) was recorded at 2, 4, 6, 8, 10,

12, 15, 19, 24, 30, 36, 48, 72, and 96 h after incubation at 39 °C. The volume of gas produced after 24 h of incubation (GP 24) was used as an index of the energy feed value of tree fodder samples (Menke, 1988). The volume of gas produced (GP) (mL 200 mg⁻¹ DM) after 24 h of incubation was used with CP content to estimate metabolizable energy (ME) concentration (MJ kg⁻¹ DM) based on the following equation reported by Menke et al. (1979) for roughage feeds:

$$ME = 2.2 + 0.1357 GP + 0.057 CP + 0.002859 CP^2 \quad (R^2 = 94\%; n = 200)$$

where,

ME = Metabolisable energy (MJ kg⁻¹ DM)

GP = Gas production after 24 h (mL 200 mg⁻¹ DM)

CP = Crude protein (%)

In vitro Degradability

At the end of incubation (96 h), the contents of each syringe were completely discarded from the syringe in the 100 mL centrifuge tube. Fermentation residues were dried at 105 °C overnight and then incinerated in a muffle furnace at 550 °C for 12 h. Loss in weight after incineration was used as a measure of ash. The *in vitro* organic matter degradability (IVOMD) at 96 h of incubation was calculated as equation below:

$$IVDMD (\%) = [(DM \text{ sample} - DM \text{ residue} - \text{blank}) / DM \text{ sample}] \times 100$$

where,

IVDMD = *In vitro* dry matter digestibility

DM = Dry matter

The tubes were centrifuged at 20,000 x g for 15 min, and 15 mL of supernatant was kept for VFA determination following the procedure described by Cottyn & Boucque (1968). First, the pellets were dried in a forced-air oven at 60 °C for 48 h to determine the residual DM weights. Then, to determine ash content, the residues were kept at 550 °C for 8 hours to estimate organic matter (OM). Finally, *in vitro*, organic matter digestibility was calculated as the OM, which disappeared from the initial weight inserted into the tube. Calculations were as follows:

$$\text{IVOMD (\%)} = [(\text{OM sample} - \text{OM residue} - \text{blank}) / \text{OM sample}] \times 100$$

where,

IVOMD = *In vitro* organic matter digestibility

OM = Organic matter

Next, the supernatant was separated from the residue. Then, the mixture obtained from *in vitro* analysis was put into a centrifuge tube and then centrifuged at 2,500 x g in the 4 °C for 30 min. Finally, the supernatants were transferred into a vial in 4 replicates each treatment and stored in the -20 °C freezer for the next procedure.

Analysis of volatile fatty acid (VFA) using Gas Chromatography (GC).

The samples were thawed for 1 h before arranging the vial in the GC. Analyses were conducted on a 6820-gas chromatograph system from Agilent Technologies (USA). The instruments were prepared with a free fatty acid phase (FFAP) capillary column, 30 m x 250 µm x 0.25 µm (Quadrex Corporation, USA) and using carrier gas that could flow nitrogen gas at 1.0 mL/minute with the flame ionization detector (FID). The temperature was programmed using 60–200 °C (20 °C/min, 10 min) with the injector—250 °C and detector—300 °C. The injector was equipped with a glass liner of glass wool to separate dirt particles from the sample. The samples were dosed by an HT 300A automatic dosing device (Agilent Technology, USA) at an injection size of 1 µl using the split method and a 30:1 splitting ratio and the analysis time is approximately 15 min.

Statistical Analysis

To assess the replacement and inclusion effect of Napier silage and soybean meal with *A. filiculoides* meal on the nutrient composition, GP, IVDMD, IVOMD, and ME of Napier silage TMR, a 5 x 4 factorial analysis of variance (ANOVA) was conducted. The means and standard error of means (SEM) for five different inclusions of *A. filiculoides* in the Napier silage TMR as a function of the two factors are presented in Tables 2 and 3. In addition, the *F* test and Duncan's test for post-hoc comparisons (*p*<0.05) were applied. All

statistical analyses were performed using the SPSS (version 25) software package.

RESULTS

An actual nutrients composition value of TMRs has shown in Table 2. Soybean meal and Napier silage had become the main source of protein and fiber in this TMR. The inclusion of *A. filiculoides* meal into the TMR did not affect ($p>0.05$) their DM, CP, and ash compared to the control. However, the inclusion of 23.0% of *A. filiculoides* meal (T4) replacing 4.0% of soybean meal and 19.0% of Napier silage from the TMR had significantly affected the values of CF, OM, and EE compared to the treatment that consisted of 0% *A. filiculoides* meal inclusion. The values of CF and OM was significantly higher ($p<0.05$) while EE had reduced significantly compared to the control. Besides, the values of NDF, ADF, and ADL showed an increment ($p<0.05$) at the range of 9.3%–21.0%, with the

10% inclusion of *A. filiculoides* meal (T2) replacing 3 % of soybean meal and 13% of Napier silage.

The effect of replacing Napier silage and soybean meal with *A. filiculoides* meal into the ruminant diet on the cumulative *in vitro* gases production, IVDMD, IVOMD, and ME was as shown in Table 3 and Figure 1. At 6% of *A. filiculoides* (T1), cumulative *in vitro* gas production at 24, 48, and 96 h was significantly higher than T2, T3, and T4 during the incubation period. However, after 48 h incubation, the gas production was still significantly increased at the higher inclusion treatments (T2, T3, and T4) instead of T1, which had nearly reached a plateau after that period. From the result, the highest volume of gasses was produced at 6% inclusion (T1), and the lowest was obtained from 23% inclusion (T4) which is 261.2 mL g⁻¹ DM and 228.3 mL g⁻¹ DM, respectively.

Table 2

Nutrient composition and fiber components (%/DM basis) of the total mixed rations (TMR) with the different inclusion percentage of Azolla filiculoides meal

Indices	TMR				
	Control (n = 4)	T1 (n = 4)	T2 (n = 4)	T3 (n = 4)	T4 (n = 4)
Nutrient composition					
Dry matter (%)	58.5 ± 2.7 ^a	57.8 ± 1.8 ^a	58.8 ± 2.8 ^a	64.0 ± 1.4 ^a	66.5 ± 2.1 ^a
Organic matter (%)	31.1 ± 3.3 ^c	34.8 ± 0.8 ^{bc}	35.7 ± 2.9 ^{abc}	38.9 ± 1.5 ^{ab}	42.2 ± 2.1 ^a
Crude protein (%)	15.2 ± 0.3 ^a	14.5 ± 0.6 ^a	14.9 ± 1.3 ^a	15.2 ± 0.4 ^a	15.4 ± 0.2 ^a
Crude fiber (%)	27.2 ± 0.3 ^b	26.8 ± 0.1 ^b	28.4 ± 0.6 ^{ab}	29.0 ± 0.3 ^{ab}	30.3 ± 0.5 ^a
Ether extract (%)	6.3 ± 0.2 ^a	6.2 ± 0.1 ^a	6.1 ± 0.1 ^a	5.2 ± 0.6 ^{bc}	4.2 ± 0.6 ^c
Ash (%)	27.5 ± 0.7 ^a	23.0 ± 1.8 ^a	23.1 ± 1.5 ^a	25.1 ± 1.6 ^a	24.1 ± 1.0 ^a

Table 2 (Continue)

Indices	TMR				
	Control (n = 4)	T1 (n = 4)	T2 (n = 4)	T3 (n = 4)	T4 (n = 4)
Fiber components					
NDF (%)	31.0 ± 0.6 ^c	32.5 ± 0.5 ^{bc}	33.7 ± 0.6 ^b	36.3 ± 0.5 ^a	37.5 ± 0.1 ^a
ADF (%)	24.6 ± 0.4 ^b	25.0 ± 0.2 ^b	25.0 ± 0.2 ^b	26.6 ± 0.3 ^a	26.9 ± 0.5 ^a
ADL (%)	12.6 ± 0.5 ^b	13.0 ± 0.4 ^{ab}	14.0 ± 0.3 ^{ab}	14.1 ± 0.2 ^{ab}	14.2 ± 0.3 ^a

Note. Control = 0% *Azolla filiculoides* meal; T1 = 6% *Azolla filiculoides* meal; T2 = 10% *Azolla filiculoides* meal; T3 = 16% *Azolla filiculoides* meal; T4 = 23% *Azolla filiculoides* meal; n = Number of samples; NDF = Neutral detergent fiber; ADF = Acid detergent fiber; ADL = Acid detergent lignin

All data are means ± standard error of the mean (S.E.M.)

^{a,b,c} Mean with different superscripts within a row are significantly different ($p < 0.05$)

IVDMD was significantly affected by the increase of *A. filiculoides* inclusion percentage in the TMR formulation. From the result, a dry matter digestibility was reduced at 12.2%–41.2% after replacing the Napier silage and soybean meal with *A. filiculoides* meal. With the inclusion, the highest dry matter digestibility was determined at 391.1 g kg⁻¹ DM at 6% inclusion (T1), and the lowest was recorded at 262.0 g kg⁻¹ DM, which is from T4. However, IVOMD and ME were not affected with the lower inclusion (T1). The inclusion of 10% (T2) and above had resulted in a significant reduction on both parameters. The highest IVOMD of the TMR with *A. filiculoides* inclusion was 453.6 g kg⁻¹ DM (T1 = 6%), and the lowest was 417.6 g kg⁻¹ DM (T4 = 23%). However, the values of ME were also directly reflected by the IVOMD. Inclusion of 6% *A. filiculoides* (T1) had produced higher energy for the metabolic process during digestion, similar with the control ($p > 0.05$) compared to a

higher percentage of inclusion. After *A. filiculoides* meal was used as a fiber and protein alternative source, the highest ME was recorded as 14.1 MJ kg⁻¹ DM (T1 = 6%), and the lowest was 11.6 MJ kg⁻¹ DM.

From this research, the concentration of total VFA and proportion of acetate, propionate, and butyrate were shown in Table 4. The basal diet (control) had produced 86.0 mM/L total VFA with the proportion of partial VFA (acetate, propionate, and butyrate) at 50.4:26.7:15.1, respectively. When viewed from all treatments, the inclusion of *A. filiculoides* meal in the ruminant feed had produced total VFA at 87.9 mM/L–120.0 mM/L. The difference in total VFA produced between treatments was significant. The value was increased as a higher percentage of *A. filiculoides* meal was used on the feed. As the inclusion percentage was increased, acetate and propionate of T3 and T4 were also enhanced significantly. However, propionate production was higher ($p < 0.05$) at T1 and T2 than T3 and T4. From

this study, T4 had produced the highest concentration of acetate and butyrate, which are 67.4 mM/L and 18.1 mM/L, respectively. Propionate in T4 had significantly decreased ($p < 0.05$) to 14.8 mM/L.

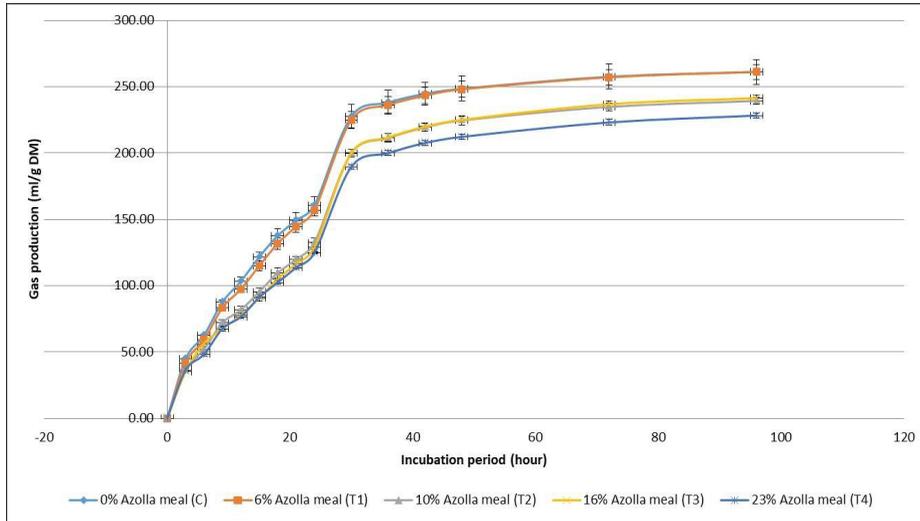


Figure 1. *In vitro* gas production profiles of total mixed rations with the different levels of *Azolla filiculoides* meal in the cattle rumen incubation

Concurrently, the acetate and propionate A:P ratio was determined, as shown in Figure 2. The ratio increased as more *A. filiculoides* meal inclusion was used to replace the portion of Napier silage and soybean meal. The A:P ratio produced from the trial was at 2.43 to 2.75, and the T1 had no significant difference with control. The treatment with more than 16% inclusion had significantly produced a higher A:P ratio than TMR without the inclusion of *A. filiculoides* meal inclusion.

Table 3

In vitro fermentation characteristics of total mixed rations (TMR) with the different levels of *Azolla filiculoides* meal during *in vitro* ruminal incubation

Parameter	Inclusion of <i>Azolla</i> meal in the TMR				
	Control (n = 4)	T1 (n = 4)	T2 (n = 4)	T3 (n = 4)	T4 (n = 4)
Gas					
GP 24	160.63 ± 6.21 ^{a,z}	157.03 ± 4.61 ^{a,z}	132.48 ± 3.24 ^{b,z}	128.73 ± 3.50 ^{b,z}	124.75 ± 0.86 ^{b,z}
GP 48	248.50 ± 9.39 ^{a,y}	248.03 ± 6.25 ^{a,y}	224.58 ± 3.51 ^{b,y}	225.03 ± 2.50 ^{b,y}	212.18 ± 1.68 ^{b,y}
GP 96	261.10 ± 9.29 ^{a,y}	261.23 ± 5.60 ^{a,y}	239.33 ± 2.86 ^{b,x}	241.35 ± 2.06 ^{b,x}	228.25 ± 1.84 ^{b,x}

Table 3 (Continue)

Parameter	Inclusion of <i>Azolla</i> meal in the TMR				
	Control (n = 4)	T1 (n = 4)	T2 (n = 4)	T3 (n = 4)	T4 (n = 4)
Degradability					
IVDMD (g/kg DM)	445.5 ± 0.84 ^a	391.1 ± 0.75 ^b	339.0 ± 1.03 ^c	315.4 ± 0.88 ^c	262.0 ± 0.84 ^d
IVOMD (g/kg DM)	542.2 ± 0.23 ^a	453.6 ± 0.83 ^a	445.0 ± 0.01 ^b	421.0 ± 0.01 ^{bc}	417.6 ± 0.01 ^c
ME (MJ/kg DM)	14.40 ± 0.45 ^a	14.08 ± 0.26 ^a	12.50 ± 0.17 ^b	12.08 ± 0.29 ^b	11.60 ± 0.18 ^b

Note. GP = Gas production (mL/g DM at 24 hours, 48 hours, and 96 hours); IVDMD = *In vitro* dry matter degradability (g/kg DM); IVOMD = *In vitro* organic matter degradability (g/kg DM); ME = Metabolizable energy content (MJ/kg DM), n = Number of samples; IVDMD = *In vitro* dry matter digestibility; IVOMD = *In vitro* organic matter digestibility; ME = Metabolizable energy

All analyses are means ± standard error of the mean (S.E.M.)

^{a,b,c} Mean with different superscripts within a row are significantly different ($p < 0.05$)

^{x,y,z} Mean with different superscripts within a column are significantly different ($p < 0.05$)

Table 4

Volatile fatty acid (VFA) profile of Napier silage total mixed ration with different inclusion percentage of *Azolla filiculoides* meal

VFA (mM/L)	Control (n = 4)		T1 (n = 4)		T2 (n = 4)		T3 (n = 4)		T4 (n = 4)	
	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
Acetate	50.4	± 1.47 ^b	52.8	± 2.04 ^b	55.3	± 2.22 ^b	64.2	± 1.49 ^a	67.4	± 2.81 ^a
Propionate	26.7	± 1.01 ^a	23.5	± 2.04 ^a	16.8	± 0.85 ^b	16.2	± 0.91 ^b	14.8	± 0.36 ^b
Butyrate	15.1	± 0.23 ^b	15.3	± 0.27 ^b	15.6	± 0.26 ^b	17.3	± 0.37 ^a	18.1	± 0.60 ^a
Total VFA	86.0	± 0.38 ^c	87.9	± 0.14 ^c	97.1	± 0.08 ^c	116.1	± 0.87 ^b	120.0	± 0.32 ^a

Note. VFA = Volatile fatty acid; Control = 0% *Azolla filiculoides* meal; T1 = 6% *Azolla filiculoides* meal; T2 = 10% *Azolla filiculoides* meal; T3 = 16% *Azolla filiculoides* meal; T4 = 23% *Azolla filiculoides* meal; n = Number of samples

All data are means ± standard error of the mean (S.E.M.)

^{a,b,c} Mean with different superscripts within a row are significantly different ($p < 0.05$)

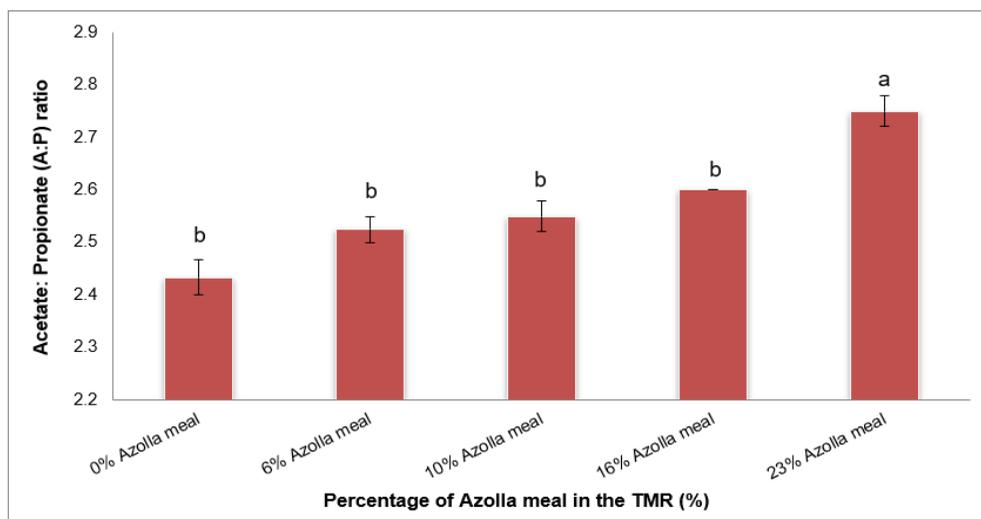


Figure 2. Acetic: propionic ratio of Napier silage total mixed ration with different inclusion percentage of *Azolla filiculoides* meal. All data are means \pm standard error of the mean (S.E.M).

^{a,b} Mean with different superscripts are significantly different ($p < 0.05$)

DISCUSSION

Inclusion of *A. filiculoides* meal at a rate of 6%–23% was able to retain the CP content at an average value of 15.0% CP kg^{-1} DM after reducing soybean meal components at the range of 1.0% to 4.0%. It can be attributed to the *Azolla*'s high CP content, consisting of CP at the range of 19.4%–24.5%. Therefore, Kamaruddin et al. (2019) verified that this species was suitable for animal feed. According to Mohammad Fitri Rimi et al. (2021), *A. filiculoides* could be cultivated by fully utilizing the organic source such as manure from the livestock waste as their nutrient supplier for growth. A significant difference has occurred in this plant's biomass production and nutrient composition depending on the source and type of manure. Thus, the protein composition produced by *A. filiculoides*

meal was adequate for ruminant requirement (Freer, 2007; NRC, 1996). However, the inclusion higher than 16% had affected the fiber and fat composition of Napier silage TMR. The highest CF values were obtained once replacing 19.0% Napier silage and 4.0% soybean meal with 23% *A. filiculoides* meal inclusion within the basal diet. As a result, it had showed an increment of 11.4% compared to control (0% *A. filiculoides* meal). However, it was lower than the CF of Napier grass forage between 33.0%–35.0% kg^{-1} DM (Haryani et al., 2018). The CF of T1, T2, T3, and T4 was lower than the CF of rice straw total mixed rations, which reached 43.3% CF kg^{-1} DM (Sarker et al., 2018). Therefore, it has been an indication of the suitability of this species as an alternative source of fiber for ruminants. Besides, CF produced through the inclusions was higher

than 15.4%, which is the optimum value to ensure an optimum acetic: propionic ratio produced at 3.0 or for the methane gas production below 6.9% MJ day⁻¹ (Luthfi et al. 2018).

Simultaneously, the inclusion of 10% *A. filiculoides* (T2) had consequent an increment of NDF at 8.7%, which had significantly declined 9.6% volume of 48 h cumulative *in vitro* gas production compared to the lower inclusion. The NDF value for *A. filiculoides*, which is 36.5%–37.6% kg⁻¹ DM (Mohammad Fitri Rimi et al., 2021), is lower than *Eichhornia crassipes* which is 65.9%–72.9% (Mako et al., 2011). Therefore, this species might enhance the feed intake of ruminants compared to other aquatic species. However, the high lignin composition of *A. filiculoides* (7.61%–9.02%) compared to *E. crassipes* and *Pistia stratiotes* (5.49% and 3.47% /kg DM) had slower digestibility once utilized at the higher percentage (Mani, 2019; Sivasankari & Ravindran, 2016).

A significant decrease could be seen in the cumulative *in vitro* gas production of TMR in line with higher inclusion than 6% into the ruminant diet. With this amount of inclusion, 157.6 mL g⁻¹ DM of cumulative *in vitro* gas production was recorded, and it was lower than 195.5 mL g⁻¹ DM, which was recorded from 40:60 TMR of sweet corn residue and rice straw (Kraiprom & Tumwasorn, 2017). However, as the cumulative *in vitro* gas production was inversely proportional with the percentage of the inclusion of *A. filiculoides* meal, Murillo-Ortiz et al. (2018) have reported

a similar effect detected on the addition of *E. crassipes* in the alfalfa hay-based diet. However, the 48-h cumulative *in vitro* gas production of 23% inclusive had been recorded higher than the 209.0 mL g⁻¹ DM, which took from Zailan et al. (2016a)'s study on common Napier. It can be attributed to an increase in the value of the fiber component, which causes a higher duration for the degradation of fiber along the rumination process. The compositions of ADF and ADL for *A. filiculoides* plants were 27.6% and 7.61% were lower than *E. crassipes*, which was determined as 77.9% and 15.4% (Ganguly et al., 2013; Hossain et al., 2015; Mohammad Fitri Rimi et al., 2021). These factors have affected the catabolism process and nutrient absorption into an animal digestion system. With the 23% inclusion of *A. filiculoides* meal, the values of IVDMD and IVOMD were 41.2% and 23.0%, respectively. Even though those values were lower than the value obtained from the 25% *E. crassipes* with alfalfa hay reported by Murillo-Ortiz et al. (2018), they were higher than IVDMD and IVOMD of common Napier, which were measured at 54.6% and 50.8%, respectively. However, those values were still lower, and the ME reached 11.6% MJ kg⁻¹ DM compared to 7.3% MJ kg⁻¹ DM for common Napier (Zailan et al., 2016a). However, the ME value of T1 (6% *A. filiculoides*) was higher than the *Mucuna* bean (Castro et al., 2003).

The increment of *A. filiculoides* meal inclusion percentage had directly affected the concentration of total VFA at the range of 86.0–120.0 mM/L was in line

with the optimum range for total VFA for ruminant, which is between 80–120 mM/L as mentioned by McDonald et al. (2010). The high VFA in T1, T2, T3, and T4 was due to the degradation of cell wall components (NDF and ADF) into VFA, which was greater than control. The higher the level of fermentability of the feed ingredient, the greater the VFA produced other than those from protein because the VFA was derived from carbohydrates and protein. In this research, partial VFA for T3 was 64.2:16.2:15.6 for acetate, propionate, and butyrate, respectively. This ratio was near the proportion of good partial VFA ratio in the rumen, which Hungate (2013) stated, which is 63:21:16. Meanwhile, Jouany and Ushida (1999) also stated that the molar proportion in the rumen of various good feed formulations for acetate was 53–72 mM/L, while propionate was 15–30 mM/L and for butyrate was 7–21 mM/L. The tendency of a higher molar proportion of acetate in the treatment with higher inclusion of *A. filiculoides* meal indicates the potential for higher energy production for livestock diet to the higher ATP production in the substrate. As the percentage of acetate was increased, together with the percentage of *A. filiculoides* meal inclusion, the range of the A: P ratio increased from 2.2 to 3.0, as mentioned by Russell (1998).

CONCLUSION

Based on this study, *Azolla filiculoides* meal was used in the ruminant diet as an alternative source of fiber and protein. Due to this species' low dry matter content, *A. filiculoides* could not be used as the

main fodder for ruminants, especially cattle. However, this species was able to produce sufficient organic matter digested in the total ruminant digestive tract and will simultaneously affect the production of metabolizable energy for the animal. Furthermore, instead of using it in fresh form, this plant was more suitable to be used in the form of dried or meal. Inclusions of *A. filiculoides* meal at the level 6% to 10% in ruminant diets will help farmers enhance their productivity through their livestock performance by utilizing an alternative source of fiber and protein such as *A. filiculoides* meal. This plant was able to be used as the inclusion with the concentrate and Napier silage at 6% to replace 5% of Napier and 1% of soybean meal. A digestibility study should be conducted to determine the optimum inclusion of *A. filiculoides* between 6% to 10% in the TMR feed with or without fermentation treatment.

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