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The Silage Quality of *Pennisetum purpureum* Cultivar Gamma Umami Mixed with *Calliandra calothyrsus* and *Lactiplantibacillus plantarum*

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ABSTRACT

This study aimed to determine the effect of *Calliandra calothyrsus* supplementation and inoculation of *Lactiplantibacillus plantarum (L. plantarum)* on *Penisetum purpureum* cv. Gamma Umami grass fermentative and chemical quality and nutrient degradability. The study used a completely randomized design with 3 × 3 factorial patterns. The first factor was *Calliandra* supplementation levels at 10%, 20%, and 30%; the second was *L. plantarum* inoculation levels at 0%, 2%, and 4%. The variables measured included chemical fermentation profiles, chemical composition, and rumen fermentation and degradability parameters. The result showed that a higher level of *C. calothyrsus* supplementation concomitant increased silage pH and NH_3-N concentration ($p<0.05$), while *L. plantarum* inoculation significantly decreased the silage pH and ammonia concentration ($p<0.05$). The silage contents of dry matter, organic matter, crude protein, and ether extract in silage significantly ($p<0.05$) increased. Inoculation of *L. plantarum* decreased ($p<0.05$) crude fiber, ether extract, and total tannin content of silage. The total volatile fatty acids, acetate, propionate concentrations, and rumen microbial protein synthesis were significantly increased with *Calliandra* supplementation ($p<0.05$). *L. plantarum* inoculation treatment only increased the proportion of acetate ($p<0.05$) and tended to increase the volatile fatty acids of rumen fluid, the proportion of acetate, propionate, and butyrate. The rumen ammonia concentration decreased with *Calliandra* supplementation and *L. plantarum* inoculation. It is concluded that 30% *Calliandra* supplementation and 2% *L. plantarum* inoculation and their combination were the treatments that produced the best chemical fermentation, rumen fermentation, and degradability parameters.

Keywords: Calliandra calothyrsus; Lactiplantibacillus plantarum; P. Purpureum cv. Gamma Umami; silage

INTRODUCTION

Pennisetum purpureum cv. Gamma Umami (*P. purpureum* cv. Gamma Umami) is used as superior forage because of its high level of productivity. This grass was developed at the Faculty of Animal Science Universitas Gadjah Mada and has received a variety list mark (889/pvhp/2020) for protecting agricultural plant varieties from the Indonesian Ministry of Agriculture and can support feed adequacy. *P. purpureum* cv. Gamma Umami grass is available throughout the year because of its high tolerance to drought or lack of water sources and even in abundance during the rainy season; however, the nutrient content, especially protein, is relatively low (Sanjaya *et al.*, 2022). To address this problem, especially to meet the adequate protein needs of ruminants, in the context of feeding in the form of a mixture of silage feed, it is necessary to increase the protein content, one of which is supplementation using Calliandra (*Calliandra calothyrsus*).

Forage preservation has been performed, mainly as the grass is cultivated singly or in a mixture of several types of grass and produces good-quality silage. The final quality of forage preservation depends on the initial quality, and one factor affecting plant nutrient content is plant age or maturity at harvest time (McDonald *et al.*, 2011). The mature plant has a decreased proportion of nonstructural carbohydrates (NSC) but an increased proportion of structural carbohydrates. During the fermentation process, NSC, such as sugars and starches, are transformed into lactic acid by lactic acid bacteria such as *L. plantarum* (Zhang

et al., 2022). It is important to ensure that the forage used for silage has enough NSC. When the amount of NSC is low, the lactic acid bacteria may not have enough substrate to produce adequate lactic acid. If necessary, additional carbohydrates can be added to the silage to increase the NSC content.

Preserving forage with the addition of legumes can indeed be challenging. Xue *et al.* (2020) suggest that using legumes can increase silage protein content but can also cause undesirable fermentation, proteolysis, and poorly utilized nitrogen by ruminants. According to Ni *et al.* (2018), legumes in silage contribute to a high buffering capacity and make it more difficult to suppress pH during ensilage compared to grass species under natural fermentation conditions; legumes have a lower sugar content than grass species. The buffering capacity in legumes is 500-550 mE/kg of DM (McDonald *et al.,* 1991). The legume addition may prevent the pH from decreasing to the level expected for the silage process to take place properly, which is between pH 3.8 to 4.2. As other researchers reported, legume silage with a DM content of approximately <35%-55% results in a pH value of approximately 4.3-5.0 (Kung *et al.,* 2018).

The silage quality is determined not only by anaerobic conditions but also by the degree of acidity. A high degree of acidity (low pH) is determined by the number of lactic acid bacteria (LAB) colonization and the consequent production of lactic acid. Elevated lactic acid production will reduce silage acidity (pH) and help inhibit the growth and development of spoilage microbes and pathogens, which are detrimental because they use nutrients in silage (Soundharrajan *et al.,* 2021). An essential factor in making silage is the moisture content of the raw material. Silage with high moisture content (>80%) has a slimy texture and is soft and moldy, whereas silage with low moisture content (<30%) has a dry texture and is overgrown with fungus. Forage with high water content can impair fermentation (Guo *et al.,* 2013).

Lactic acid bacteria strain *Lactiplantibacillus plantarum* (*L. plantarum*) is a lactic acid bacteria (LAB) strain that usually grows when forage plants are fermented (epiphyte bacteria). The applicant rate of *L. plantarum* in silage can vary depending on the specific purpose of the fermentation. However, a standard guideline is that well-preserved silage requires no less than 10^5 cfu/g FM of LAB, which includes *L. plantarum* (Dong *et al.,* 2022). Another Study revealed that LAB inoculant doses ranged from 0 cfu/g, 1 x 10^6 cfu/g, 1 x 10^7 cfu/g, and 1 x 10^8 cfu/g on three tropical forages (cassava foliage, *P. purpureum*, and *B. papyrifera*) with the result that the addition of *L. plantarum* improved the fermentation quality and bacterial community of silage, which reduced part of *mycotoxin* content and abundance of harmful bacteria (Liu *et al.,* 2022).

Therefore, it is necessary to study the effect of Calliandra mixed with *P. purpureum* cv. Gamma Umami grass as one of the new varieties in this grass group. To our knowledge, a high proportion of legumes can increase the nutritional value of silage but can also reduce its quality; therefore, adding inoculants to enrich the fermentation results is necessary. There is no information regarding the preservation strategy of *P. purpureum* cv. Gamma Umami from the tropics was applied with Calliandra and *L. plantarum* inoculation. Therefore, this study was conducted to observe whether *P. purpureum* cv. Gamma Umami with Calliandra supplementation and *L. plantarum* inoculation in mixed silage on fermentation characteristics of silage, nutritional quality, digestibility, and rumen fermentation profile.

MATERIALS AND METHODS

Ethical Clearance

The research ethics committee of the Faculty of Veterinary Medicine Universitas Gadjah Mada approved this study, with Ethical Approval number 027/EC-FKH/Eks./2022.

Experimental Design

The study used a completely randomized design with a 3 x 3 factorial pattern. The first factor was Calliandra supplementation, and the second was *L. plantarum* inoculation. The following treatments were used: 10%, 20%, or 30% Calliandra supplementation on fresh matter (FM) basis of *P. purpureum* cv. Gamma Umami, and 0%, 2%, or 4% *L. plantarum* inoculation. Inoculum was prepared with *L. plantarum* concentrations of 0 cfu/g FM forage for 0% inoculation treatment, 2 x 10^5 cfu/g for 2% inoculation treatment, and 4 x 10^5 cfu/g FM forage for 4% inoculation treatment. Each treatment was replicated five times and fermented for 21 days in a sterilized room with daily temperatures of 24 °C – 27 °C and about 55%-65% relative humidity. Room sterilization was carried out by emptying the room of equipment not used during the fermentation process, and the entire surface of the room was sprayed with 90% alcohol. The silo used was stored in a jar with a 2 kg capacity.

Forage Harvested and Inoculation Preparation

The grass *(P. purpureum* cv. Gamma Umami) used in this study was cut from the forage field, which was 60 days old, at the Faculty of Animal Science Universitas Gadjah Mada. Calliandra was obtained from the Garden of the Regional Technical Implementation Unit of Agriculture Services of the Special Region of Yogyakarta. The Inoculants of *L. plantarum* were a commercial product from the National Research and Innovation Agency (BRIN, Indonesia), containing *L. plantarum* concentration of 10^{10} cfu/L.

The grass was cut to a size of 2–5 cm. Wilting was done by aerating for ±4 days until the moisture content was 65% and the dry matter (DM) reached 35%. Wilting was done by placing grass standing in an open warehouse. Calliandra was harvested in the edible parts two days before use. The ingredient composition of the silage was grass with 35% dry matter (DM), 90% organic matter (OM), 11.2% crude protein (CP), 30.3% crude fiber (CF), 3.03% ether extract (EE), while Calliandra contained 60% DM, 93.55% OM, 23.78% CP, 17% CF,

4.8% EE, and 6.20% total tannins (TT) after two days withering.

Chemical Composition, Fermentation Quality, and *In Vitro* Analysis Procedures

The DM, OM, CF, CP, and EE silage contents were determined using AOAC (2005), and total tannin content followed the procedure Wiryawan *et al.* (1999). The pH value was measured with a pH meter (Hanna brand) (Nahm, 1992). Analysis of the ammonia nitrogen (NH_3-N) concentration followed the procedure of Chaney & Marbach (1962). *In vitro* digestibility was analyzed using the methods developed by Tilley & Terry (1963).

A total of 0.5 g of *Calliandra calothyrsus* leaves was taken for total tannin analysis. The sample was finely crushed, and 20 mL of 70% acetone solution containing 0.1 ascorbic acid was added. Then, centrifugation was performed for 10 minutes at 7000 rpm at 4 °C. The supernatant sediment was re-extracted with 70% acetone solution twice and centrifuged to retrieve the supernatant. The supernatant obtained was roto-vaporized to evaporate the acetone. The resulting rotavapor was extracted with diethyl ether 3 times in a ratio of 1:1 using a column separator to separate chlorophyll from the water fraction. Finally, the water fraction was frozen by freeze drying to obtain tannins, which were then calculated in concentration.

In vitro tubes were filled with samples consisting of rumen fluid and *McDougall solution* with 1:4 ratios. *McDougall solution* was prepared for 1 liter; the ingredients and method of preparation were as follows: weighed 9.8 g of $NaHCO_3$, 10 g of $Na_2HPO_4\ _{12}H_2O$, 0.57 g of KCl, 0.47 g of NaCl, 0.12 g of $MgSO_4\ 7H_2O$. The ingredients that have been weighed are then dissolved with 500 mL of distilled water in a beaker glass (1000 mL capacity) (solution A). Dissolution was carried out at 39 °C and using a magnetic stirrer to speed up the process. Furthermore, about 5.3 g of $CaCl_2$ was put into a graduated glass and dissolved with 100 mL of distilled water (solution B). Subsequently, 1 mL of Solution B was added to solution A and stirred until homogeneous (solution C). Finally, distilled water was added to Solution 3 until the volume became 1000 mL (McDougall, 1948). Carbon dioxide gas (CO_2) was provided simultaneously to enable the anaerobic condition in tubes that will be incubated. Incubation was conducted in the water bath at 39-40 °C for 48 hours.

Rumen fermentation parameters, including NH_3-N concentration in rumen fluid, were measured following the technique of Chaney & Marbach (1962). *In vitro* rumen fluid of as much as 1 mL was mixed with distilled water to a volume of 5 mL, then centrifuged at 3000 rpm for 15 minutes, and then 2 mL was taken to be mixed with 1 mL of 10% sodium tungstate and 1 mL of 1N H_2SO_4. The mixed solution was then centrifuged at 10,000 rpm for 10 minutes. The sample liquid added 2.5 mL of LC and 2.5 mL of LD solution in another tube. The final step is incubation at 40 °C for 30 minutes using a water bath. After the blue color is formed, the reading is done on a spectrophotometer with a wavelength of 630 nm.

The concentration of volatile fatty acids (VFA) was measured following the method of Filípek & Dvořák (2009). The VFA was determined by adding 25% metaphosphoric acid at a ratio of 4 : 1 (v/v). This mixture was centrifuged at 3000 g for 10 minutes, and 0.5 mL of 20 mM/L methyl n-valeric acid was added as an internal standard. Concentrations of VFA were determined via a gas chromatograph (model GC-8A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and CP-FFAP CB column (Agilent Technologies, Inc. Santa Clara, CA, USA) (25 m x 0.32 mm inner diameter, 0.30 μm film thickness).

The Lowry method determined Rumen microbial protein synthesis (MPS) (Plummer, 1971). A total of 1 mL of *in vitro* rumen fluid from each treatment was placed in a tube, and 5 mL of lowry B, the solution, remained for 10 minutes. Subsequently, lowry A solution was added as a volume of 0.5 mL and allowed to settle for 30 minutes. Then, the absorbance was recorded at a wavelength of 750 nm using a UV-VIS *Spectrophotometer* T60U.

Data Analysis

The data obtained from this study were analyzed using the general linear model (GLM) procedure of IBM SPSS version 20 at a significance level of 5%. The statistical model used was the analysis of variance (ANOVA):

$$yijk = \mu + \alpha i + \beta j + \alpha\beta(ij) + \varepsilon ij$$

Where yijk represents the observed value of each individual, μ denotes the overall mean, αi represents the effect of the Calliandra supplementation factor, βj represents the effect of the *L. plantarum* inoculation factor, $\alpha\beta(ij)$ signifies the interaction between these two factors, and εij denotes the residual error (variation among replicates for each treatment).

RESULTS

Silage Chemical Composition

As shown in Table 1, Calliandra supplementation and its interactions significantly ($p<0.05$) affect the DM content of *P. purpureum* cv. Gamma Umami silage. However, inoculation of *L. plantarum* did not affect it. Calliandra supplementation at 10% produced the lowest DM content of 23.65%, significantly less than that produced with the 20% and 30% supplementation treatments of 24.65% and 25.10% DM, respectively ($p<0.05$).

The DM and OM contents did not differ significantly ($p>0.05$) because of the influence of the *L. plantarum* inoculation. However, different results were obtained with 30% Calliandra supplementation, which produced the highest DM content (25.10% vs. 24.65%; 23.65%) and OM content (88.77% vs. 88.28%; 87.31%, $p<0.05$) compared with other group treatments. Each

treatment factor and their combined effect significantly influenced the CP, CF, and EE contents (p<0.05; Table 1).

The CP content was influenced by the level of supplementation and the dose of inoculation, and both showed a linear trend of increasing CP content with increasing levels of supplementation and increasing doses of *L. plantarum* inoculation (p<0.05). Treatment with 30% Calliandra supplementation produced the highest CP content (17.13%) compared to the other treatments. The optimal combination effect between the two treatment factors that produced the highest CP of 17.71% (p<0.05) was obtained with 30% Calliandra supplementation and 2% inoculation dose.

A positive response was also shown in the CF content, where increasing the supplementation and inoculation dose and their interactions reduced the CF content of grass silage (p<0.05). Similarly, the treatment factors significantly affected the EE contents and were between 2.09% and 2.71% (p<0.05).

The mean of total tannin across silage were 1.30%, 1.19%, 1.16%, 1.79%, 1.75%, 1,71%, 2.10%, 1.54%, and 1.91%, respectively (Table 1). Based on partial effect, an increase in Calliandra supplementation levels increased total tannin content in the silage (1.93% vs. 1.75% vs. 1.22%, respectively, p<0.05). Likewise, *L. plantarum* inoculation dose significantly reduced the total tannin content of silage, whereas 4% and 2% inoculation doses had a similar total tannin content of 1.60% compared to silage without inoculation treatment, which contained 1.70% total tannin content (p<0.05). Nonetheless, despite each treatment factor significantly affecting total tannin content, no interaction was observed between both treatments.

Silage Fermentative Quality

The pH value of the *P. purpureum* cv. Gamma Umami silage significantly (p<0.05) increased with Calliandra addition (Table 2). The 10% Calliandra supplementation resulted in a pH of 3.72, substantially different from that of the 20% and 30% supplementation treatments at a pH of 3.80 and 3.95 (p<0.05), respectively. Furthermore, a significant (p<0.05) difference in pH value occurred due to the influence of *L. plantarum* inoculation dose (p<0.05). The pH value concomitantly decreased with the increase in the inoculation dose. Treatment with 4% *L. plantarum* inoculation significantly reduced the pH value of the silage to the lowest value obtained (pH 3.65).

A significant effect was shown by the interaction of the two treatment factors (p<0.05). The test results showed that the lowest pH value was obtained with 10% Calliandra supplementation and 4% *L. plantarum* inoculation dose (pH 3.72) (Table 2). However, the pH value was not significantly different from that found with the interaction of 20% or 30% Calliandra supplementation treatment at a dose of 4% *L. plantarum* inoculation.

The concentration of NH_3-N silage was significantly (p<0.05) increased with the increasing levels of Calliandra supplementation (p<0.05). Calliandra supplementation at 10% level resulted in significantly (p<0.05) lower NH_3-N concentration (5.26 mg/100 g) compared to Calliandra supplementation at 20% (5.50 mg/100 g) and 30% supplementation (5.88 mg/100 g). For all doses of *L. plantarum* inoculation, the *P. purpureum* cv. Gamma Umami grass silage showed significant concentrations

Table 1. Nutrient composition of *Pennisetum purpureum* cv. Gamma Umami grass silage with different levels of *Calliandra calothyrsus* and different doses of *Lactiplantibacillus plantarum* inoculants

Variables	Treatments									SEM	p-value		
	C10			C20			C30						
	B0	B2	B4	B0	B2	B4	B0	B2	B4		C	B	C*B
Dry matter (%)	23.54^{a}	23.66^{a}	23.77^{a}	24.77^{c}	24.41^{b}	24.77^{c}	25.05^{d}	25.18^{d}	25.07^{d}	0.043	<0.001	0.152	0.012
Organic matter (% DM)	87.09	87.55	87.3	88.21	88.38	88.29	89.37	89.37	88.81	0.135	<0.001	0.874	0.079
Crude protein (% DM)	13.22^{a}	13.83^{c}	13.57^{b}	14.68^{d}	15.71^{e}	14.65^{d}	16.86^{f}	17.71^{g}	16.83^{f}	0.041	<0.001	<0.001	<0.001
Crude fiber (% DM)	26.67^{c}	25.61^{b}	26.59^{c}	25.85^{b}	25.74^{b}	25.59^{b}	25.72^{b}	23.77^{a}	25.73^{b}	0.064	<0.001	<0.001	<0.001
Ether extract (% DM)	2.09^{a}	2.19^{ab}	2.16^{ab}	2.43^{b}	2.28^{ab}	2.33^{ab}	2.71^{c}	2.32^{ab}	2.23^{ab}	0.048	0.002	0.046	0.025
Total tannin (%)	1.30	1.19	1.16	1.79	1.75	1.71	2.10	1.85	1.91	0.027	<0.001	0.017	0.661

Note: Means in the same row with different superscripts differ significantly (p<0.05); C10= 10% Calliandra supplementation; C20= 20% Calliandra supplementation; C30= 30% Calliandra supplementation; B0= 0% dose of *L. plantarum*, B2= 2% dose of *L. plantarum*; B4= 4% dose of *L. plantarum*; DM= dry matter; SEM= standard error of mean; C= *C. calothyrsus*; B= *L. plantarum*.

Table 2. The pH value and ammonia (NH_3-N) concentration of *Pennisetum purpureum* cv. Gamma Umami grass silage with different levels of *Calliandra calothyrsus* and different doses of *Lactiplantibacillus plantarum* inoculants

Variables	Treatments									SEM	p-value		
	C10			C20			C30						
	B0	B2	B4	B0	B2	B4	B0	B2	B4		C	B	C*B
pH value	3.87^{de}	3.69^{abc}	3.58^{a}	3.98^{e}	3.80^{bcd}	3.66^{ab}	4.29^{f}	3.84^{cde}	3.68^{abcd}	0.035	<0.001	<0.001	0.036
NH_3-N silage (mg/100 g)	5.79^{cd}	4.60^{a}	5.41^{bc}	5.92^{d}	5.37^{b}	5.21^{b}	6.17^{d}	5.41^{bc}	6.07^{d}	0.076	<0.001	<0.001	0.011

Note: Means in the same row with different superscripts differ significantly (p<0.05); C10= 10% Calliandra supplementation; C20= 20% Calliandra supplementation; C30= 30% Calliandra supplementation; B0= 0% dose of *L. plantarum*, B2= 2% dose of *L. plantarum*; B4= 4% dose of *L. plantarum*; NH_3-N= ammonia; SEM= standard error of mean; C= *C. calothyrsus*; B= *L. plantarum*.

of NH_3-N silage (p<0.05). Among the inoculation doses, the lowest concentration of NH_3-N was with 2% inoculation, which produced 5.12 mg/100 g. The negative interaction of both treatment factors showed a significant effect.

Digestibility and Rumen Fermentation Characteristics

Table 3 shows that Calliandra supplementation and doses *of L. plantarum* inoculation and their interaction did not affect the rumen pH produced with *P. purpureum* cv. Gamma Umami grass silage. The resulting rumen pH ranged from 7.18 to 7.42 (p<0.05). However, a positive effect was obtained when the supplementation and inoculation dose increased, increasing the total VFA and the concentrations of acetic acid, propionate acid, and acetate: propionate ratio in the rumen fluid (p<0.05). A significant effect was only present with the Calliandra supplementation factor for butyric acid.

The current result shows that the digestibility of dry matter (DMD) and organic matter (OMD) increased concomitantly with the increase of Calliandra supplementation and *L. plantarum* dose (p<0.05). Likewise, the interaction effect significantly affected the DMD and OMD of silage (p<0.05). The interaction effect showed that the best digestibility was obtained at the treatment dose of 2% *L. plantarum* inoculation at all levels of Calliandra supplementation with average DMD of 53.04%, 54.00%, and 48.92%, and OMD values of 50.86%, 53,63%, and 47.48% respectively (p<0.05). Partially, the DMD and OMD values with Calliandra supplementation at 10% were 51.13% and 49.21%, while 20% were 51.30% and 49.62%, while 30% were 49.16% and 47.41% (p<0.05).

The total VFA concentration obtained due to Calliandra supplementation ranged from 49.69 mmol/L to 55.25 mmol/L, with 20% supplementing treatment producing the highest total VFA concentration (p<0.05). In comparison, the effect of inoculation dose treatment ranged from 51.24 mmol/L to 53.71 mmol/L of rumen fluid, with the highest VFA concentration obtained at the 4% inoculation dose (p<0.05). There was a significant interaction between two factors on total VFA concentration (p<0.05). The highest total VFA concentration was obtained in combination with 20% Calliandra supplemented and 2% inoculation *L. plantarum* (56.57 mmol/L) compared to all combination treatments (p<0.05).

The results also showed that the partial concentrations of acetic acid experienced a linear increasing trend as the level of supplementation and inoculation dose increased (p<0.05). The acetic acid obtained in our study ranged from 55.39% to 57.56% of molar (p<0.05). The analogous pattern was also demonstrated by the *L. plantarum* inoculation treatment, which provided the highest acetic acid concentration of 56.93% molar at a 4% dose of inoculation than 2%, which produced acetic acid of 55.39% of molar (p<0.05). However, neither treatment had any interaction effect on acetic acid concentration (Table 3).

Table 3 showed that increasing Calliandra supplementation and dose *L. plantarum* inoculation increased propionic acid concentrations (p<0.05). The highest propionic acid concentration was obtained in the 30% Calliandra supplementation, which dramatically decreased in the 20% and 10% supplementation treatments (35.03% of molar vs. 32.51% and 31.62% of molar, respectively, p<0.05). Regarding *L. plantarum* inoculation treatment, inoculated doses of 2% and 4% showed no difference, but both showed significant differences with 0% inoculation treatment (33.49% and 33.32% of molar vs. 32.35% of molar, respectively, p<0.05).

Table 3. Degradability and rumen fermentation characteristics of *Pennisetum purpureum* cv. Gamma Umami silage with different levels of *Calliandra calothyrsus* and different doses of *Lactiplantibacillus plantarum* inoculants

Variables	Treatments									SEM	p-value		
	C10			C20			C30						
	B0	B2	B4	B0	B2	B4	B0	B2	B4		C	B	C*B
Rumen pH	7.25	7.42	7.31	7.26	7.18	7.26	7.28	7.35	7.34	0.064	0.556	0.825	0.839
Dry matter digestibility (%)	48.50^{b}	53.04^{e}	51.86^{d}	45.95^{a}	54.00^{e}	53.95^{e}	47.88^{b}	48.92^{b}	50.67^{c}	0.397	<0.001	<0.001	<0.001
Organic matter digestibility (%)	46.67^{bc}	50.86^{fg}	50.11^{ef}	43.93^{a}	53.63^{h}	52.30^{gh}	45.86^{b}	47.48^{cd}	48.91^{de}	0.519	<0.001	<0.001	<0.001
VFA total (mmol/L)	47.55^{a}	48.49^{b}	53.03^{d}	53.62^{e}	56.57^{h}	55.56^{g}	52.54^{c}	54.31^{f}	53.15^{d}	0.064	<0.001	<0.001	<0.001
Acetate (% of molar)	55.26	54.68	56.52	55.56	54.34	56.54	57.8	57.16	57.73	0.456	<0.001	0.002	0.392
Propionate (% of molar)	32.46^{b}	31.05^{a}	31.35^{a}	31.10^{a}	32.87^{bc}	33.58^{c}	33.52^{c}	36.03^{d}	35.56^{d}	0.263	<0.001	<0.001	<0.001
Butirate (% of molar)	10.81	10.4	10.82	9.78	10.06	10.05	9.93	10.27	10.71	0.274	0.017	0.269	0.509
Acetat: Propionate ratio	1.70^{cd}	1.76^{ef}	1.80^{f}	1.79^{f}	1.65^{bc}	1.68^{cd}	1.72^{de}	1.58^{a}	1.62^{ab}	0.018	<0.001	<0.001	<0.001
NH_3-N (mmol/L)	25.51^{c}	23.97^{c}	12.86^{a}	23.69^{c}	16.68^{b}	13.16^{a}	23.82^{c}	15.71^{ab}	13.38^{a}	1.022	<0.001	<0.001	0.002
MPS (% DM)	7.89	7.90	7.91	8.54	8.81	8.94	9.39	9.78	9.85	0.165	<0.001	0.128	0.686

Note: Means in the same row with different superscripts differ significantly (p<0.05); C10= 10% Calliandra supplementation; C20= 20% Calliandra supplementation; C30= 30% Calliandra supplementation; B0= 0% dose of *L. plantarum*, B2= 2% dose of *L. plantarum*; B4= 4% dose of *L. plantarum*; VFA= volatile fatty acid; MPS= microbial protein synthesis; SEM= standard error of mean; C= *C. calothyrsus*; B= *L. plantarum*.

The interaction of both treatment factors produced the highest propionic acid concentration obtained when combined with Calliandra supplementation of 30% and 2% inoculation dose, providing propionic acid of 36.03% of molar ($p<0.05$). Except for 30% Calliandra supplementation and 4% *L. plantarum* inoculation dose, that treatment showed a significant difference with propionic acid concentration in the other treatments ($p<0.05$).

Application of different levels of Calliandra supplementation and doses of *L. plantarum* inoculation in *P. purpureum* cv. Gamma Umami grass silage resulted in significant differences in acetate: propionate ratio (A: P ratio) ($p<0.05$). The highest A: P ratio was found in the 10% supplementation treatment, followed by 20% supplementation, and the lowest in the 30% supplementation treatment (1.76, 1.71, and 1.64, respectively, $p<0.05$). On the other hand, silage without *L. plantarum* inoculation significantly produced the highest A: P ratio compared to the other treatments (1.74, 1.70, and 1.67, respectively, $p<0.05$). An interaction effect was found in the A: P ratio with Calliandra application level at all inoculation doses of *L. plantarum*, where the highest value was found in the combination of 10% supplementation treatment at all inoculation doses ($p<0.05$; Table 3).

Our current study showed that treatment and their interaction significantly affected the NH_3-N of rumen fluid ($p<0.05$). The NH_3-N of rumen fluid ranged from 12.86 mmol/L to 25.51 mmol/L. Based on the partial effect, the increase of Calliandra supplementation linearly decreased the NH_3-N concentration of silage (20.35 mmol/L to 17.64 mmol/L; $p<0.05$). A similar phenomenon also occurred due to increasing the inoculation dose of *L. plantarum* (24.00 mmol/L to 13.13 mmol/L; $p<0.05$). On the other hand, the lowest concentration of NH_3-N was found in the interaction of all levels of Calliandra supplementation and the inoculation dose of *L. plantarum* 2%-4% ($p<0.05$), while for the interaction effect without the inoculation, treatment resulted in significantly higher NH_3-N concentrations.

Analysis of variance showed that Calliandra supplementation significantly influenced rumen microbial protein synthesis (MPS) ($p<0.05$). In contrast, the inoculation dose of *L. plantarum* and its interaction had no effect on rumen microbial protein synthesis within the range of MPS production 7.89% DM to 9.85% DM (Table 3). Partially, the highest MPS production was obtained in the 30% Calliandra supplementation treatment, followed by the 20% supplementation treatment, and the lowest MPS production was obtained in the 10% supplementation treatment (9.98% DM vs. 8.78% DM and 8.90% DM, respectively, $p<0.05$).

DISCUSSION

Silage Chemical Composition

Increasing the levels of Calliandra supplementation concomitant increased the DM and OM of the *P. Purpureum* cv. Gamma Umami grass silage ($p<0.05$, Table 1). Mixing several feed ingredients provides advantages, such as increasing the nutrient content; in this case, the CP content of silage linearly increases (see Table 1). Increasing the CP content of the silage increases the OM content and ultimately improves the content of the DM silage. Wang *et al.* (2017) reported an increased DM content with a mixture of corn stover and different portions of legume herbage after 45 days of ensiling.

The OM content showed a linear relationship with the DM content. Calliandra supplementation at 30% produced the highest OM content of 88.87% ($p<0.05$). The increase in OM silage content because of Calliandra supplementation was caused by the contribution of OM from Calliandra itself. Unfortunately, the OM content was not affected by the inoculation dose of *L. plantarum* inoculation and their interaction ($p>0.05$). Using *L. plantarum* in Bengal grass silage (*Panicum maximum*) with an incubation period of 15 days also produced an OM content that did not differ from that in silage that was not inoculated (Adesoji *et al.,* 2010). Furthermore, using *L. buchneri* inoculum combined with *L. plantarum* increased the aerobic stability of silage and inhibited yeast activity, reduced pH, NH_3-N concentration, and loss during fermentation. However, it showed no significant difference in DM, OM, and neutral detergent fiber (NDF) contents (Filya, 2003).

Calliandra supplementation in *P. purpureum* cv. Gamma Umami grass silage significantly affected the CP content ($p<0.05$). The CP content of the silage increased with increasing levels of Calliandra supplementation and significantly differed between supplementation levels. These results agree with a previous study, which reported that 37% Calliandra addition in cactus silage produced silage with the highest N content of 40 g/kg DM, significantly different from other treatments (Gusha *et al.,* 2015). The increase in Calliandra supplementation caused an increase in their CP content; Calliandra has a high CP content of 23.51%, and the higher supplementation contributes to the increase in CP content. These occurred as an associative effect of nutrients from feed ingredients that have been mixed. McDonald *et al.* (2011) explained that the nitrogen (N) content was higher in mixed silage than in silage prepared from cereals.

L. plantarum inoculation did not affect silage CP content ($p>0.05$). The resulting CP content was between 13.22% to 17.71% in all treatments. Hu *et al.* (2009) reported a lack of effect of *L. plantarum* inoculation on the CP content of corn silage. *L. plantarum* inoculation in silage preparation was primarily aimed at helping achieve a rapid decrease in pH and optimizing the silage process rather than increasing the nutritional content of the silage. The difference in CP content can be attributed to the low pH and NH_3-N concentration (Table 1) in the whole silage treatment that indirectly produced the low proteolytic activity.

Calliandra supplementation in the *P. purpureum* cv. Gamma Umami grass silage significantly affected the CF content ($p<0.05$). Calliandra supplementation at 30% significantly decreased CF content (25.07% DM) compared with that at 20% and 10% levels (25.72% and 26.29% DM, respectively; $p<0.05$). The low content of CF at 30% supplementation was caused by the increase in

the portion of Calliandra to basal material that reduced the proportion of grass used and thus decreased the CF content it contributed. Another researcher also reported the same trend (Wang *et al.,* 2017; Sutaryono *et al.,* 2023). They showed a decrease in the CF content of corn stover silage by 10% to 30%, in line with the increase in legume administration.

The low content of CF in the treatment with 2% *L. plantarum* may be due to hemicellulose hydrolysis during the silage process, where almost half of the hemicellulose can be degraded during the silage process (Putra *et al.,* 2017; Li *et al.,* 2019). Inoculation of *L. plantarum* in corn silage of different maturity stages showed decreased NDF content (Haghparvar *et al.,* 2012). The decrease in CF content may be caused by degradation by plant or bacterial enzymes and hydrolysis by organic acids during the fermentation process (Widiyastuti *et al.,* 2014; Li *et al.,* 2019).

The current result showed that the *Calliandra calothyrsus* supplementation and *L. plantarum* inoculation and their combination significantly affected the EE content of the grass silage ($p<0.05$). The content of EE in all treatments was between 2.09% to 2.71%. The EE content in these treatments remained within the normal EE acceptance range in the rumen, as more than 5% fat in the feed will interfere with rumen biological processes. A high EE feed content will interfere with the activity of rumen microbes, especially cellulose-digesting bacteria. In addition, the high content of EE in the feed will also interfere with the VFA absorption process. Raes *et al.* (2004) explained that the availability of fat in feed is not only a source of energy but is also needed to support several biological functions of the rumen to ensure healthy performance.

The significantly low EE content in the silage that received the 4% dose of *L. plantarum* inoculation was due to the EE in the silage used by *L. plantarum* during the ensiling process. McDonald *et al.* (1991) reported that the reduced content of unsaturated fatty acids in silage was due to the biohydrogenation process during ensilage. Furthermore, the use of *L. plantarum* as an inoculant was shown a decrease in the concentration of C18:3n-3 in grass silage (Boufaïed *et al.,* 2003). However, in another study, a reduction in EE content in barley silage that received LAB inoculation did not show any difference with the treatment without the addition of LAB inoculation (3.63% vs 4.08% DM, respectively) (Kim *et al.,* 2015).

The total tannin (TT) content of Calliandra used in the study was 6.20% after two days of withering. Total tannin concentration increased directly to the increase in Calliandra supplementation level ($p<0.05$). It was understood that the increased addition of Calliandra, besides contributing to the increase in silage protein content, also contributed to the increase in silage tannin content. This agrees with Gao *et al.* (2022), who noted that when legumes are ensiled, the tannins they contain become part of the silage. Contrary to the Calliandra supplementation, *L. plantarum* inoculation reduced the TT content of *P. purpureum* cv. Gamma Umami grass silage ($p<0.05$). The decrease in TT content of silage in this study is presumably due to the ability of *L. plantarum* to reduce tannin by producing the *tannase* enzyme. In their study, Tefa *et al.* (2019) found that *L. plantarum* could reduce the tannin content of *Canna edulis Kerr* from 2.53 mg/mL to 0.84 mg/mL under optimum conditions.

The interaction effect of Calliandra supplementation and *L. plantarum inoculation* influenced the TT content of *P. purpureum* cv. Gamma Umami grass silage ($p<0.05$). The interaction effect in this case at least provides benefits among others: Calliandra plays a role in increasing the CP content of silage, and *L. plantarum* in improving the overall fermentation quality of silage, but also contributed by reducing the amount of TT input from Calliandra through its *tannase* enzyme activity. However, the influential effect of *L. plantarum* on reducing tannins and other anti-nutritional factors may depend on various factors, including the specific strain of *L. plantarum* used, the composition of the diet, and the physiological condition of the ruminant (Li *et al.,* 2022; Wang *et al.,* 2022). Therefore, further research may be required to optimize the use of *L. plantarum* in reducing tannins in mix or sole legume silage for specific applications.

Silage Fermentative Quality

The silage pH value determines the success rate and reflects the fermentation dynamics during the ensiling process (Zhang *et al.,* 2017; Mu *et al.,* 2020). At a critical pH, lactic acid is the dominant acidic product that inhibits the growth of other bacteria. Successful ensiling of silage containing legumes is quite challenging to achieve. Legumes contain high amounts of protein, which act as a buffering capacity, and are low in water-soluble carbohydrates (WSC) (Dewhurst *et al.,* 2003; Liu *et al.,* 2012). Low pH value obtained in this study with both the inoculated and uninoculated treatments was thought to be due to the suitability of the *L. plantarum* or LAB epiphytic inoculant source with the silage material or forage. These enabled the adaptation process and inoculant colonization of the silage material to proceed relatively quickly based on the overall pH value of the silage produced in all treatments (Table 2).

Well-preserved silage should have a pH value of at least 4.5 (McDonald *et al.,* 1991). Combining both factors proved to decrease the concentration NH_3-N with a pH value of 3.87–3.88 in all treatments, thus meeting the criteria of well-preserved silage. These results agree with previously reported values that the inoculation of *Lactobacillus* species affected the pH of silage ranging from 4.15 to 5.53 (Paradhipta *et al.,* 2019; Li *et al.,* 2020; Wu *et al.,* 2022). Low pH values indicated that lactic acid production sufficiently suppressed the pH and inhibited microbial activity, thereby preserving the organic matter. *Enterobacteria*, *Clostridium*, *Listeria*, and other harmful organisms can continue to grow, perform further fermentation, and produce various metabolic products if the lactic acid formation is insufficient to lower the pH to a level that inhibits them (Ávila & Carvalho, 2020). The pH value obtained in our study was lower than that found in a combination of Guinea grass and Stylo-legume silage (pH 4.45) (Bureenok *et al.,* 2016).

The silage NH_3-N concentration was similar in all treatments, ranging from 4.60 to 6.17 mg/100 g (Table 2). This condition shows that the level of Calliandra supplementation increased the concentration of NH_3-N silage ($p<0.05$), with the lowest concentration obtained with a 10% level of Calliandra supplementation (5.26 mg/100 g). The NH_3-N concentration increases concomitantly with the increase in the pH value of the *P. purpureum* cv. Gamma Umami grass silage. These conditions illustrate that the protein content in Calliandra can act as a pH buffer to inhibit the rapid decrease in pH at the beginning of the fermentation phase. The higher the pH of the resulting silage, the higher the proteolytic activity. Muhandiram *et al.* (2023) stated that a rapid decrease in pH can help inhibit proteolysis, thereby maintaining the protein content of the silage. Low pH values at the beginning of the ensiling process help prevent protein breakdown into NH_3-N. Our result was slightly higher than found in the mixed silage of high moisture amaranth and rice straw treated with *L. plantarum* NH_3-N of (3.07 mg/100 g) (Kim *et al.*, 2017b), which might be due to different silage base materials used in each of these studies. However, the concentration of NH_3-N obtained in this study met the critical attention of NH_3-N in the silage (<10 g/100 g of total N) (Lima *et al.*, 2010).

Alternatively, the increase in the NH_3-N concentration may be due to the amount of dissolved N obtained from supplementation with Calliandra, which caused an increase in the NH_3-N concentration in silage, which indicates the proportion of protein degraded during the ensiling process. The concentration of NH_3-N is closely related to the number of products and the type of fermentation during the silage process. Thus, the concentration of NH_3-N is associated with the level of protein degradation both by plant enzymes and microbial enzyme activity, which acts as a secondary fermenter by *Enterobacteria*, *Clostridia* (butyric acid producer), and yeasts (ethanol producer) (Kim *et al.*, 2021). However, the efficiency of rumen microbial N-synthesis can be increased by supplementing the silage with N-protein rather than non-protein nitrogen (NPN); thus, reduced proteolysis during ensilage is essential (Muhandiram *et al.*, 2023).

Rumen Fermentation Profile

The effect of treatments on the pH of *in vitro* rumen fluid is shown in Table 3. Calliandra supplementation, *L. plantarum* inoculation, and the interaction between both factors did not affect rumen pH. Adding Calliandra silage at 30%, 40%, and 60% to the basal feed of rice straw in bulls dairy cows also did not affect the pH compared with the control treatment (Giang *et al.*, 2016). In this study, the rumen pH value at all levels of Calliandra addition ranged from 7.24 to 7.3; however, the *in vitro* rumen pH value obtained was relatively higher than the ideal pH of rumen fluid of 5.5 to 6.9 (McDonald *et al.*, 2011). The increased range of rumen pH values was thought to be caused by the type of feed used that contained low energy or nonstructural carbohydrates, which then affected the low production of VFA.

Table 3 demonstrated that the DMD of *P. purpureum* cv. Gamma umami grass silage increased linearly with increasing levels of Calliandra supplementation ($p<0.05$). The same increasing trend was observed for the OMD. The increased digestibility of silage in our results is thought to be due to the increased availability of CP in the silage. This condition intensifies rumen microbial activity to degrade silage during digestibility testing. Similar results reported by Sutaryono *et al.* (2023) showed an increase in the digestibility of corn stover silage concomitant with an increase in the Leucaena portion. They further explained that legumes in the feed provide a nitrogen source for rumen microbes. For rumen microbes, the availability of nitrogen, carbon, and ATP sources can encourage intensive cell multiplication (Dilaga *et al.*, 2022). Another study by Xue *et al.* (2020) under *in vitro* assessment showed that there was an increase in DMD and OMD of mixed silage of Orchardgrass and Alfalfa, which was caused by an increase of protein content and a decrease in the fiber fraction as the proportion of alfalfa was added.

Our current study also showed that the inoculation dose of *L. plantarum* significantly increased the OMD and OMD of *P. purpureum* cv. Gamma umami grass silage ($p<0.05$). Several researchers have also confirmed the increase in silage digestibility due to *L. plantarum* inoculation. For example, Cao *et al.* (2011) showed that using *L. plantarum* inoculant in vegetable waste silage increased DMD and decreased CH_4 gas production under *in vitro* conditions. Furthermore, Paradhipta *et al.* (2019) showed increased digestibility of high moisture sorghum-sudangrass silage due to inoculation of *Lactobacillus plantarum* R48-27 and *Lactobacillus buchineri* R4-26 at ratio 1:1. Inoculation of *L. plantarum* in silage at least provides benefits as a potential probiotic and its interaction with rumen microbes in improving rumen performance. Lactic acid bacteria (LAB) inoculants can improve livestock performance by changing rumen fermentation patterns (Mohammed *et al.*, 2012; Li *et al.*, 2023).

Due to interaction effects, the high DMD and OMD values are caused by the suitability between the availability of energy and protein to meet the needs of rumen microbes for these two substrates. Thus supporting the rumen fermentation process and indirectly increasing the activity of silage degradation. These conditions lead to faster rumen microbial proliferation, improving overall rumen performance. Qu *et al.* (2013) showed that higher CP content and lower fiber content in legumes than in grasses may affect digestion.

The production of VFA in the rumen is related to the carbohydrate fermentation process and is directly related to livestock productivity because most of the VFA in the rumen originates in the fermented carbohydrate feed (Kariyani *et al.*, 2021). The digestibility rate can be used to predict feed quality. Our results indicated that an increase in the level of Calliandra supplementation from 10% to 20% increased the VFA concentration. However, the 30% level of supplementation decreased the VFA concentration (Table 3). This decrease at 30% supplementation was

caused by the lower carbohydrate levels indirectly influenced by the tannins contained in Calliandra (Table 2), although the 30% treatment had a higher level of CP, which could contribute to the increase in VFA concentration when deaminated in the rumen (France & Dijkstra, 2005; Weimer, 2022). Jayanegara *et al.* (2009) indicate that the presence of tannin at 0.5 mg/mL in hay rations significantly decreased the total rate of gas production, organic matter digestibility (OMD), total VFA, and iso-VFA of rumen fluid under *in vitro* assessment.

Overall, the total VFA obtained in this study was relatively low compared with the average concentration in the rumen of 70 mM–130 mM (McDonald *et al.,* 2011). The total VFA concentration of the results of this study is consistent with Noviandi *et al.* (2014) showed the total VFA concentration in rumen fluid from a mixed feed Alfafa, *Lotus corniculatus*, and *Astragalus cicer* L. and tall fescue grass (*Festuca arundinaceae*) with a combined ratio of 25%, 50%, and 75% inclusion of legumes produced total VFA concentrations between 47.8 to 56.1 mM under *in vitro* continuous culture conditions.

The proportion of acetic acid at both 20% and 30% Calliandra supplementation levels was similar but significantly higher than that at 10% of supplemented ($p<0.05$). The concentration of acetic acid obtained in this study was slightly lower than the concentration of acetic acid (30.7 mM–31.9 mM) in a previous study that used a mixed feed of legumes (Alfafa, *Lotus corniculatus*, and *Astragalus cicer* L.) and tall fescue grass (*Festuca arundinaceae*) with a combined ratio of added legumes (to 25%), which was digested using the *in vitro* continuous culture method (Noviandi *et al.,* 2014). The difference in the amount of acetic acid between both studies was caused by differences in the quality of the forage used, where the silage feed in this study originated from a tropical area.

Increasing dose inoculation of *L. plantarum* showed a linear increase in the rumen acetic acid concentration ($p<0.05$). The increase in acetic acid concentration in this study might be attributed to the activity of *L. plantarum*, which produces organic acids, during the ensilage process and the possibility of producing enzymes from the fiber degradation group. In their study, Kim *et al.* (2017a) reported that *Lactobacillus plantarum* R48-27, a silage inoculant, can release *cellulose, xylinase, chitinase*, and *esterase enzymes*. In addition, feeding with LAB inoculants, such as *L. plantarum* species, can change the rumen fermentation pattern through their interaction with rumen microbes, increasing the amount of digested OM and ruminant performance (Hristov *et al.,* 2013).

Supplementation of 20% Calliandra and 2% inoculation of *L. plantarum* showed significant interaction in producing the highest acetic acid concentration ($p<0.05$). This interaction shows a positive supporting condition, where the addition of calliandra directly affects the increase in the proportion of degradable fiber, and on the other hand, with the inoculation of *L. plantarum*, which works to produce organic acid compounds such as lactic acid and the possibility of producing enzymes to reducing structural carbohydrates (Li *et al.,* 2022), by degrading the complex bonds of lignocellulose which in turn facilitates rumen microbes to degrade them. According to Weimer (2022), the degradation of structural carbohydrates such as cellulose and hemicellulose in the rumen by anaerobic microbes produces volatile fatty acids, including acetate. However, Ungerfeld (2020) emphasizes that other factors, such as feed fermentation rate and methanogen growth, can also influence the concentration of acetate and other volatile fatty acids in the rumen.

The proportion of propionic acid was significantly affected by the interaction of both treatment factors ($p<0.05$). Our study showed that increasing the level of calliandra supplementation led to an increase in propionic acid concentration from rumen fermentation, with 30% calliandra supplementation with 2% and 4% dose of inoculation resulting in the highest propionic acid concentration among the treatments. Calliandra is a protein source feed ingredient from the legume group. Fermentation of legumes as feed ingredients in the rumen is more directed toward producing propionic acid (Gonzalez-Garcia *et al.,* 2017; Ammar & Philippidis, 2021), which explains the low or absent variation in the proportion of butyric acid in all treatments. Chen *et al.* (2020) noted that stimulating ruminant propionate fermentation can reduce methane emissions. Unlike acetate and butyrate, propionate is a gluconeogenic VFA and thus can increase energy availability for production.

In this study, the A: P ratio of *in vitro* rumen fluid increased in the 10% Calliandra supplementation treatment at all *L. plantarum* inoculation doses but conversely decreased when the supplementation level was applied at all inoculation doses. This condition may be due to the effect of the concentration of acetic acid and propionate, which both increased in this study so that it does not provide a gap in the value of the increase in the ratio of A: P ratio of the silage. Sun *et al.* (2022) indicate that the balance of volatile fatty acids, including the ratio of acetate, butyrate, and propionate, can vary depending on the type of carbohydrate being fermented. For example, starch fermentation generally promotes propionate formation, which is associated with less methane production. This balance is essential as it can affect the energy supply to the ruminant. For example, propionate is a significant source of glucose in ruminant, which is crucial for meeting their energy needs.

Silage prepared with high protein content was identified with high NH_3-N concentration consequences feed protein degraded during the ensilage process. Our current result showed that the NH_3-N concentration is affected by Calliandra supplementation and a dose of inoculation, and their interaction was demonstrated in the normal range of NH_3-N concentration of 12.86 to 25.51 mmol/L of rumen fluid. Following McDonald *et al.* (2011), the ideal concentration of NH_3-N in rumen fluid leading to support microbial growth ranged from 10.21 to 35.76 mmol/L. Overall, our current result showed that the NH_3-N concentration was affected by Calliandra supplementation and a dose of inoculation, and their interaction was demonstrated in the normal range of NH_3-N concentration of 12.86 to 25.51 mmol/L of rumen fluid. The ideal concentration of NH_3-N in rumen fluid

leading to support microbial growth ranged from 10.21 to 35.76 mmol/L (McDonald *et al.,* 2011).

In our study, the declined two conditions probably caused NH_3-N concentration: 1) Rumen microbes efficiently incorporate ammonia to meet their N requirements for cell synthesis in line with an increased level of Calliandra supplementation, 2) limiting protein degradation activity of proteolytic bacteria caused by tannin content; as in this present study, there was an increase in total tannin content by increasing the level of Calliandra supplementation. Other researchers have also reported decreased NH_3-N concentration in line with the legume increase (Min *et al.,* 2002; Copani *et al.,* 2014; Mudhita *et al.*, 2016). Tannin compounds have two pathways in influencing rumen NH_3-N concentration, as follows: 1) reducing feed protein degradation through the formation of tannin-protein-complex formation or reducing the solubility of proteins (Min *et al.,* 2002), and 2) inhibiting the activity of proteolytic bacteria or proteolytic enzymes or inhibiting the activity of both (Patra, 2012). Nevertheless, the specific effects may depend on various factors, including the type and concentration of tannins, diet composition, and physiological condition of the ruminant (Ningrat *et al.,* 2016; Sun *et al.,* 2022).

A similar trend was also shown by dose inoculation treatment. The difference ($p<0.05$) in NH_3-N concentration of rumen fluid between silage treated with *L. plantarum* inoculation and without inoculation is thought to be closely related to the increased VFA concentration of both *L. plantarum* inoculation treatments so that it can describe the availability of energy which is then incorporated with NH_3-N through the biochemical process of rumen microbial cells. However, different results were reported by Hapsari *et al.* (2016) showed that inoculation of *L. plantarum* 1A-2 singly in elephant grass silage produced rumen NH_3-N concentrations that were not different from the treatment without inoculation or the combination treatment of *L. plantarum* 1A-2 plus formic acid.

The best interaction was shown by the treatment of 30% Calliandra supplementation with a 2% *L. plantarum* inoculation dose, which resulted in NH_3-N concentration of 15.71 mmol/L. Although the interaction of the 20% and 30% Calliandra supplemented treatment with a 4% inoculation dose resulted in lower NH_3-N concentrations of 13.16 mmol/L and 13.38 mmol/L, respectively, the efficiency of using *L. plantarum* inoculation needs to be considered. Likewise, the interaction between the 30% Calliandra supplementation treatment with uninoculated of *L. plantarum* was not chosen as the best treatment interaction due to considerations in terms of silage feed conservation in this study, as explained earlier that the NH_3-N concentration in the 30% Calliandra supplementation, and without *L. plantarum* inoculation treatment resulted in higher NH_3-N silage.

Calliandra supplementation significantly affected microbial protein synthesis (MPS) ($p<0.05$). Microbial protein synthesis increased and showed significant differences between groups as the level of Calliandra supplementation increased. In this study, increased MPS due to Calliandra supplementation occurred due to synchronization between energy and protein in the rumen fluid. This synchronization of energy and protein availability is indicated by the increase in VFA concentration and the decrease in rumen NH_3-N concentration (Table 3), indicating an increase in microbial biomass that performs degradation activities of the silage. Noviandi *et al.* (2014) reported an increase in the level of legume use in the treatment feed resulted in increased microbial protein synthesis, namely obtained microbial N of 7.64% DM at a legume-grass ratio of 25:75 and obtained 9.00% DM at a legume-grass ratio of 75:25.

On the other hand, the increase in rumen MPS is also thought to be caused by the activity of tannin compounds contained by Calliandra, which, in this case, causes a decrease in the protozoal population in the rumen fluid. Patra (2012) stressed that tannins also affect the efficiency of rumen MPS. The decrease in protozoa was followed by a decrease in microbes from the *archaea* group that act as CH_4 producers. This study indicates this condition by the low A: P ratio value produced (Table 3). The previous research by Tan *et al.* (2011) showed reduced CH_4 and CO_2 production concomitantly increased condensed tannins from *Leucaena leucocephala.*

CONCLUSION

The present study concluded that the supplementation levels of Calliandra at 20% to 30% effectively increased the chemical composition of *P. purpureum* cv. Gamma Umami grass silage. Similarly, the dose of 2% *L. plantarum* inoculation positively contributed to the quality of the resulting silage. Overall, a synergistic interaction occurred between Calliandra and *L. plantarum* in increasing the chemical composition, silage fermentation quality, and improving digestibility and rumen fermentation parameters with the optimal combination obtained at 20% Calliandra supplementation with a dose of *L. plantarum* of 4%. Further, an *in vivo* study should be performed to investigate the direct effect of the overall performance of these feeds on cattle production.

CONFLICT OF INTEREST

The authors declared there was no competing interest during the research and writing of the manuscripts.

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