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Increasing the nutritive value of a rice straw-based diet using mulberry and *Leucaena* to promote the growth performance of lambs

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Abstract

The objective of this study was to evaluate the effect of substituting urea and rice bran with mulberry or a mixture of mulberry and *Leucaena* in the diet on growth rate, feed intake, nutrient digestibility, nitrogen balance, rumen fermentation, estimated methane production, and rumen microbial protein synthesis in lambs fed a basal diet of urea-treated rice straw. The experiment consisting of three supplement treatments with six replications was arranged in a randomized block design. Eighteen lambs with an initial body weight of 14.4 ± 3.35 kg were used. The three supplement treatments were (i) urea–rice bran (UR) comprising 38.5% of the diet, (ii) mulberry foliage comprising 30% of the diet as UR substitution (Mb), and (iii) a mixture of mulberry–*Leucaena* foliage in a 1:1 ratio comprising 30% of the diet as UR substitution (MbL). All lambs were fed a basal diet of urea-treated rice straw, and the diets were formulated with iso-energy and iso-protein content. Substituting urea rice bran with either mulberry foliage or a mixture of mulberry–*Leucaena* foliage yielded similar effects on urinary and faecal nitrogen excretions, nitrogen retention, rumen fermentation, microbial nitrogen yield, and the average daily gain of lambs (71.4 g/day). Both the mulberry and the mulberry–*Leucaena* mixture supplements exhibited higher dry matter intake by 15.4% and 9.9% and neutral detergent fibre digestibility by 17.5% and 14.5%, respectively, compared to urea–rice bran supplementation. These findings indicate that mulberry or a mixture of mulberry–*Leucaena* foliage is a promising alternative to replace urea–rice bran in improving the nutritional values of the rice straw basal diet for sheep.

Keywords: digestibility, growth rate, nitrogen utilization, rumen fermentation, supplements

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Introduction

The continuous unavailability and low nutritional value of basal forage feed pose significant challenges in ruminant farming, particularly in many developing countries in the humid tropics. To address these limitations, it is crucial to fully exploit local feed sources to maximize dry matter intake

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and improve the nutritional composition of ruminant rations, which typically consist of fibrous agricultural by-products and native natural grasses. Rice straw, which is abundantly available in rice production areas, does not provide balanced nutrients for efficient microbial rumen fermentation of fibrous feed. This nutrient imbalance negatively affects the stability and functionality of rumen microbes during the fermentation process (Loor *et al*., 2016). Therefore, enhancing the nutritional value of rice straw for ruminant feeding is a crucial step towards its increased utilization, thereby reducing food competition with humans and promoting environmental sustainability in livestock production (Loor *et al*., 2016). An alternative to enhance the utilization of fibrous feed is to optimize the efficiency of rumen fermentation and synthesis of microbial protein (Wadhwa *et al*., 2016). This can be achieved through a strategic balance of energy, protein, and minerals (Wadhwa *et al*., 2016) using concentrate supplementation. Rice bran, a valuable by-product in rice production, is widely utilized as an energy source in both non-ruminant and ruminant concentrate formulations due to its year-round availability. Although ruminant animals can derive energy from the rumen fermentation of forages, incorporating tree foliage with high crude protein content and digestibility presents an alternative to concentrate supplements containing rice bran. Among these options, mulberry and *Leucaena* are notable tree foliage choices due to their high protein content. Mulberry foliage has been used as a fermentable protein and energy source in sheep diets (Doran *et al*., 2007; Yulistiani *et al*., 2015).

Mulberry has demonstrated superior quality compared to alfalfa (Doran *et al*., 2007) and has been incorporated into concentrate mixtures due to its superior digestibility (Ouyang *et al*., 2019). However, it has been reported to possess high protein degradability (Saddul *et al*., 2005), leading to protein deamination in the rumen and resulting in the loss of valuable essential amino acid sources for host animals (Preston & Leng, 1987; Bach *et al*., 2005). To solve this problem, condensed tannin (CT) supplementation can reduce crude protein (CP) degradation in the rumen and thus provide a source of undegradable crude protein (Henke *et al*., 2016).

Leucaena leaves have been reported to contain CT levels ranging from 20.0 to 131.0 g/kg (Kamsekiew, 2005; Archimede *et al*., 2015; Rira *et al*., 2015; Phesatcha & Wanapat, 2016; Pineiro-Vanquez *et al*., 2017; Gaviria-Uribe *et al*., 2020). With its high tannin content, *Leucaena* shows potential as a feeding supplement to enhance protein utilization in foliage with low tannin levels. This *Leucaena* foliage is widely cultivated and is utilized as an economical protein source for ruminants in tropical regions. Notably, it is palatable and contains more than 25% crude protein, making it a suitable protein supplement for ruminants (Giang *et al*., 2016).

Feeding *Leucaena* foliage as a protein source has been proven to increase sheep productivity (Rahman *et al*., 2015; Fernandes *et al*., 2020), goat productivity (Cowley & Roschinsky, 2019), cattle productivity (Pineiro-Vancuez *et al*., 2017), reduction in methane production (Archimede *et al*., 2015; Giang *et al*., 2016; Pineiro-Vasques *et al*., 2017; El-Zaiat *et al*., 2020), and urinary nitrogen excretion (Archimede *et al*., 2015; Pineiro-Vasques *et al*., 2017; El-Zaiat *et al*., 2020). Condensed tannin binds to dietary protein to form a tannin–protein complex that subsequently reduces protein degradation in the rumen and enhances amino acid absorption in the small intestine (Barry & McNabb, 1999). Moderate supplementation with condensed tannin (2.0–4.5% of DM) exerts a beneficial impact on protein metabolism in the rumen. As a result, reduced protein degradation leads to a decrease in nitrogen losses through urine and an increase in nitrogen excretion through faeces (Ahnert *et al*., 2015; Aguerre *et al*., 2016). Shifting nitrogen loss from urine to faeces is considered advantageous since faecal nitrogen is less volatile compared to urinary nitrogen (Castillo *et al*., 2001), resulting in lower ammonia and nitrous oxide emissions from manure (Dijkstra *et al*., 2013; van Cleef *et al*., 2022). A tannin-rich diet has been reported to decrease CH_4 emissions through a decreased methanogens population (Min *et al*., 2014; Króliczewska *et al*., 2023). Animal productivity can be enhanced by supplementing with mulberry or *Leucaena* foliages, but its combined use has not yet been thoroughly explored. The combination of highly nutritious forages (e.g, mulberry) and foliage with high CT content (e.g., *Leucaena*) should improve protein utilization, leading to increased ruminant productivity and reduced greenhouse gas emissions from CH_4 and N_2O. Reducing CH_4 emissions is crucial, as enteric CH_4 emissions contribute 17% to global CH_4 emissions (Gerber *et al*., 2013).

Rice bran and urea supplementation in animal diets has been well documented and has proven successful in enhancing the utilization of low-quality fibrous feed. In this study, we substituted rice bran and urea with mulberry and *Leucaena* feed supplements to improve the use of urea-treated rice straw basal diets in lambs. We hypothesized that mulberry supplementation would have a comparable effect to rice bran and urea in improving the utilization of urea-treated rice straw basal diets for lambs. Furthermore, we expected that incorporating a mixture of mulberry–*Leucaena* as energy and protein source supplements into the urea-treated rice straw basal diet would further enhance its performance. The objective of this study was to evaluate the effect of substituting urea rice bran with mulberry or mulberry–*Leucaena* mixture on growth rate, feed intake, nutrient digestibility, nitrogen balance, rumen fermentation, estimated methane production, and rumen microbial protein synthesis in lambs fed a basal diet of urea-treated rice straw.

Material and Methods

Urea-treated rice straw (TRS) was prepared by thoroughly spraying and mixing chopped (5 cm) rice straw with 5% urea solution (1 L/kg of straw DM) and sealed in black plastic bags (5 kg/bag) for three weeks. The treated straw was spread on a concrete floor to allow the ammonia to evaporate a day before feeding the lambs. The mulberry (*Morus Alba*) foliage was harvested from the mulberry paddock at 6-week cutting intervals. The *Leucaena* (*Leucaena leucocephala* hybrid) foliage from the paddock was harvested at 8 weeks. The harvested foliages were chopped to 5–10 cm in size and dried under the sun for 3 days. Sufficient amounts of chopped and dried foliage were prepared, thoroughly mixed, and stored for use in the experiment.

The use of the animals followed the Protocols of Animal Ethics of the Institutional Animal Care and Use Committee (IACUC) of the Universiti Putra Malaysia based on the guidelines for the care and use of laboratory animals (NRC 2011).

The study used 18 lambs aged between 3 and 6 months, with an average body weight of 14.4 ± 3.35 kg. The three supplement treatments were (i) urea-rice bran (UR) comprising 38.5% of the diet, (ii) mulberry foliage comprising 30% of the diet as a substitution for urea-rice bran (Mb), and (iii) a mixture of mulberry–*Leucaena* foliage in a 1:1 ratio comprising 30% of the diet for urea rice bran substitution (MbL). Further details of the treatments are given in Table 1. The basal diet of urea-treated rice straw (UTRS) was mixed with molasses to improve the palatability of the straw. The 18 lambs were divided into six blocks as replications based on initial body weight and arranged in a randomized complete block design. The lambs were individually penned on a slatted floor, each lamb within each block was assigned to one of the three supplement treatments.

Table 1 Composition of diet (% DM basis), nutrient content and metabolizable energy contents of the treatments

Ingredients	Supplement treatments[1]			TRS	Mulberry	Leucaena
	UR	Mb	MbL			
UTRS (%)	55.0	55.1	55.1			
Molasses (%)	6.5	4.8	4.8			
Urea (%)	2.7	1.5	1.0			
Rice bran (%)	35.8	8.6	9.1			
Mulberry (%)		30	15			
Leucaena (%)			15			
Calculated nutrient content:						
Metabolisable energy (MJ/kg DM)	8.3	8.2	8.3			
Crude protein (g/kg)	153	152	152	68	189	215
Organic matter (g/kg)	843	851	863	821	917	957
Neutral detergent fibre, NDF (g/kg)	517	565	573	758	415	451
Acid detergent fibre, ADF (g/kg)	350	402	407	579	254	283
Condensed tannin (g/kg)						131

[1] UR: Urea and rice bran supplements. Mb: Mulberry supplements. MbL: Mulberry and *Leucaena* mixture supplements. UTRS: Urea-treated rice straw

The diets were formulated in iso-nitrogenous and iso-energetic form, containing a crude protein (CP) level of 15% and a metabolizable energy (ME) content of approximately 8.32 MJ/kg (Table 1). These formulations were specifically designed to meet the lamb growth rate requirement, targeting a rate of 100 g/day, as recommended by Kearl (1982). Daily feed consumption was determined by calculating the difference between the amount of feed offered and the amount of feed left in the troughs. The feed residue from the previous day was weighed before morning feeding. The average daily gain of the lambs during 8 weeks of growth trial was obtained by weighing their weight at weekly intervals.

The feed conversion ratio was calculated by dividing the dry matter intake by the average daily gain. At the end of the growth studies, the lambs were transferred to individual metabolic crates for a 7-day digestibility study.

Daily feed intake and refusal measurements were performed during the digestibility study before morning feeding. To ensure separation, a metabolic crate was equipped with a separator to collect faecal and urine samples. A representative portion (10% of the total faecal production) was taken from the faecal sample and subjected to oven drying at 60 °C for 48 h. At the end of the collection period, the faeces were pooled for each sheep. A 10% subsample was ground, passed through a 1-mm sieve, and stored in the freezer for subsequent analysis. The daily urine excretion of each sheep was collected every morning in a bucket containing 100 ml of 10% sulfuric acid (to maintain the pH below 3). A representative sample (10% of total urine) was collected and stored in a freezer. At the end of the collection period, these samples were pooled for each sheep and kept in a freezer for urine-N and purine derivative excretion analysis. At the end of the digestibility trial, ruminal fluid was collected from each sheep 4 h after their morning feeding through a stomach tube. The pH of the fluid was immediately measured using a portable pH meter. A drop of concentrated sulfuric acid was added to the sample to stop the microbial activity. Subsequently, the ruminal fluid was centrifuged at 3,000 × *g* for 10 min. Approximately 10 ml of the resulting supernatant was stored in an airtight container at -20 °C for analysis of the concentration of ammonia (NH_3-N) and volatile fatty acid (VFA) components. Methane production was estimated using VFA proportions, applying the equation described by Moss et al. (2000).

$$CH_4 \text{ production} = 0.45 \times (\text{acetate, C2}) - 0.275 (\text{propionate, C3}) + 0.4 \times (\text{butyrate, C4}) \quad (1)$$

The feeds, residues, and faeces were subjected to analysis for dry matter (DM), organic matter (OM), and crude protein (CP) contents following the procedures of AOAC (2012). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to the methods described by Van Soest et al. (1991). Rumen ammonia nitrogen (NH_3-N) concentrations were determined using steam distillation and titration methods. Gas chromatography (Agilent Technologies, Palo Alto, CA, USA) was employed to determine the total and individual volatile fatty acids (VFA), following the procedure described by Cottyn and Bouque (1968). High-performance liquid chromatography (HPLC) based on the method by Balcells et al. (1992) was utilized to measure the contents of urinary purine derivatives (PD), including allantoin, uric acid, xanthine, and hypoxanthine. The PD were quantified in a single run using allopurinol as an internal standard. Microbial-N production was estimated based on PD excretion using the equations of Chen *et al*. (1990).

$$\text{Microbial-N production (gN/day)} = 0.727 \times x \quad (2)$$

where: x = microbial PD absorbed (mM/day) after duodenal and intestinal digestion.

$$\text{Urinary PD output} = 0.84x + (0.15w^{0.75}\ e^{-0.25x}) \quad (3)$$

where: w = the weight of sheep.

The experiment used a randomized complete block design consisting of three supplement treatments (UR, Mb, and MbL) with six replicates (Table 1). The statistical analysis was conducted using the general linear model procedures, following a randomized complete block design with the model:

$$Yij = \mu + Ti + Bj + Eij \quad (4)$$

where: Yij = response variable, μ = overall mean, Ti =treatment effect, Bi = block effect, and Eij = random error associated with each observation. The results were presented as mean values with the standard error of the means. All differences among treatments means were compared using Duncan's multiple range test. Statistical significance was considered at *P* <0.05. All statistical analyses were performed using R 3.6.1 (R Core Team, 2019).

Results and Discussion

The dry matter intake (DMI), expressed as a percentage of body weight, showed that the mulberry (Mb) and the mulberry and *Leucaena* mixture (MbL) supplements were similar (*P* >0.05) (4.56

vs 4.43% BW) but were higher (P <0.05) than the urea and rice bran supplement (UR) (3.95% BW) (Table 2). This suggests that supplementation with mulberry and mulberry–*Leucaena* mixture stimulates total DMI in the urea-treated rice straw basal diet. This finding agrees with a study conducted by Anbarasu *et al*. (2004), which showed that replacing 50% of protein concentrates with a leaf meal mixture (*Leucaena*, mulberry, and *Arachidachta indica*) increased total DMI, despite the bulky nature of the feed. Archimede *et al*. (2015) and Rira *et al*. (2015) also reported an increase in DMI in sheep fed tropical grass (*Dichanthium* sp) hay when supplemented with tannin-rich plants (*Leucaena*, *Gliricidia* and Manihot) in the form of leaf meal pellets, attributed to the higher protein content of the diet. In the present study, the MbL diet supplemented with 15% *Leucaena* had a tannin content of 1.95%, which did not reduce DMI and even increased DMI. This result is consistent with a previous study on heifers fed *Penisetum purpureum* grass supplemented with *Leucaena* that contained a low level of tannin content (<2% DM), which did not affect DMI (Pineiro-Vasquez *et al*., 2017). The previous study using *Lotus corniculatus* showed total condensed tannins of 3–4% in the form of forage fed to ruminants did not have any adverse effects on DMI (Barry & McNab, 1999). However, DMI decreased when tannins were supplemented with 3% tannin extract (Dschaak *et al*., 2011). The decrease in DMI observed in diets containing tannins can be attributed to insufficient rumen degradable protein available for microbial growth (Waghorn, 2008) and reduced palatability due to the astringency of tannins (Landau *et al*., 2000; Makkar, 2003). In the present study, the absence of a tannin effect on the DM intake of the MbL diet can be attributed to the low tannin content (1.95%) and the lack of any impact on the palatability of *Leucaena* foliage. The similarity in total dry matter intake (DMI) between Mb (without *Leucaena* supplementation) and MbL (with *Leucaena* supplementation) indicates that the tannin level of 1.95% in the MbL diet does not adversely affect DMI.

Table 2 Mean values of DMI and nutrient digestibility in lambs fed on different supplement treatments

Variables[2]	**Supplement treatments**[1]			**S.E.M.**	**P values**
	UR	**Mb**	**MbL**		
Intake					
Total DMI (g/day)	682.2	756.3	737.8	27.205	0.6453
DMI (% BW)	3.95^b	4.56^a	4.43^a	0.088	0.0107
DMI g/$BW^{0.75}$	80.2^b	91.7^a	89.2^a	1.857	0.0147
Digestibility (g/kg DM)					
DM	529^a	529^a	483^b	0.84	0.0024
OM	567^a	573^a	522^b	9.49	0.0034
CP	738	721	721	26.67	0.8240
NDF	411^b	483^a	471^a	20.72	0.0475
ADF	326^b	352^b	414^a	14.86	0.0041

Means with different superscript in the same row are significantly different (P <0.05)

[1] UR: urea and rice bran supplements, Mb: Mulberry supplementation, MbL: Mulberry and *Leucaena* mixture supplementation, S.E.M.: standard error mean

[2] DMI: dry matter intake; DM, dry matter; OM, organic matter; CP, crude protein; NDF neutral detergent fibre; ADF, acid detergent fibre

The digestibility of the dry matter (DM) of the mulberry–*Leucaena* mixture diet (MbL) was lower (483 g/kg) (P <0.05 than the UR and Mb diets achieving 529 g/kg each. The digestibility of organic matter (OM) of the UR and Mb diets was similar (567 vs. 573 g/kg) but was higher than the MbL diet (522 g/kg) (Table 2). The comparable digestibility of DM and OM between UR and Mb diets suggests that mulberry can effectively replace fermentable carbohydrates and proteins provided by urea and rice bran supplementation. A previous study (Yulistiani *et al*., 2015) demonstrated that mulberry foliage can serve as a fermentable energy and protein source in the urea-treated rice straw basal diet, replacing urea and rice bran supplements. However, the inclusion of 50% *Leucaena* in the diet (MbL) reduced the digestibility of DM and OM. Replacement of mulberry with *Leucaena* at 50%, which corresponds to *Leucaena* supplemented at 15% (MbL) in the total diet, was expected to increase nutrient digestibility, but it decreased DM and OM digestibility. This suggests that there could be an interaction between *Leucaena* and mulberry that contributes to the lower digestibility of OM and DM observed in the MbL diet.

The digestibility of crude protein (CP) was similar in the three dietary treatments (P >0.05) with an average of 726.6 g/kg (Table 2). This similarity indicates that the quality of the protein provided by mulberry (Mb) or the mixture of mulberry and *Leucaena* (MbL) leaves is comparable to that of urea rice bran (UR) supplements. The presence of tannins from *Leucaena* in the MbL diet did not affect protein digestibility. This finding is consistent with previous studies that demonstrate that tannin content in *Acacia nilotica*, when used as a supplement in small quantities for grazing sheep, did not show a marked difference in protein digestibility compared to a diet supplemented with concentrate or *Albizia* that does not have tannin content (Bhatta *et al*., 2005) and supplementation with *Leucaena* in pellet form to *Dichanthium* sp. grass (Archimede *et al*., 2015). Furthermore, Pineiro-Vasquez *et al*. (2017) reported an increase in CP digestibility with high levels of *Leucaena* supplementation (from 20 to 80% DM) containing low condensed tannins (20 g/kg) in a basal diet of *Penisetum purpureum* grass.

In contrast to the digestibility of crude protein (CP), digestibility of neutral detergent fibre (NDF) of the MbL diet was higher compared to the UR diet (471 g/kg vs 411 g/kg) (P <0.05), but the MbL was comparable to the Mb diet (483 g/kg) (Table 2). The lower digestibility of fibres in the UR diet may be attributed to the presence of fermentable energy sources from rice bran. Higher levels of fermentable energy, particularly starch, can lead to rapid fermentation by amylolytic bacteria, which fibre increases their population, and a subsequent reduction in the population of cellulolytic bacteria, leading to a decrease in fibre degradation (Heldt *et al*., 1999). The digestibility of NDF of the MbL diet was similar to that of the Mb diet (Table 2), indicating that the tannin content derived from *Leucaena* had no impact on the fibre digestibility of the MbL diet. The effects of dietary tannin levels on fibre and protein digestibility have been inconsistent in previous studies. Some studies have reported a decrease in NDF and CP digestibility with the addition of tannin extract at 4% (Ahnert *et al*., 2015) and 3% (Henke *et al*., 2016) intraruminal infusion. Archimede *et al*. (2015) indicated that only NDF digestibility was reduced when the total tannin content of the diet reached 3.3% through *Leucaena* pellet supplementation at 44%. El-Zaiat *et al*. (2020) also observed a reduction in NDF and CP digestibility when the tannin content in the diet reached 1.7% by feeding of *Leucaena* foliage at a level of 25% of the diet.

In our study, the *Leucaena* content, which contained 13.1% condensed tannins (CT), was mixed with Mulberry in a 1:1 ratio, resulting in a total CT content of 1.9% in the diet. This level of tannin did not adversely affect NDF digestibility but tended to increase ADF digestibility (Table 2), suggesting that the tannin level in the MbL diet did not negatively affect fibre digestibility. According to Mueller-Harvey (2006), ruminants fed on a diet containing tannins generally lead to a decrease in fibre digestibility owing to the formation of complex bindings between tannins and natural polymers, such as proteins or carbohydrates, which potentially reduce their digestibility in the ruminant's digestive tract.

Mulberry supplementation at 30% in the Mb diet had higher NDF digestibility than UR (Table 2). Mulberry fibre has previously been reported to have high digestibility (Saddul *et al*., 2005; Doran *et al*., 2007). Therefore, mulberry can be used to replace concentrate without any negative effect on nutrient digestibility when incorporated into sheep diets up to 75% in a grass hay basal diet (Gebru *et al*., 2017) and up to 60% in a whole corn plant silage basal diet (Ouyang *et al*., 2019). Additionally, mulberry leaf meal can completely replace concentrate in rice straw basal diets for beef cattle (Syahrir *et al*., 2012).

The nitrogen intake by the lambs in the three diets was similar (P >0.05), averaging 15.8 g/head/day (Table 3). This similarity can be attributed to the formulation of the diet treatments to be iso-protein and iso-energy. Furthermore, nitrogen excretion through urine and faeces did not differ (P >0.05) among the three diets, with average percentages of 34.3% and 28.8%, respectively (Table 3). The results disagree with previous studies, where tannin supplementation in the diet had a positive effect on N utilization, as evidenced by a shift in N excretion from urine to faeces, indicating a reduction in urine N excretion and an increase in faecal N excretion (Ahnert *et al*., 2015; Archimede *et al*., 2015; Yang *et al*., 2016).

The effect of condensed tannin supplementation on nutrient digestibility and N utilization was associated with variations in the level, source, and supplement form, as well as its interaction with diet composition, and the adaptation time of rumen microbes (Makkar, 2003). The reason for the difference between our study and results reported in previous studies can be explained by the lack of the ability of tannins to protect proteins from rumen degradation due to the natural form of tannins in the herbage (hay foliage) and the relatively low tannin content in the diet (1.9%) in our study (Table 1). This finding aligns with the results reported by Nguyen *et al*. (2017), where N excretion in both urine and faeces increased with an increasing level of supplementation with Leucaena silage (30 to 60%) with a tannin content of 0.84–1.68%. Pelleted *Leucaena* supplement at 44% containing 3.3% tannin in a diet of *Dichanthium spp*, decreased urine excretion without affecting faecal N excretion in sheep (Archimede *et al*., 2015). According to Ahnert *et al.* (2015), quebracho tannin supplement in extract form at 1% added to a mixture concentrate and grass hay basal diet was able to reduce N excretion in urine while increasing N excretion in faeces. Further, a tannic acid supplement at 1.3% to a mixture of concentrate and corn silage resulted in a shift of N excretion from urine to faeces (Yang *et al*., 2016). Reduced protein degradation in the rumen attributed to supplementation with extract tannins was indicated by low urinary N excretion and low concentration of ruminal NH_3N (Dawson *et al*., 1999; Dschaak *et al*., 2011). Furthermore, tannins were reported also to reduce amino acids deamination as indicated by the lower concentration of branch chain volatile fatty acid (BCVFA) when the mixture of hydrolysable and condensed tannin were supplemented in the diet (Augere *et al*., 2016).

The MbL diet treatment appears to contain insufficient tannins to effectively protect the protein from rumen degradation to protect the protein from rumen degradation. If tannins are present in sufficient amounts, they can form stable tannin–protein complexes through hydrogen bonding, which exhibit resistance to microbial degradation in the rumen at pH levels between 5 and 7 (Makkar, 2003). Typically, a decrease in the degradation of crude protein (CP) in the rumen is associated with a reduction in nitrogen (N) losses through urine (Castillo *et al*., 2001). Protein degradation in the rumen generates ammonia, which serves as a nitrogen source for rumen microbes. However, excessive ammonia production occurs when the protein is highly degradable, beyond the capacity of the rumen microbes to utilize it, leading to elevated levels of rumen ammonia. Excess NH_3-N, which is not incorporated during microbial synthesis, is absorbed from the rumen, converted into urea in the liver, and subsequently excreted in the urine (McDonald *et al*., 2002). The similarity of N excretion through urine and faeces resulted in similar retention of N (average 37% from N intake or 6.21g/d) in the three diet treatments (Table 3). Positive N retention indicates a net gain of N within the animals and may support sheep production, as indicated by the increase in the average daily gain of the lambs (Table 6).

Table 3 Nitrogen (N) utilization in lambs fed on different supplement treatments

Variables	**Supplement treatments**[1]			**S.E.M.**	**P values**
	UR	**Mb**	**MbL**		
N Intake (g/d)	16.3	15.8	15.3	0.379	0.7978
N Intake (g/kg $BW^{0.75}$)	1.82^a	1.71^b	1.69^b	0.025	0.006
N excretion					
Faecal N (g/d)	4.3	4.4	4.3	0.142	0.9805
Faecal N (% of intake)	26.9	27.9	31.1	2.209	0.8240
Urinary N (g/d)	4.85	5.85	5.0	0.501	0.5004
Urinary N (% of intake)	31.0	36.9	35.1	2.749	0.1746
N Absorption (g/d)	12.0	11.4	11.0	0.448	0.6468
N Retention (g/d)	7.10	5.51	6.03	0.733	0.1765
N Retention (% of intake)	42.1	35.2	33.7	3.86	0.2965
N retention (% from absorption)	59.6	48.5	53.7	5.072	0.1889

Means with different superscript in the same row are significantly different ($P < 0.05$), S.E.M.: standard error mean.
[1] UR: urea and rice bran supplement, Mb: Mulberry supplement, MbL: Mulberry and *Leucaena* mixture supplement

The ruminal pH of the lambs fed the three diet treatments was similar ($P > 0.05$) (Table 4), with an average rumen pH of 6.7. This pH value falls within the optimal range (6.7–7.0) for cellulolytic activity and microbial protein synthesis. It appears that all diet treatments in this study did not have any inhibitory effect on fibre fermentation (Table 2) or synthesis of microbial protein in the rumen (Table 5). This finding is consistent with previous studies investigating the effects of mulberry leaf supplementation in concentrate (Ouyang *et al*., 2019). *Leucaena* pellet supplementation in a rice straw basal diet (Khy *et al*., 2012; Rira *et al*., 2015), and CT supplementation that did not affect rumen pH (Henke *et al*., 2016). The problem of cellulolysis may occur when the pH of the rumen drops below 6.1 (Mould *et al*., 1983).

Ruminal ammonia nitrogen (NH_3-N) was not affected ($P > 0.05$) by diet treatments (Table 4). Microbial degradation of the protein diet in the rumen leads to the production of intermediate metabolites, such as NH_3, which support the fermentation activity in the rumen. The rumen ammonia nitrogen (NH_3-N) concentration was comparable across all diets, averaging 23.9 mg/100ml (Table 4). This suggests that the tannins present in *Leucaena* in the MbL diet were insufficient to protect the protein of the forages from degradation. The observed concentration of NH_3-N in the rumen (23.9 mg/100ml) in this study was substantially higher than the optimal levels required for normal rumen microbial function (5 to 8 mg/100ml) (Satter & Slyter, 1974) and fibre digestion (15 to 20 mg/100ml) (Preston & Leng, 1987). Consequently, the elevated concentration of ruminal NH_3-N indicates a rapid protein fermentation. The BCVFA is recognized as a marker of protein degradation (Apajalathi *et al*., 2019). The similarity concentration of BCVFA (iso-butyric and iso-valeric acid) (Table 4) between the MbL and Mb diets further confirms the lack of protein protection from *Leucaena* tannins. Rira *et al*. (2015) reported similar findings in a study comparing *Leucaena* supplementation, high in tannin content, and *Gliricidia*, low in tannin content, in a basal diet of native grass. They observed that the tannins derived from *Leucaena* were insufficient to protect the protein from degradation, as evidenced by the similarity in the concentration of ruminal ammonia and BCVFA.

Table 4 Rumen fermentation parameters of lambs fed on different supplement treatments

Variables	**Supplement treatments**[1]			**S.E.M.**	**P values**
	UR	**Mb**	**MbL**		
pH	6.7	6.8	6.8	0.08	0.2523
NH_3-N (mg/100ml)	22.3	24.0	25.6	1.43	0.1111
Total VFA (mM)	101.6^{b}	103.0^{a}	102.9^{a}	0.0295	0.0001
Molar proportion (mol/100mol)					
Acetic	56.4^{b}	66.9^{a}	66.7^{a}	1.47	0.0001
Propionic	35.5^{a}	22.7^{b}	23.2^{b}	1.51	0.0001
Iso-butyric	0.58	0.80	0.71	0.13	0.1328
Butyric	5.80^{b}	7.46^{a}	7.35^{a}	0.86	0.0026
Iso-valeric	0.58	0.79	0.81	0.13	0.1110
Valeric	0.96	1.36	1.15	0.32	0.0597
Acetic/propionic	1.68^{b}	3.05^{a}	2.94^{a}	0.167	0.0001
CH_4 production (mol/100mol)	18.0^{b}	26.9^{a}	26.6^{a}	0.760	0.0002

Means with different superscript in the same row are significantly different ($P < 0.05$), S.E.M.: standard error mean
[1] UR: urea and rice bran supplements, Mb: Mulberry supplementation, MbL: Mulberry and *Leucaena* mixture supplementation

The Mb and MbL diets showed the same effect in increasing total VFA (103.0–102.9 mM) and were higher ($P < 0.05$) than the UR diet (101.6 mM) (Table 4). Concentration of VFA is an indication of the efficiency of feed fermentation in the rumen. Fermentation of the Mb and MbL diets is better than UR as revealed by the high concentration of VFA in the former two diets. The higher feed fermentation of the Mb and MbL diets, each comprising 30% mulberry or mulberry–*Leucaena* mixture, provide highly fermentable structural carbohydrates from mulberry. This helps sustain nutrient supply in the rumen (Doran *et al*., 2007). Similar results were reported by Syahrir *et al*. (2012), which showed that higher VFA production was achieved when mulberry replaced 50% of concentrate feed. Ouyang *et al*. (2019), on the other hand, did not find any difference in total VFA production when mulberry leaf meal was included in the concentrate up to 60%. The present study and the studies by Syahrir *et al*. (2012) and Ouyang *et al*. (2019) indicated that mulberry and *Leucaena* are suitable sources of energy and protein for ruminants. The tannins of *Leucaena* in the MbL diet did not affect fibre fermentation in the rumen, as indicated by similar total VFA in diets with Mb. Aguere *et al*. (2016) reported that supplementation with hydrolysable tannin and CT in a total mixed ration did not affect total VFA production.

The proportion of acetate and the ratio of acetic to propionic acid in the MbL diet was similar ($P > 0.05$) to the Mb diet but was higher ($P < 0.05$) than the UR diet (Table 5). The higher proportion of acetic acid in Mb and MbL was related to the higher digestibility of NDF of these diets (Table 2). Energy source in the form of structural carbohydrate produces acetate when it is fermented in the rumen (Firkins *et al*., 2006). A higher acetate content in Mb and MbL diets indicates that a mulberry and mulberry–*Leucaena* mixture, which contains a higher fibre content, can be used as alternative energy and protein sources. Henke *et al*. (2016) reported that Quebracho tannin extract (QTE) supplementation decreased fibre degradation, resulting in a lower proportion of acetate and a decreasing acetic-to-propionic ratio. This study indicated that tannin concentrations in *Leucaena* in the MbL diet did not affect fibre digestion.

The Mb and MbL diet treatments had estimated CH_4 production of 26.9 and 26.6 mol/100mol, respectively, higher ($P < 0.05$) than the UR diet (18.0 mol/100mol) (Table 4). This is due to their higher acetate concentration and the lower A:P ratio. The partial concentration of VFA affects CH_4 production in which CH_4 emissions decrease at lower concentration of acetate and higher concentration of propionate (Monteny *et al*., 2006). The diet with a forage-based diet increased CH_4 emission (Wallace *et al*., 2014) due to fibrolysis providing H_2 as a substrate for methanogenesis in forming acetate from pyruvate (Moss *et al*., 2000). In the rumen, methanogens use H_2 to reduce CO_2 to CH_4 (Moss *et al*., 2000). Production of VFA with a lower acetate: propionate ratio decreases the availability of H_2 in the rumen, which in turn reduces CH_4 formation (van Nevel & Demeyer, 1996). Therefore, the reduction in fibre digestibility of the diet reduced CH_4 production (Min *et al*., 2020). Methane production can be reduced using tannins to lower fibre digestibility and the size of the methanogen population (Min *et al*., 2014; Christensen *et al*., 2017). In the present study, it appears that *Leucaena* tannins were unable to reduce fibre degradation in the rumen, thereby CH_4 production did not decrease. However, Giang *et al*. (2016) reported that supplementation of *Leucaena* silage at 30 and 60% to the rice straw basal diet increased protein content and feed fermentation to reduce methane emission. The reduction in methane attributed to the *Leucaena* supplement is likely a result of the different forms in which *Leucaena* was provided. In the current study, *Leucaena* foliage hay was used, while previous studies have offered *Leucaena* in the form of silage (Giang *et al*., 2016). Furthermore, the application of tannins in an extract form obtained from *Acasia mernsii* at 3% DM or 2% DM reduced methane emissions (Deninger *et al*., 2020). In the current study, the tannin supply from *Leucaena* was approximately 1.9%, which might have been insufficient to reduce methane production in the MbL diet.

Table 5 Excretion of urinary purine derivatives (PD) (mM/d) and estimated daily microbial N supply in lambs fed on different supplement treatments

Variables[2]	Supplement treatments[1]			S.E.M.	P values
	UR	Mb	MbL		
PD excretion (mM/d)					
Allantoin	8.66	9.65	8.84	1.144	0.3600
Uric acid	1.018	2.08	1.35	0.739	0.2450
Hypoxanthine and xanthine	0.86	1.02	1.47	0.21	0.1108
Total	10.46	12.75	11.63	1.57	0.6899
PD excretion mM/W^{075}/d	1.71	1.32	1.27	0.15	0.7199
Proportion of PD excretion					
Allantoin	0.82	0.80	0.77	0.018	0.1437
Uric acid	0.10	0.11	0.11	0.012	0.8339
Hypoxanthine and xanthine	0.08^b	0.08^b	0.12^a	0.011	0.013
DOMI (kg/d)	0.31	0.36	0.34	0.024	0.3601
DOMR (kg/d)	0.20	0.23	0.22	0.016	0.3601
MNS (g N/d)	9.61	10.43	10.1	1.37	0.6878
EMNS (g N/kg DOMR)	44.8	44.8	44.5	5.09	0.9576

Means with different superscript in the same row are significantly different (P <0.05); S.E.M.: standard error mean
[1]UR: urea and rice bran supplements, Mb: Mulberry supplementation, MbL: Mulberry and *Leucaena* mixture supplementation, [2]PD: purine derivative, DOMI: digestible organic matter intake, DOMR: digestible organic matter fermented in the rumen, MNS: microbial nitrogen supply, EMNS: efficiency microbial nitrogen supply

The effects of diet treatments on the excretion of urinary PD and the estimated microbial nitrogen supply (MNS) were similar (P >0.05) (Table 5). Total PD excretion ranged from 10.46 to 12.75 mM/day with the highest value in the Mb and the lowest in the UR diet. A similar trend was observed for MNS with the value ranging from 9.61–10.43 gN/day. The similarity of PD excretion in all treatments indicated that the urea rice bran or mulberry or mulberry and *Leucaena* mixture supplement provided similar nutrient availability for synthesizing ruminal microbial crude protein (MCP). According to Henke *et al*. (2016), PD excretion indicates MCP synthesis. On the contrary, Henke *et al*. (2016) reported that 1.5% quebracho tannin extract (QTE) supplementation reduced PD excretion. Ahnert *et al*. (2015) also reported a linear decrease in PD excretion with increasing levels (from 1 to 6%) of QTE infused into the rumen. Supplementation with QTE caused reduction in total tract digestibility of CP and carbohydrate can reduce substrate availability for MCP synthesis (Ahnert *et al*., 2015). The balance between protein degradation rate and NH_3-N assimilation for rumen bacterial synthesis was reflected in the ruminal NH_3-N concentration (Apajalathi *et al*., 2019). Comparable microbial nitrogen synthesis (MNS) across all diets (Table 5) was attributed to the similarity between digestible organic matter intake (DOMI) and NH_3-N concentration in our study. Consequently, the three diet treatments provided an equal supply of microbial nitrogen (MNS), which supplied the same amino acids (AA) to the lambs, resulting in a similar average daily gain (ADG) (Table 6). Based on the finding in the similarity of three diet treatments in producing lambs ADG, it is suggested that the mulberry or mulberry–*Leucaena* mixture is a potential alternative to replace up to 76% of rice bran and 44% of urea in the rice straw basal diet.

The effects of different diet treatments on average daily gain (ADG) and feed conversion ratio (FCR) were statistically similar (P >0.05) (Table 6). The average daily gain (ADG) of diet treatments ranged from 69.6 to 73.2 g/head, with an overall average of 71.4 g/day. Additionally, the FCR of urea rice bran, mulberry, and mulberry–*Leucaena* mixture supplements was 10.02, 11.29, and 10.74, respectively. These results indicate that the tannin content in the diet did not have a marked impact on the daily body weight gain. It seems that the tannin level used in this study (1.9%) was slightly below the optimal range for sheep (2–3%) (Min & Solaiman, 2018), explains the lack of effect on ADG in lambs. Similar ADG (Table 6) and total dry matter intake (DMI) (Table 2) among the three diet treatments indicate an efficient feed conversion ratio (FCR) (Table 6), suggesting a similar efficiency in the utilization of feed by lambs.

Table 6 Average body weight and daily weight gain in lambs fed on different supplement treatments

Variables	**Supplement treatments**[1]			**S.E.M.**	**P values**
	UR	**Mb**	**MbL**		
Initial body weight (kg)	14.8	13.9	14.5	0.371	0.8747
Final body weight (kg)	18.9	17.8	18.5	0.523	0.8742
Average daily gain (g/d)	73.2	69.6	71.4	8.626	0.9858
FCR (Feed conversion ratio)	9.31	10.87	10.33	1.1321	0.6801

[1] UR: urea and rice bran supplements, Mb: Mulberry supplementation, MbL: Mulberry and *Leucaena* mixture supplementation, S.E.M.: standard error mean

The positive and similar ADG between Mb and MbL supplements indicate that both diets were able to supply glucose and N and resulted in similar MCP synthesis and VFA production that meet the lamb requirements for growth. Ruminants required glucose, nitrogen (N) to ensure a sufficient supply of microbial protein synthesis, VFA production to meet the requirement for the maintenance, and production of the animal (Min *et al*., 2020). The ADG of lamb fed the Mb and MbL diets was 69.6 and 71.4 g/head/day, respectively, which are statistically comparable to the control diet (UR, 73.2 g/head/day). The implication of this study is that mulberry or mulberry–*Leucaena* mixture offered an alternative source of feed supplementation, as a protein and energy source, to increase lamb growth. The ADG in the present study is similar to the result reported by Worknesh & Getachew (2017), namely 69.2 g/day, which utilized a Rhodes grass hay basal diet supplemented with 40% *Leucaena* hay foliage. Asaolu *et al*., (2012) reported a lower ADG (15.5 g/h/day) than the current study when West African Dwarf goats were fed an air-dried *Leucaena* supplementation at 40% to the cassava peel basal diet. The lower ADG (47.2 g/head/day) was also reported by Yadete (2014) when *Leucaena* foliage hay supplementation at 33% to urea treated straw basal diets. The results of previous studies and the present study showed that the ADG of sheep attributed by *Leucaena* supplementation to basal diets varies depending on the animal species, breed, diet composition, and levels of supplementation.

The diet used in the present study was formulated to meet the nitrogen and energy requirement for a growth rate of 100 g/day for lambs, as recommended by Kearl (1982). However, the digestible crude protein (DCP) intake was only 8.1 g/kg $BW^{0.75}$/day, which was lower than the recommended intake (10.1 g/kg $BW^{0.75}$/day), whereas the ME intake (8.5 MJ/kg) was higher than the recommended intake (5.9 MJ/kg) (Kearl, 1982). The lower intake of DCP could be due to the selective feeding of lambs since the diet was offered in the loose form (non-pelleted) consisting of a mixture of basal urea-treated rice straw and supplements. Therefore, the lambs select the preferred materials from the diet. Although protein consumption was lower than the recommendation (Kearl,1982), protein consumption of the diets in the current study was not the limiting factor for microbial growth. Evidence is shown by the high efficiency microbial protein supply (EMPS) (Table 6) of all diets. However, previous studies suggested that digestible rumen undegradable protein (RUP) supplementation is needed when the fermentation condition of the rumen has been optimized (Klopfenstein, 1996; Leng, 1997; Henke *et al*., 2016). The digestible RUP is required for the synthesis of tissue protein. The present study showed that the concentration of ammonia in the rumen (Table 4) was high, exceeding the acceptable level for optimal fibre digestion, suggesting that the protein content of the diets was degraded in the rumen, resulting in a limited protein feed that passes through the intestine. The lack of digestible RUP probably caused the lambs' lower body weight gain. In the present study, an attempt was made to reduce protein degradation in rumen by mixing mulberry with *Leucaena* as a supplement in urea treated rice straw basal diet to supply digestible RUP and promote the growth rate of lambs.

Conclusions

Mulberry and mulberry–*Leucaena* mixture substitution of urea rice bran both have a similar effect on nutrient digestibility, N utilization, rumen fermentation characteristics, microbial protein synthesis and body weight gain of lambs fed on urea-treated rice straw basal diet. Mulberry supplement at 30% or 30% mulberry–*Leucaena* mixture in a 1:1 ratio in the total diet provides fermentable energy and protein sources in the rice straw basal diet to increase lamb growth. The supplement of the mulberry or mulberry–*Leucaena* mixture resulted in increased lamb body weight. Mirroring the impact of supplementation with rice bran and urea. This suggests the potential of mulberry or mulberry–*Leucaena* mixture to replace rice bran and urea up to 76% and 44%, respectively, in the rice straw basal diet. The mulberry–*Leucaena* mixture supplement can reduce the mulberry rate from 30% to 15% and compensate for it with 15% *Leucaena* in the diet to supply tannins in the diet, appears insufficient to reduce protein degradation in the rumen. Therefore, it is suggested to increase the proportion of *Leucaena* composition in formulating the diet of lambs for future research.

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Authors' contributions
DY designed the experiment, collected the data, conducted the statistical analyses, interpretation of the results, and wrote the initial draft of this manuscript; ZAJ developed the original hypotheses, designed the experiments, interpreting the results, and finalized the manuscript. JBL designed the experiments, interpreting the results, and finalized the manuscript. HY read, edited, and approved the final manuscript, AN contributed interpretation of the results, and finalized the manuscript, FS contributed data analysis, and interpreted the results.

Conflict of interest declaration
The authors have no conflicts of interest to declare.
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