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Nutritional compositions of Katuk leaves and their supplementation to hays of different quality: an *in vitro* study

R.R. Nurdianti[1,2,3], R. S. Nuryana[2], A. Handoko[2], I. Hernaman[2], D. Ramdani[2,3], A. Jayanegara[3,4], U. Dickhoefer[1,5], C. Böttger[6] and K.-H. Südekum[6]

[1]Institute of Agricultural Sciences in the Tropics (Hans-Ruthenberg-Institute), University of Hohenheim, 70599 Stuttgart, Germany; [2]Faculty of Animal Husbandry, University of Padjadjaran, 45363 Sumedang, Indonesia; [3]Faculty of Animal Science, Animal Feed and Nutrition Modelling (AFENUE) Research Group, IPB University, 16680 Bogor, Indonesia; [4]Department of Nutrition and Feed Technology, IPB University, 16680 Bogor, Indonesia; [5]Institute of Animal Nutrition and Physiology, Kiel University, 24098 Kiel, Germany and [6]Institute of Animal Science, University of Bonn, 53115 Bonn, Germany.

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Corresponding author:
R.R. Nurdianti; Email: risma_rizkia.nurdianti@uni-hohenheim.de

Abstract

Katuk leaves (*Sauropus androgynus* (L.) Merr.; KL) are widely consumed by breast-feeding Indonesian mothers as it has been reported to increase breast milk production. It is hypothesized that supplementing KL in diets might increase crude protein (CP) concentration and fibre digestibility in the diet. The KL had high CP and non-fibre carbohydrate concentrations (333 and 332 g/kg dry matter; DM, respectively), but low neutral detergent fibre assayed with heat, a stable amylase and expressed exclusive of residual ash (aNDFom; 200 g/kg DM). Fibre digestibility linearly increased with increasing of KL supplementation in low-quality hay (LQH) diets. The KL did not contain a considerable amount of tannins. In LQH diets, gas production after 24 h incubation (GP_{24}) linearly increased with increasing of KL supplementation ($P < 0.001$). Meanwhile, GP_{24} linearly decreased with increasing of KL supplementation in medium- and high-quality hays (MQH and HQH; $P < 0.001$). Metabolizable energy tended to linearly increase in LQH diets, but tended to linearly decrease with increasing of KL supplementation in MQH and HQH diets ($P = 0.078$). Therefore, this study suggested that KL can be a potential supplement in the ruminant diet due to its abundant dietary proteins but low fibre concentration in its leaves. However, further studies (e.g. *in vitro* or *in vivo*) investigating other rumen parameters after incubation should be performed to validate how KL can be supplemented in the diet of ruminant livestock.

Introduction

Dairy cattle in developing countries often produce less milk and have shorter lactation periods than cattle in other regions. Poor animal performance in small-scale dairy systems in developing countries is the result of factors such as climate (high ambient temperature and humidity), low-quality feed, low levels of concentrate supplementation, low genetic potential for milk production of multi-purpose animals (in addition to milk and meat these cattle also often provide draught power) and high incidence of diseases (Food and Agriculture Organization, 2014). In tropical and sub-tropical developing countries, there is a gap between available and required animal feeds. Typically, ruminants in those countries are fed lignified forages and crop residues that are low in available energy and nitrogen (N; Nasser *et al.*, 2009).

Sauropus androgynus (L.) Merr. (Katuk) is a perennial shrub found growing wild in South East Asia and widely cultivated in Indonesia and Malaysia (Padmavathi and Rao, 1990). Katuk, a plant species of the Euphorbiaceae family, is rich in fatty acids, flavonoids and polyphenols as the main bioactive constituents (Zhang *et al.*, 2020) and is commonly utilized as a medicinal herb in the treatment of diabetes, cancer, inflammation, microbial infection, ulcers, obesity and allergies (Paul and Beena Anto, 2011; Zhang *et al.*, 2020). However, it is not recommended to consume excessive amount of freshly uncooked Katuk leaves (KL) over a period of time, because it could be associated with the occurrence of bronchiolitis obliterans disease (Zhang *et al.*, 2020). Consumption of extract of KL is considered to increase milk production in human mothers during lactation up to 50.7 g/100 g (Sa'roni *et al.*, 2012). Supplementing KL in the diet might also increase the milk yield of ruminants. In addition, Noach *et al.* (2020) reported that KL inclusion (with or without Zn bio complex) in a concentrate diet of late pregnant Ettawah could improve the milk production and birth weight of kid during kidding period.

Evaluating the nutritive value of the available local feed resources is important to assess their potential role in the nutrition of livestock (Taghizadeh *et al*., 2008) which can influence the livestock productivity (Geremew *et al*., 2017). For this, *in vitro* techniques are commonly used to screen a variety of feedstuffs that are not available in sufficient quantity for *in vivo* experiments (Getachew *et al*., 2002). Menke and Steingass (1988) reported a strong correlation between metabolizable energy (ME) values measured *in vivo* and predicted from 24 h *in vitro* gas production (GP) and chemical composition of feeds. *In vitro* GP is an alternative technique used to determine the nutritive value of feedstuffs, since rate and extent of degradation and rumen fermentation can be simply determined by measurement of cumulative GP (Sommart *et al*., 2000). *In vitro* GP technique is less expensive, easy to determine and appropriate for use in developing countries (Salamatazar *et al*., 2012). Fibre digestibility can also be determined using a modified *in vitro* technique (Tilley and Terry, 1963; Robertson and Van Soest, 1981). With respect to KL as animal feed supplement, limited information is available on their nutritive value and ruminal degradation. Therefore, the present study aimed at testing a hypothesis that the nutritive value of KL and their supplementation to hays of different quality could improve the crude protein (CP) concentration, fibre digestibility and ME, especially in low-quality hay diet. The preliminary findings of this study were published in abstract form (Nurdianti *et al*., 2017).

Materials and methods

Sample collection and experimental design

Approximately 0.5 kg of fresh leaves of KL without stem were harvested for the sample collection. Samples of KL with old maturity were collected from 80–90 cm herbaceous-shrub plant in Bandung, Indonesia. In Bandung, annual precipitation ranges from 2500 to 3000 mm and temperature ranges from 24 to 28 °C (BMKG, 2013).

After collection, KL samples were stored at 4 °C overnight, oven dried at 60 °C for 24 h (F115, Binder GmbH, Tuttlingen, Germany), and ground to pass a 1 mm sieve using a laboratory mill (model SM100, Retsch GmbH, Haan, Germany). As a substrate for KL to be added to, three grass hays from temperate regions (grown in Germany and Switzerland) were selected based on their CP concentration to represent a wide range of qualities: hay with low quality (LQH), hay with medium quality (MQH) and hay with high quality (HQH). Hays were ground to pass a 1 mm sieve using the same mill as for KL. Samples of KL and each grass hay were incubated alone or in KL + grass hay combinations with four different inclusion levels of KL (i.e. 0, 50, 100 and 200 g/kg dry matter (DM)). The cows used as donor animals were housed in accordance with the German Animal Welfare legislation. All procedures for animal handling within the present study were performed according to the National Committee for the Protection of Animals Used for Scientific Purposes for the Federal Republic of Germany. The experimental diets were arranged in a completely randomized design in six replicates. The feedstuff combinations, i.e. diets, are presented in Table 1.

Chemical analyses

Chemical composition of the individual sample was determined according to the Association of German Agricultural Analytic and Research Institutes (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, 2007). The DM concentration was determined by oven-drying at 105 °C for 24 h (method 3.1) and ash concentration was determined by incinerating samples at 550 °C for 5 h (method 8.1). The N concentration was determined by Kjeldahl procedure (method 4.1.2) and CP was calculated as N×6.25. Ether extract (EE) concentration was analysed using method 5.1.1. Neutral detergent fibre, assayed with a heat-stable amylase and expressed exclusive of residual ash (aNDFom), acid detergent fibre, expressed exclusive of residual ash (ADFom) and lignin determined by solubilization of cellulose with sulphuric acid (Lignin (sa)) concentrations were measured using an ANKOM200 Fibre Analyzer (Ankom Technology Corp., Macedon, NY, USA) according to methods 6.5.1, 6.5.2 and 6.5.3, respectively. Sodium sulphite was used for the aNDFom analysis. Concentration of hemicelluloses was determined by subtracting ADFom concentration from aNDFom concentration and concentration of cellulose by subtracting Lignin (sa) concentration from ADFom concentration (Rinne *et al*., 1997). All samples were analysed in duplicate for each chemical constituent and analysis repeated, if the coefficient of variation exceeded 5%.

Non-fibre carbohydrates (NFC) were calculated according to National Research Council (2001), as:

$$\mathrm{NFC} = 1000 - (\mathrm{aNDFom} + \mathrm{CP} + \mathrm{EE} + \mathrm{ash}) \quad (1)$$

where all nutrient concentrations are in g/kg DM.

Tannin bioassay

To investigate whether the KL sample contained tannins or not, an indirect approach was selected. Katuk leaves were evaluated using polyethylene glycol (PEG) supplementation in an *in vitro* GP experiment as mentioned by Jayanegara *et al*. (2009). The PEG has a high affinity for tannins and makes tannins inactive by binding to them. Hence, KL samples (220–250 mg) were incubated without and with 750 mg PEG supplementation with 10 ml of rumen fluid and 20 ml of buffer solution (Menke *et al*., 1979) using Hohenheim gas test (HGT; Makkar *et al*., 1995).

The syringes were placed in a rotor inside the incubator (39 °C) with about one rotation per minute. The GP was recorded after 48 h of incubation (GP_{48}). Blanks were prepared by incubating syringes containing rumen fluid and buffer solution, but without any sample. Katuk leave samples were incubated in triplicate in two different *in vitro* incubation runs. The difference between the *in vitro* GP with and without PEG supplementation is an indicator of tannin effect. The increase in gas on addition of PEG is a measure of tannin activity, where the protocol used was similar to that described in Makkar *et al*. (1995).

Fibre fractionation and in vitro fibre digestibility

The fibre fractionation and *in vitro* fibre digestibility experiment were evaluated using the modified Tilley and Terry technique (Tilley and Terry, 1963; Robertson and Van Soest, 1981). For this, rumen fluid was collected from two rumen-fistulated Jersey cows, fed on a total mixed ration containing grass silage (180 g/kg DM), corn silage (167 g/kg DM), grass hay (184 g/kg DM), barley straw (21 g/kg DM), and concentrate mixture (426 g/kg DM), urea (2 g/kg DM) and mineral mixture (20 g/kg DM). The total mixed ration had a forage to concentrate ratio of 55:45 (on DM basis) and contained 148 g CP/kg DM. Rumen fluid was collected immediately before morning feeding and

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Table 1. Ingredient composition and calculated chemical composition ($n = 2$) of the experimental diets

	Forage												
	Low-quality hay				Medium-quality hay				High-quality hay				
	Katuk leaves (g/kg DM)												
Level	0	50	100	200	0	50	100	200	0	50	100	200	1000
Ingredient composition of diets (g/kg DM)													
Katuk leaves	0	50	100	200	0	50	100	200	0	50	100	200	1000
Low-quality grass hay	1000	950	900	800	0	0	0	0	0	0	0	0	0
Medium-quality grass hay	0	0	0	0	1000	950	900	800	0	0	0	0	0
High-quality grass hay	0	0	0	0	0	0	0	0	1000	950	900	800	0
Chemical composition of the diets (g/kg DM)													
Dry matter (g/kg FM)	928	927	926	925	922	921	921	920	907	907	907	908	911
Ash	56	59	62	67	86	87	88	91	102	102	103	104	111
Crude protein	45	60	74	103	89	101	113	137	147	156	166	184	333
Ether extract	3.6	4.7	5.7	7.8	13	13.6	14.2	15.3	30	29.7	29.5	28.9	24.5
aNDFom	700	675	650	600	550	533	515	480	512	496	481	450	200
ADFom	450	437	424	398	301	296	290	279	280	276	271	262	192
Lignin (sa)	33.9	32.8	31.7	29.5	31.9	30.9	29.9	27.9	31.8	30.8	29.8	27.8	11.9
Hemicelluloses[a]	250	238	226	202	249	237	225	201	232	221	210	187	8.0
Cellulose[a]	416	404	393	369	269	265	260	251	248	245	241	235	180
NFC[a]	195	202	208	222	263	266	270	276	209	215	221	234	332

ADFom, acid detergent fibre expressed exclusive of residual ash; aNDFom, neutral detergent fibre assayed with a heat-stable amylase and expressed exclusive of residual ash; DM, dry matter; FM, fresh matter; Lignin (sa), lignin determined by solubilization of cellulose with sulphuric acid; NFC, non-fibrous carbohydrate.
[a]Cellulose = ADFom – Lignin (sa); hemicellulose = aNDFom – ADFom; NFC = 1000 – (aNDFom + crude protein + ether extract + ash).

strained through two layers of cheesecloth into pre-warmed and isolated bottle. All laboratory handling of rumen fluid was carried out under a continuous flow of CO_2. The rumen fluid of both cows was mixed, homogenized and filtered using a nylon net (pore size 100 μm).

The ground initial sample (500 mg) was weighed into a 250 ml flask (Goering and Van Soest, 1970). The rumen fluid (10 ml) and the buffer solution (40 ml) were added to each flask. The flasks were then placed in a preheated (39 °C) water bath under CO_2 positive pressure to ensure anaerobiosis, and incubated for 240 h, which corresponds to the maximum extent of fibre digestion in an anaerobic environment *in vitro* (Fox *et al.*, 2004; Raffrenato and Van Amburgh, 2010; Raffrenato *et al.*, 2018). Re-inoculation of the flasks with 10 ml rumen fluid and 40 ml buffer solution was conducted after 120 h of incubation to preserve the microbial activity during the whole incubation process (Palmonari *et al.*, 2014). Blanks were prepared by incubating flasks containing buffer and rumen fluid but without any sample to correct for any feed particles introduced into the *in vitro* system with the rumen fluid (Raffrenato *et al.*, 2018). Each sample was incubated in triplicate in two runs resulting in six observations per experimental diet.

At the end of the incubation, the whole content of each flask was moved to a 600 ml beaker that was covered by a round cold-water condenser to minimize evaporation (Mertens, 2002) and determine the aNDFom concentration of the residue (Goering and Van Soest, 1970). About 0.5 g of sodium sulphite and 50 ml of neutral detergent solution were added to each refluxing beaker and refluxed for 60 min at boiling temperature to create vigorous particle movement. After refluxing, the content of each beaker was filtered through crucibles (40 μm porosity, Duran™ Borosilicate Glass Filter Crucibles number 2, DWK Life Sciences, Wertheim, Germany) and the water removed with a vacuum pump. Filtered residues were dried in a forced-air oven (105 °C) for 3 h, and the weights of the crucibles were recorded. Ash correction was done by incineration of the residue at 550 °C for 4 h.

The *in vitro* neutral detergent fibre digestibility (IVNDFD of the incubated samples after 240 h; $IVNDFD_{240}$) was calculated as

$$IVNDFD_{240} = \frac{aNDFom_r - aNDFom_b}{aNDFom_i} \quad (2)$$

where $aNDFom_r$ is the residual aNDFom after 240 h *in vitro* fermentation (g/kg DM), $aNDFom_b$ is the blank correction after 240 h *in vitro* fermentation (g/kg DM) and $aNDFom_i$ represents the initial aNDFom concentration from samples (g/kg DM).

The uNDF concentration (g/kg DM) after 240 h *in vitro* fermentation ($uNDF_{240}$) was calculated as

$$uNDF_{240}\,(g/kg\ DM) = \frac{(1000 - IVNDFD_{240})\ x\ aNDFom_I}{1000} \quad (3)$$

where $aNDFom_i$ (g/kg DM) is the aNDFom concentration of the sample (g/kg DM) and $IVNDFD_{240}$ is *in vitro* fibre digestibility (IVNDFD) of the incubated samples after 240 h.

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The pdNDF concentration (g/kg DM) was calculated as

$$\text{pdNDF (g/kg DM)} = \text{aNDFom}_i - \text{uNDF}_{240} \quad (4)$$

where aNDFom_i (g/kg DM) is the aNDFom concentration of the sample (g/kg DM) and uNDF_{240} is the uNDF in the residue after 240 h *in vitro* fermentation (g/kg DM).

In vitro *gas production, metabolizable energy and net energy for lactation*

The HGT was performed according to Menke and Steingass (1988) but modified regarding the incubation duration. Rumen fluid was collected from two castrated male adult Blackface sheep, fed on a standard diet of grass hay, a commercial compound feed and barley (650, 200 and 150 g/kg DM, respectively) that covered maintenance energy requirements. The animals never received tanniferous and/or tropical feeds before. Rumen fluid was collected immediately before feeding and strained through two layers of cheesecloth into pre-warmed and isolated bottle. All laboratory handling of rumen fluid was carried out under a continuous flow of CO_2.

The ground samples (220–250 mg) of the air-dried feedstuffs and the respective mixtures were accurately weighed into 100 ml glass syringes and the syringe pistons were lubricated with Vaseline and inserted into the syringes. Triplicates of syringes without substrate (blanks) and of standard hay and concentrate were included as laboratory controls. According to Menke and Steingass (1988), GP from the blank was subtracted from all samples incubated to obtain the net GP. Subsequently, GP from the hay standard was divided by the measured net value of the hay standard to provide the correction factor. Similarly, GP from the concentrate standard was divided by the measured net GP of the concentrate standard. The average value of correction factor and concentrate standard was used for the adjustment. Each sample was incubated in triplicate in two different *in vitro* incubation runs. Incubations were repeated when gas volumes of the standards deviated by more than 10% and when coefficient of variation between repetitions exceeded 5% from the reference values.

Syringes were filled with 30 ml of medium consisting of rumen fluid (10 ml) and 20 buffer solution (20 ml) as described by Menke and Steingass (1988), except that the concentration of $NaHCO_3$ was reduced to 33 g/l and that of $(NH_4)HCO_3$ increased to 6 g/l to prevent a shortage in N during prolonged incubation times. The syringes were placed in a rotor inside the incubator (39 °C) with about one rotation per minute. The GP was recorded after 2, 4, 6, 8, 12, 16, 24, 36, 48, 60, 72 and 96 h of incubation. The ME and net energy for lactation (NEL) values were calculated according to Menke and Steingass (1988) as:

$$\text{ME} = 2.20 + 0.1357\ \text{GP} + 0.0057\ \text{CP} + 0.0002859\ \text{EE}^2 \quad (5)$$

$$\text{NEL} = 0.54 + 0.0959\ \text{GP} + 0.0038\ \text{CP} + 0.0001733\ \text{EE}^2 \quad (6)$$

where GP is the net GP from 200 mg dry sample after 24 h of incubation (GP_{24}) and after being corrected from its correction factor for the day-to-day variation in the activity of rumen fluid (ml) is expressed in ml/200 mg DM, and ash, CP and EE concentrations are expressed as g/kg DM.

Data analyses

To describe the dynamics of GP over time, the following Gompertz function (Schofield *et al.*, 1994) was chosen:

$$\text{GP} = A \exp\left\{-\exp\left[1 + \frac{b}{A}(\text{LAG} - t)\right]\right\} \quad (7)$$

where A is the theoretical maximum of GP, b is the maximum rate of GP (ml/h) that occurs at the point of inflection of the curve, LAG is the lag time (h) which is defined as the time-axis intercept of a tangent line at the point of inflection, and t is time (h). The parameters A, b and LAG were estimated by non-linear regression analysis (PROC NLIN; SAS 9.4, SAS Institute Inc., Cary, North Carolina, USA).

The fibre fractions, *in vitro* fibre digestibility, GP (i.e. 12, 24, 48 and 96 h of incubation), ME and NEL values were analysed using a mixed procedure (PROC MIXED) by SAS 9.4 (SAS Institute Inc.) according to:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\,\beta)_{ij} + R_k + e_{ijk} \quad (8)$$

where Y_{ijk} = the dependent variable; μ = the overall mean; α_i = the effect of hays of different quality (i.e. LQH, MQH and HQH); β_j = the inclusion levels of KL (i.e. 0, 50, 100 and 200 g/kg DM); $(\alpha\ \beta)_{ij}$ = the interaction of grass hay with different quality and inclusion levels of KL; R_k = the random effect of run; and e_{ijk} = the residual error. Linear effects of level were tested by orthogonal polynomial contrasts using the CONTRAST statement. Differences between means with $P < 0.050$ were accepted as statistically significant, and differences with $0.050 < P < 0.100$ were considered to represent tendencies to significance.

Results

Chemical composition

The calculated chemical composition varied among experimental diets (Table 1). Initial sample of KL had higher CP and NFC concentrations than experimental diets. Meanwhile, LQH diet without KL supplementation had lower CP and NFC concentrations among experimental diets. Initial sample of KL had lower aNDFom, ADFom, Lignin (sa), hemicelluloses and cellulose concentrations compared to experimental diets. Meanwhile, LQH diet without KL supplementation had higher aNDFom, ADFom Lignin (sa), hemicelluloses and cellulose concentrations compared to other experimental diets.

Fibre fractions and in vitro *fibre digestibility*

The concentrations of undigested aNDFom after 240 h incubation (uNDF_{240}) and potentially digestible aNDFom (pdNDF; both in g/kg DM) as well as the fibre digestibility differed between the grass hays (i.e. LQH, MQH and HQH; P = <0.001 for all variables; Table 2). Moreover, the uNDF_{240} and pdNDF concentrations differed between levels of KL supplementation ($P = 0.001$ and $P < 0.001$, respectively). The HQH supplemented with 200 g/kg DM basis of KL had a much lower uNDF_{240} concentration than other experimental diets. Meanwhile, MQH diet without

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Table 2. Calculated fibre fractions, i.e. $uNDF_{240}$ and pdNDF (g/kg DM), and fibre digestibility, i.e. $IVNDFD_{240}$, of the experimental diets ($n = 3$)

Variable	Hay										*P* value[a]			
	Low-quality hay			Medium-quality hay			High-quality hay			SEM	*H*	*L*	*H*×*L*	CL
KL level (g/kg DM)	0	50	100	200	0	50	100	200	0	50	100	200					
$uNDF_{240}$ (g/kg DM)	267	256	245	223	85	83	81	78	82	80	78	75	6.666	<0.001	0.001	0.014	<0.001
pdNDF (g/kg DM)	433	419	405	377	464	449	433	402	429	416	402	376	6.666	<0.001	<0.001	0.984	<0.001
$IVNDFD_{240}$	0.618	0.621	0.623	0.628	0.844	0.835	0.826	0.809	0.839	0.830	0.822	0.805	0.0123	<0.001	0.026	0.083	0.004

aNDFom, neutral detergent fibre assayed with a heat-stable amylase and expressed exclusive of residual ash; DM, dry matter; $IVNDFD_{240}$, *in vitro* neutral detergent fibre digestibility of the incubated samples after 240 h; KL, Katuk leaves; pdNDF, potentially digestible neutral detergent fibre; SEM, standard error mean; $uNDF_{240}$, undigested neutral detergent fibre estimated after 240 h of *in vitro* incubation.
[a]Probability of the effects of *H*, hays of different quality; *L*, inclusion levels of Katuk leaves; *H*×*L*, interaction of hays of different quality and inclusion levels of Katuk leaves; CL, linear effect of inclusion levels of Katuk leaves.

supplementation of KL had highest pdNDF concentration and fibre digestibility among all experimental diets, i.e. MQH diet with KL supplementation, all LQH diet and all HQH diet. The interaction between grass hay with different quality and inclusion level of KL was significant for $uNDF_{240}$ concentration ($P = 0.014$), but tended to be significant for fibre digestibility ($P = 0.083$). In all diets, $uNDF_{240}$ and pdNDF concentrations linearly decreased with increasing level of KL in the diet ($P < 0.01$). Meanwhile, fibre digestibility linearly increased with increasing KL level in LQH diet, but not in MQH and HQH diets ($P = 0.04$).

Tannin bioassay

Addition of PEG to KL did not affect the cumulative GP_{24} (Fig. 1), averaging 36 ml/200 mg DM. Similarly, the GP_{48} of KL did not differ irrespective of whether it was incubated with PEG or without PEG.

In vitro *gas production, metabolizable energy and net energy for lactation*

The cumulative GP differed between experimental diets at all incubation times, i.e. 12 h, 24 h, 48 h and 96 h ($P < 0.001$, for all variables; Table 3). Mean total GP during 12 h of incubation (GP_{12}) ranged from 21.9 to 35.7 ml/200 mg DM. The interaction between grass hay with different quality and inclusion level of KL was significant for GP_{12} ($P = 0.007$). In all experimental diets, GP_{12} did not linearly increase with increasing of KL supplementation in the diet ($P = 0.410$).

Total volume of GP_{24} ranged from 33.3 to 49.6 ml/200 mg DM. The interaction between grass hay with different quality and inclusion level of KL was significant for GP_{24} ($P = 0.004$). In LQH diets, GP_{24} linearly increased with increasing of KL supplementation in the diet ($P < 0.001$). Meanwhile, GP_{24} linearly decreased with increasing of KL supplementation in MQH and HQH diets ($P < 0.001$).

Total volume of GP_{48} ranged from 42.6 to 57.6 ml/200 mg DM. There was no interaction between grass hay with different quality and inclusion level of KL for GP_{48} ($P = 0.101$). In all experimental diets, GP_{48} linearly decreased with increasing of KL supplementation in the diet ($P < 0.001$).

Total volume of gas produced during 96 h (GP_{96}) ranged from 48.4 to 62.2 ml/200 mg DM. There was no interaction between grass hay with different quality and inclusion level of KL for GP_{96} ($P = 0.410$). In all experimental diets, GP_{96} linearly decreased with increasing level of KL in the diet ($P < 0.001$).

The ME and NEL values of experimental diets varied widely (Table 4). The calculated ME and NEL values differed between experimental diets ($P < 0.001$, for all variables). The interaction between grass hay with different quality and inclusion level of KL affected ME and NEL values ($P < 0.01$, $P = 0.001$ and $P = 0.001$, respectively). In LQH diets, ME tended to linearly increase with increasing of KL supplementation in the diet ($P = 0.078$). Meanwhile, ME tended to linearly decrease with increasing of KL supplementation in MQH and HQH diets ($P < 0.01$). In all experimental diets, NEL was not observed to linearly increase or decrease with increasing of KL supplementation in the diet ($P = 0.161$).

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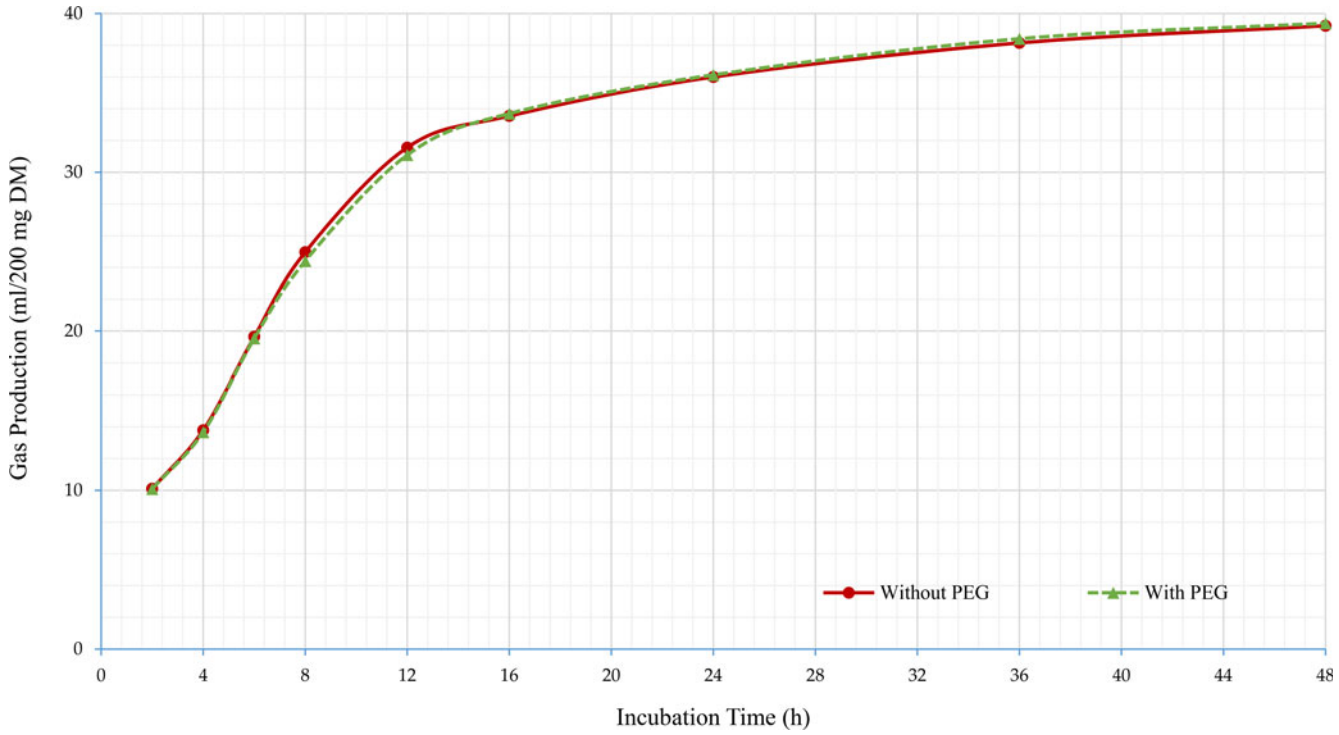

Figure 1. Effect of polyethylene glycol (PEG) treatment on the cumulative gas production (ml/200 mg dry matter; DM) during 48 h (h) of incubation of Katuk leaves.

Discussion

Chemical composition

According to the results of this study, KL had higher CP concentration (333 g/kg DM) than the three hays (i.e. LQH, MQH and HQH), which were grasses from temperate region. The CP concentration of KL was also greater than mean CP concentrations of tropical grasses and tropical forage legumes as determined in a previous study (79 and 198 g/kg DM, respectively; Nurdianti *et al*., 2019). Piliang and Djojosoebagio (1991) reported a CP concentration of KL of about 257 g/kg DM. Meanwhile, Yang and Guo (2002) found that CP concentration of KL grown in South China reached up to 485 g/kg DM. Moreover, a study from Naveena *et al.* (2020) has been conducted to evaluate the nutritional composition of basal whorl leaves and terminal whorl leaves of Katuk which reported that basal whorl leaves are more enriched with nutrients than terminal whorl leaves. The authors further mentioned that the CP concentration of KL increased with the age of the plant. The present research and some previous studies showed that CP concentrations of KL exceed the requirements of ruminants, for instance, of small ruminants for maintenance and growth (110–130 g/kg DM of CP concentration; National Research Council, 2007) and even of dairy cattle for maintenance and lactation (119 g/kg DM of CP concentration; National Research Council, 2001), which makes it valuable as high-protein supplement particularly to low-protein forage diets.

The aNDFom concentration of KL was lower than of the three hays (i.e. LQH, MQH and HQH) in the present study and those reported for tropical forage legumes or tropical grasses in the literature (374 and 592 g/kg DM, respectively; Nurdianti *et al*., 2019). Hence, supplementing KL would decrease the aNDFom concentration in the high-fibre diets. Villalba *et al.* (2021) mentioned that NFC is also important in the ruminant nutrition as it can offer energy adequately for an efficient microbial protein synthesis (Shabi *et al*., 1998). The present study reported that supplementation of KL in the diet can increase NFC concentration in all experimental diets. Batajoo and Shaver (1994) summarized that for cows producing over 40 kg of milk, the diet should contain NFC concentration about more than 300 g/kg DM, yet little benefit was reported if the diets contain 420 g/kg DM over 360 g/kg DM of NFC concentration. In previous study, Villalba *et al.* (2021) mentioned that the decreased dietary fibre and increased NFC concentration might lead to increased intake, increased meat and milk in ruminant production and decreased methane enteric emissions and carbon dioxide. The present study showed that KL inclusion can increase CP and NFC concentrations, while reducing aNDFom concentrations in ruminant diets based on forage grasses, and that it may thus be a valuable supplement.

Tannin bioassay

According to Makkar *et al.* (1995), PEG might bind to tannins and thereby reduce their anti-nutritive activity. In response to the deactivation of secondary compounds, nutrient fermentation might improve and *in vitro* GP might increase (Batajoo and Shaver, 1994; Nocek, 1997). The greater the increase in the GP, the higher the suppressive activity of tannin in the feeds (Jayanegara *et al*., 2009).

A study of Selvi and Bhaskar (2012) reported that the KL contain substances such as sterols, resins, tannins, saponins, alkaloids, flavonoids, terpenoids, cardiac glycosides, phenols and catechols. Previous studies reported that KL contain per 88.7 mg tannins (Singh *et al*., 2011) and 580 mg alkaloid papaverine (Bender and Ismail, 1975; Padmavathi and Rao, 1990) per 100 g DM of KL, as well as 11.5 mg gallic acid equivalents of total phenolics

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Table 3. Kinetics cumulative gas production of the experimental diets ($n = 3$)

Variable	Hay													*P* value[a]			
	Low-quality hay				Medium-quality hay				High-quality hay								
KL level (g/kg DM)	0	50	100	200	0	50	100	200	0	50	100	200	SEM	*H*	*L*	*H*×*L*	CL
Cumulative GP (ml/200 mg DM)																	
GP_{12}	21.9	22.7	23.2	24.1	35.7	34.8	34.8	34.7	34.1	33.6	33.2	33.3	0.664	<0.001	0.506	0.007	0.410
GP_{24}	33.4	33.3	33.8	34.0	49.6	48.2	47.7	46.4	46.0	45.3	44.6	43.7	0.875	<0.001	0.001	0.004	<0.001
GP_{48}	43.1	42.6	42.6	42.6	57.6	55.9	55.5	53.9	52.5	51.3	50.8	49.5	0.833	<0.001	<0.001	0.101	<0.001
GP_{96}	50.1	49.3	48.8	48.4	62.2	60.8	60.1	58.3	56.2	55.0	54.5	52.9	0.800	<0.001	<0.001	0.410	<0.001
Parameters of Gompertz function																	
A[a] (ml/200 mg DM)	48.3 (1.2)	47.5 (1.4)	46.9 (2.5)	46.4 (1.4)	59.8 (1.6)	58.2 (0.3)	57.6 (2.1)	56.0 (1.3)	54.0 (0.2)	52.7 (0.4)	52.3 (1.2)	50.7 (0.6)					
b[a] (ml/h)	1.15 (0.0)	1.14 (0.1)	1.19 (0.0)	1.22 (0.0)	2.20 (0.1)	2.16 (0.1)	2.14 (0.1)	2.12 (0.1)	2.24 (0.1)	2.26 (0.1)	2.17 (0.2)	2.17 (0.1)					
LAG[a] (h)	−6.10 (0.1)	−6.65 (0.6)	−6.38 (0.3)	−6.61 (0.2)	−3.77 (0.1)	−3.69 (0.2)	−3.87 (0.1)	−3.69 (0.2)	−2.89 (0.1)	−2.64 (0.2)	−2.98 (0.3)	−3.18 (0.0)					

DM, dry matter; GP, gas production; GP_{12}, gas production during 12 h of incubation; GP_{24}, gas production during 24 h of incubation; GP_{48}, gas production during 48 h of incubation; GP_{96}, gas production during 96 h of incubation; KL, Katuk leaves; SEM, standard error mean.

[a]*A*, theoretical maximum of gas production (ml/200 mg dry matter); *b*, maximum rate of gas production (ml/h) that occurs at the point of inflection of the curve; LAG, lag time (h), which is defined as the time-axis intercept of a tangent line at the point of inflection; *t*, time (h). Probability of the effects of *H*, hays of different quality; *L*, inclusion levels of Katuk leaves; *H*×*L*, interaction of hays of different quality and inclusion levels of Katuk leaves; CL, linear effect of inclusion levels of Katuk leaves.

Table 4. Calculated metabolizable energy (MJ/kg DM) and net energy for lactation concentrations of the experimental diets (MJ/kg DM)

Variable	Hay											P value[a]			
	Low-quality hay			Medium-quality hay			High-quality hay								
KL level (g/kg DM)	0	50	100	200	0	50	100	200	0	50	100	200	SEM	*H*	*L*	*H*×*L*	CL
ME (MJ/kg DM)	7.0	7.1	7.2	7.4	9.5	9.4	9.4	9.4	9.5	9.5	9.5	9.4	0.119	<0.001	0.207	0.001	0.078
NEL (MJ/kg DM)	3.9	4.0	4.1	4.2	5.7	5.6	5.6	5.6	5.7	5.6	5.6	5.6	0.084	<0.001	0.324	0.001	0.161

DM, dry matter; KL, Katuk leaves; ME, metabolizable energy; NEL, net energy for lactation; SEM, standard error mean.
[a]Probability of the effects of *H*, hays of different quality; *L*, inclusion levels of Katuk leaves; *H*×*L*, interaction of hays of different quality and inclusion levels of Katuk leaves; CL, linear effect of inclusion levels of Katuk leaves.

and 10.4 mg rutin equivalents of total flavonoids (Maisuthisakul *et al*., 2008) per gram db. However, in contrast to these results, the present study showed that PEG addition did not increase GP of KL indicating that KL did not contain a considerable amount of tannins.

Fibre fractions and in vitro *fibre digestibility*

Fibre digestibility is important in assessing forage quality (Ward, 2001). Fibre digestibility and pdNDF concentration can be calculated by subtracting the $uNDF_{240}$ concentration from total aNDFom concentration (Cotanch *et al*., 2014; Nurdianti *et al*., 2019). The $uNDF_{240}$ concentration is the functional fibre fraction that influences physical effectiveness, gut fill and digestion/passage dynamics of forages (Cotanch *et al*., 2014; Harper and McNeill, 2015). Fustini *et al*. (2017) reported that voluntary feed intake is influenced and improved by forage fibre digestibility and its pdNDF concentration (when it represents up to 500 g/kg DM basis of the ration).

The $uNDF_{240}$ concentration of KL in the present study was much lower than reported for tropical forage legumes and grasses (294 and 231 g/kg DM; Nurdianti *et al*., 2019). On the other hand, the pdNDF concentration of KL interestingly was much higher compared to tropical forage legumes and grasses (134 and 360 g/kg DM, respectively; Nurdianti *et al*., 2019), which shows that fibre of KL is more digestible than that of other tropical forages. Therefore, the inclusion of KL in diets with hays of different quality decreased the concentration of $uNDF_{240}$, but might increase the fibre digestibility of the diet.

In vitro *gas production, metabolizable energy and net energy for lactation*

In the exponential model, the GP rate depends on substrate availability for fermentation after a lag time has been reached (Ørskov and McDonald, 1979; McDonald, 1981). According to López *et al*. (2007), the lag time might be affected by some factors such as the nature of the feedstuff incubated, the microbial species inoculated and the quantity of inoculum added. In the present study, the Gompertz model was the most suitable curve shapes until the end of incubation. According to Lavrenčič *et al*. (1997), the Gompertz model assumes that the specific GP rate is proportional to microbial mass, which in turn depends on the concentration of digestible substrate. Moreover, the present study showed negative lag time which is similar to the finding of Jijai *et al*. (2016) which indicates that the accelerated growth of the initial anaerobic process in most batches was facilitated by favourable substrate conditions, resulting in a significant reduction in the time required to reach the exponential phase.

Related to gas volume and *in vitro* GP characteristics, Menke *et al*. (1979) suggested that GP_{24} has indirect relationship with organic matter digestibility and ME in feedstuffs due to the stochiometric relationships between organic matter degradation, short-chain fatty acid (SCFA) yield and GP. Therefore, ME and SCFA yield may be predicted from *in vitro* GP (Batajoo and Shaver, 1994). Yet, GP derived from protein fermentation is relatively small as compared to that from carbohydrate fermentation (Makkar, 2004). Hence, the high CP concentration of KL in the present study rather than a poor substrate degradability might have contributed to their low GP as well as the linear decline with increasing KL inclusion level in the grass hay diet.

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The estimation of the ME values can be used for the purpose of ration formulation and for other purposes in setting economic value of feeds (Getachew *et al.*, 2002). The ME value of KL was 9.52 MJ/kg DM which is good compared with typical values of feedstuffs commonly fed to cattle, such as alfalfa, barley silage, corn silage, cotton seed and sorghum silage (8.20, 8.49, 9.74 and 7.49 MJ/kg DM, respectively; National Research Council, 2001) which can give benefit economically when supplementing KL in the diet. Meanwhile, the NEL value of KL was 5.62 MJ/kg DM which is sufficient as compared to some feedstuffs commonly fed to cattle, such as alfalfa and barley grain (6.36 and 7.78 MJ/kg DM, respectively; National Research Council, 2001).

Conclusion

Katuk leaves have relatively high CP concentrations with low concentration of well digestible fibre. There was no indication that KL contained a considerable amount of tannins, hampering carbohydrate fermentation. In LQH diets, increasing KL supplementation linearly increases GP_{24} and calculated ME, with no or even negative effects in MQH and HQH diets. Hence, KL can be a potential supplement feed for ruminant livestock, in particular when fed in addition to LQH. However, further studies (e.g. *in vitro* or *in vivo*) investigating other rumen parameters after incubation are needed to validate the current observations.

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Ethical standards. The study was conducted according to the guidelines of Institute of Animal Science of the University of Bonn and the Institute of Agricultural Sciences in the Tropics (Hans-Ruthenberg Institute) of the University of Hohenheim. All procedures for animal handling within the present study were performed according to the National Committee for the Protection of Animals Used for Scientific Purposes for the Federal Republic of Germany.

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