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Sugar, Acid Soluble Polysaccharide, and Total Phenolic Contents in Tropical Legumes and Their Relationships with *In Vitro* Nutrient Fermentability

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ABSTRACT

Tropical legume is a type of C4 plant that has been adaptive to hot environments. Therefore, tropical legumes require energy reserves in the form of sugar and starch. This study aimed to explain the relationship between sugar, starch, and tannin contents of tropical legumes and their *in vitro* **fermentation profiles. Samples of** *Bauhinia purpurea, Pterocarpus indicus, Tamarindus indica, Calopogonium mucunoides, Macroptilium atropurpureum***, and** *Stylosanthes guianensis* **were tested by proximate analysis, Van Soest, and** *in vitro* **nutrient fermentability. The** *in vitro* **fermentability consisted of dry matter degradability (DMD), organic matter degradability (OMD), volatile fatty acid (VFA), methane and ammonia concentrations, and gas production. The gas production kinetics were determined as gas production from soluble nutrient (a), maximum gas production (a+b), and rate of gas production (c). All samples were tested using the orthogonal contrast test to compare** *in vitro* **fermentability characteristics between the different types of legumes and their tannin contents. Results revealed that the average content of the legumes dry matter examined was 22% with CP content of 15% -28%. The legumes in this study had average content of 3.74% sugar, 21.86% ASP, and 0.3% total phenolics (dry matter basis). Acid soluble polysaccharides content in legumes had a positive correlation with degradability, ammonia concentration, and total gas production ($p<0.05$). However, sugar content did not have a correlation with the other** *in vitro* **fermentation characteristics. The exploration of sugar and acid soluble polysaccharides content in tropical legumes might be profitable as these nutrients could increase feeding efficiency. Sugar did not have a significant relationship to the characteristics of fermentation, on the contrary, acid soluble polysaccharides had a very close relationship to the characteristics of fermentation. The total phenolics had a close relationship with the production of VFA and ammonia. Tree legumes have better digestibility than shrub legumes.**

Keywords: fermentation; sugar; legume; acid soluble polysaccharides; tropics

INTRODUCTION

One effort to maintain the sustainability of ruminant livestock business is maintaining the availability of feed. Climate change is an issue in realizing food security efforts. The negative impacts that can occur due to climate change are drought, changes in rainfall, and an abundance of CO_2 concentrations in the air. One anticipation of the abundance of CO_2 concentrations is to explore plants that have an efficient photosynthesis process. The C4 plant is a type of plant that can bind CO_2 through phosphoenolpyruvate (PEP) enzyme to form four carbons compound (oxaloacetic acid). The C4 plants have higher photosynthetic rates than C3 plants, especially in high light intensity. Furthermore, C4 plants can generally grow in hot regions, for example, tropical plants, one of which is tropical legumes.

Sugar and starch are non-structural carbohydrates that are stored by plants as energy reserves. The C4 plants produce more energy in the process of photosynthesis compared to C3 plants, because tropical plants have higher starch content and are stored in leaves (Hall, 2014). Sugar and starch content in plants is important to be analyzed in order to determine the potential of a plant in meeting the nutrient requirements for these plants and the animals that consume them. Forages

(grasses and legumes) contain structural carbohydrates (cellulose and hemicellulose) and non-structural carbohydrates. Sugar and starch are found in the contents of plant cells, while cellulose and hemicellulose are found in plant cell walls. Dickhoefer *et al*. (2018) studied that starch derived from legumes can avoid rumen degradation so that it can be digested and absorbed optimally in the small intestine. The legume starch granules are resistant to hydrolysis of amylase due to the degree of crystallinity (Alcázar-alay & Meireles, 2015).

Tropical legume is one type of forage used as a source of protein for livestock. In general, tropical legumes are adaptive to tropical climates. Junior *et al*. (2020) stated legumes could be divided into two types, namely tree legumes and shrub legumes based on the growth characteristics. There are several species of tropical legumes that have not been explored and studied on the relationship of nutritional values to *in vitro* parameters, for example, *Bauhinia purpurea, Pterocarpus indicus, Tamarindus indica, Calopogonium mucunoides*, *Macroptilium atropurpureum*, and *Stylosanthes guianensis*. Legumes are identical with higher protein content and degradability compared to grass (Heinritz *et al*. 2012). In general, legumes contain anti-nutritive components. The presence of anti-nutritive components within normal limits can increase the availability of rumen undegradable protein (RUP) so that the available protein that is easily absorbed by the small intestine will increase. Grabber (2009) studied the prediction of rumen undegradable protein value in forage legumes containing protein-binding polyphenols. The tannin content in legumes can alter rumen degradable protein (RDP) into RUP so that it can reduce methane gas production from rumen fermentation in ruminants (Focant *et al*., 2018).

In the *in vitro* fermentation parameters, Pino & Heinrichs (2016) explained that the starch component in the feed will produce a VFA product that significantly increased at 0 to 4 hours (reached 180 mM) after feeding, then the VFA production decreases. This is in accordance with a study by Hatew *et al.* (2015), which showed that the use of slowly fermentable starch produced methane of 48.4 mL/g OM incubated, while with the same level and measurement time, rapidly fermentable starch produced methane gas of 36.8 mL/g OM incubated. In the previous study, most of sugar and starch analysis were used for food grade raw materials, but in this study, they were analyzed from feedstuffs to increase ruminant feed efficiency by understanding sugar and starch contents of legumes. To our knowledge, sugar and starch contents in tropical legumes, particularly originated from South East Asian countries have not been investigated and reported. This study aimed to explain the relationship between sugar, starch, and tannin contents of tropical legumes and their *in vitro* fermentation profiles.

MATERIALS AND METHODS

Sample Preparation

The screened legume data were collected, then 6 types of legumes (*B. purpurea, P. indicus, T. indica, C. mucunoides, M. atropurpureum*, and *S. guianensis*) were obtained which had not been explored in terms of sugar, starch, and tannin contents and their relationships to *in vitro* nutrient fermentability. A total of 2 kg of legumes that can be consumed by livestock (edible parts) from each type of legume were collected. The tree legumes collection was performed by cutting the young leaves and stems, while the shrub legumes collection was performed by cutting the crowns. Cutting was performed with a length of ±5 cm. Plants were air dried in a greenhouse for 2-3 days, then followed by drying with an oven at 60^{o}C for 1 day. Dried plants were ground by using a grinder with a screen diameter of 1 mm.

Procedure of Chemical Composition Analysis

Proximate analysis (AOAC, 2005) and Van Soest *et al*. (1991) were used to determine the nutrient compositions of six legumes. The proximate analysis consisted of dry matter (DM), ash, crude protein (CP), ether extract (EE), crude fiber (CF), and nitrogen-free extract (NFE). Dry matter and ash measurements were carried out using a 105 ^{o}C oven and a 550-600^{o}C furnace, respectively. Crude fiber fraction analysis (Van Soest *et al*., 1991) consists of Neutral detergent fiber (NDF), Acid detergent fiber (ADF), and Acid detergent lignin (ADL).

Analysis of Sugar, Starch, and Total Phenolic Compounds

Total sugar content analysis used the Anthrone method, as was suggested by Bawazeer *et al*. (2017). Acid hydrolyzed polysaccharides content was analyzed by using the method of Duan *et al*. (2012) and its modification.

The total phenolic content analysis used the Petchidurai *et al*. (2019) method. Total phenolic compound content analysis was carried out by adding 10 mg of leaf powder with distilled water to a volume of 100 mL. Then, the solution was put into a 10 mL volumetric flask and 1 mL of Folin Ciocalteu reagent was added, then the solution was shaken and waited for 5 minutes. After that, 2 mL of 15% Na_2CO_3 was added to the solution and the solution was shaken homogeneously and waited for 5 minutes. The solution was added with distilled water to exactly 10 mL and then measured at wavelengths from 500 to 900 nm.

Analysis of *In Vitro* Rumen Fermentation Characteristics

In vitro gas production was evaluated according to the method by Menke & Steingass (1988). An amount of 200 mg sample dry matter was put into a lubricated syringe. Previously, the lower end of the syringe was closed by using a clip. A buffer solution was produced by combining ingredients, which consisted of rumen liquid under anaerobic conditions at 39°C, micro minerals, macro minerals, buffer solutions, resazurin solutions, reducing solutions (must be prepared at any time), and distilled water. The buffer solution was mixed with rumen liquid at a ratio of 2:1. The mixture

was prepared and put in 39°C water, and then the mixture was stirred continuously under anaerobic conditions. A total of 30 mL of rumen buffer solution was put into each tube containing the sample. Each treatment was analyzed in four replications and three incubations. Waterbath shaker was maintained at 39°C. Observation of total gas production was carried out and recorded at 0, 3, 6, 9, 12, 24, 48, and 72 incubation hours.

The gas production kinetic was estimated with an exponential equation (Orskov & McDonald, 1979):

$p = a + b (1 - e^{-ct})$

where p was cumulative gas production at t hour, a was gas production from the soluble fraction, b was gas production from the insoluble fraction but can be fermented, and c was the rate of gas production.

After an incubation period of 24 hours, each legume sample was taken as many as 2 syringes, then the sample was analyzed to determine the degradabilities of DM and OM, and rumen fluid (for VFA and NH_3 analysis). VFA determination used gas chromatography (Hewlett Packard 6890 GC system) at 24 hours incubation time according to the Azizi *et al*. (2020) procedure. Then, ammonia was determined according to Cabeza *et al*. (2018).

The substrate was filtered to separate from the buffer solution by using sintered glass. Vacuum pump filtering was performed to remove water residues. Then, the substrate was put into a 105°C oven for 24 hours. Then, the sintered glass was removed from the oven and put into a desiccator before being weighed. After that, the sintered glass was put into a 550°C - 600°C oven for 3 hours and put in a desiccator before weighing was performed.

Estimated methane gas production was based on the calculation of Moss *et al*. (2000):

$CH_4 = 0.45\ C_2 - 0.275\ C_3 + 0.40\ C_4$

where C2 was acetate, C3 was propionate, and C4 was butyrate.

Statistical Analysis

The data obtained were statistically analyzed by using analysis of variance (ANOVA) with Randomized Complete Block Design (RCBD) with the rumen incubation time as the block. If there were significant differences, then Duncan test was conducted (Riadi, 2016). An orthogonal contrast test was performed as a comparison method between groups of plants. Treatment groups were differentiated based on tannin content and type of legumes. Data analysis was performed using SPSS 16.0 software.

RESULTS

Nutrient Composition

Table 1 presents the results that averages DM and CP contents of tree legume were higher than those of shrub legume. The range of CP contents in all legumes tested was 16%-28% of DM. *B. purpurea* had the highest DM content. CP content of *S. guianensis* was 16.74% that was relatively lower than that of the other legumes. Based on the data presented, *S. guianensis* had the highest CF content (42.18%) compared to the other legume types, and the average CF content of tree legume was lower than that of shrub legume. *M. atropurpureum* had the highest proportion content of NDF and ADF. On the other hand, *T. indica* relatively had the lowest NDF and ADF contents.

Sugar, Acid Soluble Polysaccharides (ASP), and Total Phenolics Contents

Table 2 shows that sugar content is lower than starch content. *C. mucunoides* had the highest content of sugar, whereas *S. guianensis* had the lowest content of sugar. The ASP contents of some legume species (Table 2) were lower than the ASP content in cereals. Tannin is an anti-nutritive compound that is commonly found in

Table 1. Nutrient composition of tropical legumes (% DM)

Nutrients	Tropical legumes					
	BP	PI	TI	CM	MA	SG
Common name	Bunga kupu	Angsana	Asem	Kalopo	Siratro	Stilo
Type of legumes	Tree	Tree	Tree	Shrub	Shrub	Shrub
DM	24.74	24.31	27.28	18.80	21.04	20.64
Ash	11.18	5.93	10.13	6.67	6.81	5.83
CP	28.95	28.91	15.89	19.69	17.87	16.74
EE	2.45	0.87	2.95	2.20	1.22	0.62
CF	23.09	25.20	19.75	28.65	28.65	42.18
NFE	34.33	39.09	51.28	42.79	42.66	34.63
NDF	57.57	67.19	52.79	67.96	80.62	73.46
ADF	29.29	35.51	30.60	35.82	49.54	48.86
ADL	0.27	0.54	0.44	0.45	0.51	0.33

Note: BP= *Bauhinia purpurea*, PI= *Pterocarpus indicus*, TI= *Tamarindus indica*, CM= *Calopogonium mucunoides*, MA= *Macroptilium atropurpureum*, SG= *Stylosanthes guianensis*, DM= dry matter, CP= crude protein, EE= ether extract, CF= crude fiber, NFE= nitrogen-free extract, NDF= neutral detergent fiber, ADF= acid detergent fiber, ADL= acid detergent lignin.

legumes. Table 2 shows the total phenolics content of the six legume plants is ≤0.5% of DM. The averages of sugar and ASP were 3.74% and 21.86%, respectively.

In Vitro Ruminal Fermentation Characteristics

T. indica legume which had a high starch content of 26.33% of DM and low fiber fraction (NDF and ADF) produced a high rate of gas production of 0.067 mL/hour ($p<0.05$) and the high degradability percentage ($p<0.001$). Total VFA content was more than 30 mM in all samples, in order of proportion from the largest to the smallest one was C_2 (acetate), C_3 (propionate), and C_4 (butyrate). Acetate proportion of those samples was more than 72% of total VFA and propionate was more than 16%. All samples produced methane concentration below 12 mM. The ammonia produced in the experiments varied on 13 up to 18 mmol/L (Table 3).

During the incubation period of 12 to 72 hours, gas production increased (Table 4). *S. guianensis* had the highest total gas production at each incubation time. Otherwise, *M. atropurpureum* had the lowest total gas production at each incubation time. *T. indica* had the highest production of dissolved fraction gas (a) and the rate of gas production (c) (Table 5). Table 6 determined that sugar and protein contents did not significantly correlate with *in vitro* parameters. In contrast, starch and tannin contents had a significant correlation with ammonia production. All *in vitro* parameters were significantly different ($p<0.05$) between plants with high and low tannins, except the maximum gas production and the rate of gas production.

Table 2. Sugar, starch, and tannin contents in legumes

Legumes	Category of legume	Sugar (% DM)	Starch (% DM)	Tannin (% DM)
BP	Low tannin	3.38	16.52	0.14
PI	High tannin	3.43	21.86	0.50
TI	High tannin	3.22	26.33	0.35
CM	Low tannin	8.77	23.49	0.14
MA	High tannin	2.09	18.27	0.40
SG	Low tannin	1.55	24.69	0.19

Note: BP= *Bauhinia purpurea*, PI= *Pterocarpus indicus*, TI= *Tamarindus indica*, CM= *Calopogonium mucunoides*, MA= *Macroptilium atropurpureum*, SG= *Stylosanthes guianensis*, DM= dry matter.

DISCUSSION

A legume can substitute another legume by viewing the degradability parameter. The percentages of OMD of several legumes are equivalent to that

Table 3. Percentage of degradability, VFA concentration, methane gas production, and ammonia of tropical legumes

Variables	Tropical legumes						SEM	p value
	BP	PI	TI	CM	MA	SG		
DMD (%)	46.02±0.43^{b}	42.42±1.39^{c}	54.25±1.29^{a}	46.68±0.45^{b}	30.03±0.30^{d}	48.22±0.13^{b}	1.67	<0.001
OMD (%)	51.13±0.05^{b}	53.72±0.08^{ab}	56.18±0.18^{a}	52.35±0.75^{b}	36.67±0.55^{c}	51.28±0.13^{b}	1.66	<0.001
VFA (mM)	33.77±0.18^{b}	31.78±0.54^{c}	31.62±1.28^{c}	33.29±0.59^{b}	30.82±0.58^{c}	35.69±0.18^{a}	0.42	<0.01
- C2 (% total VFA)	72.87±0.00^{c}	74.28±0.00^{b}	75.47±0.01^{b}	72.53±0.01^{c}	77.52±0.00^{a}	74.79±0.01^{b}	0.42	<0.001
- C3 (% total VFA)	18.94±0.01^{a}	16.74±0.01^{bc}	17.08±0.01^{bc}	18.11±0.01^{ab}	16.08±0.00^{c}	17.90±0.01^{ab}	0.27	<0.05
- C4 (% total VFA)	8.19±0.00^{bc}	8.99±0.00^{ab}	7.45±0.00^{c}	9.36±0.01^{a}	6.40±0.00^{d}	7.32±0.00^{c}	0.26	<0.001
- C2:C3	3.85±0.13^{c}	4.44±0.17^{ab}	4.43±0.20^{ab}	4.01±0.18^{c}	4.82±0.07^{a}	4.18±0.18^{bc}	0.10	<0.001
CH_4 (mM)	10.42±0.17^{b}	10.30±0.27^{b}	10.20±0.56^{b}	10.46±0.23^{b}	10.18±0.22^{b}	11.29±0.19^{a}	0.17	<0.001
NH_3 (mmol/L)	13.42±1.03^{d}	18.95±1.05^{a}	17.85±1.16^{ab}	17.61±0.77^{ab}	16.50±1.04^{bc}	15.56±1.25^{c}	0.65	<0.001

Note: Means in the same column with different superscripts differ significantly ($p<0.05$). BP= *Bauhinia purpurea*, PI= *Pterocarpus indicus*, TI= *Tamarindus indica*, CM= *Calopogonium mucunoides*, MA= *Macroptilium atropurpureum*, SG= *Stylosanthes guianensis*, DMD= dry matter degradability; OMD= organic matter degradability; VFA= volatile fatty acid; C2= acetate; C3= propionate; C4= butyrate; CH4= methane, NH3= ammonia; SEM= standard error of mean.

Table 4. Total gas production of tropical legumes at 12, 24, 48, and 72 hours of incubation

Tropical legumes	Time of incubation (hour)			
	12	24	48	72
Bauhinia purpurea	18.67±0.47^{c}	27.83±0.94^{c}	34.33±1.03^{b}	37.00±1.63^{bc}
Pterocarpus indicus	19.25±1.43^{ab}	28.92±2.97^{bc}	34.08±3.78^{b}	36.17±4.03^{bc}
Tamarindus indica	22.08±1.36^{a}	31.17±3.01^{ab}	35.83±3.47^{b}	37.25±3.68^{bc}
Calopogonium mucunoides	20.33±1.84^{b}	30.33±2.90^{abc}	36.67±3.68^{ab}	39.50±4.30^{b}
Macroptilium atropurpureum	11.33±1.31^{d}	19.33±2.66^{d}	27.83±3.47^{c}	31.33±3.06^{c}
Stylosanthes guianensis	19.67±1.25^{bc}	32.00±2.55^{a}	39.17±2.95^{a}	45.92±6.99^{a}
SEM	0.89	1.08	1.06	1.35
P Value	<0.01	<0.01	<0.01	<0.01

Note: Means in the same column with different superscripts differ significantly ($p<0.05$). BP= *Bauhinia purpurea*, PI= *Pterocarpus indicus*, TI= *Tamarindus indica*, CM= *Calopogonium mucunoides*, MA= *Macroptilium atropurpureum*, SG= *Stylosanthes guianensis*, a= Production of dissolved fraction gas, a+b= maximum gas production at t which limits to infinity (asymptote), c= rate of gas production, SEM= standard error of mean.

Table 5. Production of dissolved fraction gas (a), maximum gas (a+b), and rate of gas production (c) of tropical legumes

Tropical legumes	a (mL)	a+b (mL)	c (mL/hour)
BP	3.57±0.43^{c}	37.79±0.42^{b}	0.05±0.003^{b}
PI	2.97±0.73^{d}	36.53±0.71^{b}	0.06±0.01^{ab}
TI	4.77±0.60^{a}	37.52±0.59^{b}	0.067±0.01^{a}
CM	4.48±0.64^{b}	40.20±0.63^{b}	0.052±0.004^{b}
MA	2.89±0.66^{e}	36.61±1.05^{b}	0.027±0.003^{c}
SG	1.02±1.62^{f}	48.03±1.64^{a}	0.045±0.01^{b}
SEM	0.30	1.56	0.00
P value	<0.05	<0.05	<0.01

Note: Means in the same column with different superscripts differ significantly (p<0.05). BP= *Bauhinia purpurea*, PI= *Pterocarpus indicus*, TI= *Tamarindus indica*, CM= *Calopogonium mucunoides*, MA= *Macroptilium atropurpureum*, SG= *Stylosanthes guianensis*, a= Production of dissolved fraction gas, a+b= maximum gas production at t which limits to infinity (asymptote), c= rate of gas production, SEM= standard error of mean.

Table 6. Correlation matrix (r) between content of sugar and starch and *in vitro* fermentation characteristics

Variables	Sugar	Starch	Tannin	Protein
DMD	0.203^{ns}	0.649***	-0.380^{ns}	-0.108^{ns}
OMD	0.293^{ns}	0.58**	-0.182^{ns}	0.179^{ns}
VFA	-0.009^{ns}	0.155^{ns}	-0.692**	-0.027^{ns}
CH_4	-0.186^{ns}	0.229^{ns}	-0.390^{ns}	-0.215^{ns}
NH_3	0.259^{ns}	0.515*	0.572*	-0.154^{ns}
Gas	0.238^{ns}	0.60**	-0.313^{ns}	0.005^{ns}
a+b	-0.063^{ns}	0.282^{ns}	-0.330^{ns}	-0.233^{ns}
c	0.211^{ns}	0.476*	0.066^{ns}	0.160^{ns}

Note: ns= No significant differences; *= significantly different (p<0.05); **= significantly different (p<0.01); ***= very significantly different (p<0.001); DMD= dry matter degradability; OMD = organic matter degradability; VFA = volatile fatty acid; CH_4= methane; NH_3= ammonia; a+b= maximum gas production at t which limits to infinity (asymptote); c= rate of gas production.

of *Calliandra calothyrsus* (51.2%), but lower than that of *Leucaena leucocephala* (64.4%) (Jayanegara *et al*., 2011). Observation of methane gas production showed that *S. guianensis* produced the highest methane production compared to the other five legumes. There was a positive correlation with a low correlation coefficient between legume ASP and CH_4 production. Methane emission has been an important parameter to be considered for a sustainable animal production system since it contributes significantly to the global warming problem (Jayanegara *et al*., 2018). Therefore, selecting a high quality legume species with a low methane emission has a great potential for animal feeding with primary importance.

Data on the accumulation of C2, C3, and C4 (VFA) were in the range of 30-36 mM, that is relatively lower than the result of another *in vitro* study that is in the range of 42.9-64.7 mM (Weimer *et al*., 2011). This low accumulation of VFA may be caused by the decreasing ruminal microbes activity. Fabro *et al*. (2020) stated that rumen liquid has an impact on gas production. *S. guianensis* produced the highest average total gas production which was in line with the total concentration of VFA produced. *S. guianensis* legume has a relatively high ASP content compared to the other legumes, with respect to that, *S. guianensis* produces high gas and VFA. This result is related to the presence of ASP which is a substrate or food for ruminal microbes in their activities. Gas will be produced as a result of a ruminal microbe's metabolic activity. Starch is a raw material for VFA formation by ruminal microbes. In addition, the total phenolics content of *S. guianensis* is relatively low, so the availability of protein that can be fermented by rumen microbes can increase. In contrast, *M. atropurpureum* produced the lowest average total gas production, which was in line with the low concentration of VFA produced. It can be explained that the total gas production directly became the parameter of substrate degradation, then it would be reflected in the concentration of the fermentation product.

Ammonia is needed by ruminants as a precursor for the synthesis and formation of microbial proteins. Rumen microbes do not have the ability to utilize amino acids directly. Protein is the main factor in influencing ammonia production after being degraded and fermented by rumen microbes. Feed protein is hydrolyzed by proteolytic enzymes, that is produced by proteolytic bacteria, then it is converted into amino acid, and subsequently fermented by catabolism reaction to generate ammonia. The acid soluble polysaccharides content in legumes was positively correlated (r= 0.515, p<0.05) with ammonia production. Lu *et al*. (2019) stated that energy rich-diet induces a significant increase in rumen microbial protein yield.

Stylosanthes giuanensis produced the highest maximum gas from shrub legume. The profile of each legume varies according to gas production characteristics. The maximum gas produced by *M. atropurpureum* is low because the production rate was only 0.027 mL/hour. *P. indicus* also had a gas production rate of 0.06 mL/hour, although it produced a maximum gas which was relatively the same as *M. atropurpureum*. It can be caused by crude fiber (NDF and ADF) and protein content. Boga *et al*. (2014) stated that nutrient content and soil salinity could affect *in vitro* gas production values. Figure 1 shows that each legume species had different gas production characteristics. *M. atropurpureum* had consistently low gas production compared to the other plants. *T. indica* had high gas production at the beginning of incubation but decreased along the incubation time. In contrast, the gas production of *S. guianensis* was high at the beginning until 72 hours of incubation.

Sugar is a carbohydrate component composed of monosaccharides and disaccharides. The relatively soluble sugar content (Ahmed *et al*., 2013) causes the sugar did not have a significant correlation with *in vitro* nutrient fermentability. Acid soluble polysaccharides had a positive correlation with the degradability of dry matter and organic substrates (Table 6).

As much as 80% of ruminant protein requirements are met by microbial protein synthesis. Rumen microbes utilize sources of NPN (non-protein nitrogen) to be converted into ammonia (NH_3) through a deamination process. Rumen microbes require starch as an energy

source in the deamination process. Furthermore, ammonia is converted into proteins that can be utilized by host animals through the process of microbial protein synthesis. This process is carried out by rumen microbes. Similar to the deamination process, rumen microbes also need energy sources to support microbial protein synthesis activities. Starch is a source of non-structural carbohydrates that are easily utilized by the rumen microbes compared to structural carbohydrates (cellulose and hemicellulose). This study showed that the presence of ASP was positively correlated with the amount of ammonia produced.

Rumen microbes digest starch in anaerobic conditions. Jayanegara *et al.* (2018) mentioned that one of the fermentation products by rumen microbes is gas, such as carbon dioxide (CO_2) and methane (CH_4). The results showed a positive correlation between the presence of starch and total gas produced at 24 hours incubation time. Total gas production at a certain time determines the rate of gas production. Another factor that can influence the rate of gas production is the type of substrate digested. Moreover, Gallo *et al*. (2018) revealed that the particle size of the substrate influenced the rate of gas production.

The presence of tannin measured as total phenolics in tropical legumes has both positive and negative sides. Tannin is a limiting factor for protein degradability. Tannin can bind to protein and reduce the availability of protein (Kondo *et al*., 2014). Tannin binds to protein to form complex bonds of tannin proteins. Buccioni *et al*. (2015) stated that tannin protects protein from degradation by rumen microbes (protease enzyme). Tannin converts RDP to RUP. In other words, the presence of tannins reduces the availability of nutrients fermented by rumen microbes. The biological activity of tannin determines the amount of tannin protection against the substrate. The results (Table 5) showed that tannin had a negative correlation with total VFA production. Total VFA production is a product of fermentation by microbes in the rumen. Therefore, if the availability of nutrients in the rumen is limited (due to tannin), the VFA produced is also limited.

One positive side of tannin is the decline in methane gas production. Several studies (Bueno *et al*., 2015; Jayanegara *et al*., 2015; Szczechowiak *et al*., 2016) showed that tannin could reduce methane gas production in the digestive system of ruminants. The mechanism of tannin in reducing methane gas production can be categorized as a direct and indirect mechanism. The direct mechanism occurs because tannin can inhibit the activity of metanobacterium bacteria (methanogenesis). An indirect mechanism occurs when tannins inhibit the digestion of fiber in feed because fiber digestion can produce hydrogen (H_2). In addition to producing methane, metanobacterium bacteria play a role in digesting cellulose, by working with cellulolytic bacteria. Metanobacterium activity can be reduced by utilizing hydrogen for the other activities, such as biohydrogenation. The reaction of hydrogen and carbon dioxide (CO_2) produces methane and water (Castagninoa *et al*., 2014).

Tannin changes the protein that is easily degraded by rumen microbes (Rumen Degradable Protein) into protein that is not easily degraded by rumen microbes (Rumen Undegradable Protein). In this case, tannin is binding to protein so that the protein directly bypasses the small intestine. NPN content (non-protein nitrogen) in fresh legumes was approximately 10-15% of CP (mainly peptides, free amino acids, and nitrates), and this amount of NPN is an indicator of an increase in the value of crude protein (Solati *et al*., 2018). In this study, CP levels did not have a significant correlation with ammonia and total phenolics ($p>0.05$). High CP content influences the amount of NPN. Tannin has an important role in protecting plants against insects and fungi, as well as protecting the plant germination (Barbehenn *et al*., 2011).

Orthogonal contrast test was performed to see whether there was a difference between tree and shrub legumes and legumes with moderate and low tannin content in each of *in vitro* fermentation parameters. Statistically, the significant differences appear because the data in treatment has a good uniformity (Payadnya & Jayantika, 2018). Almost all parameters present significant differences because in one group, tree legumes or shrub legumes show a good uniformity as well as a legume with low and moderate tannin. Otherwise, ammonia level does not present a significant difference between a tree and shrub legume, it appears because the data have high variation. Consequently, it can enlarge error (Payadnya & Jayantika, 2018).

CONCLUSION

Sugar content of legumes did not have a significant relationship to the characteristics of fermentation, and on the contrary, starch had a very close relationship to the parameters of fermentation (total gas production, rate of gas production, and degradability of dry and organic matter and ammonia). The tannin content measured as total phenolics had a close relationship with the production of VFA and ammonia. Tree legumes have better digestibility than shrub legumes.

CONFLICT OF INTEREST

Anuraga Jayanegara and Anjas Asmara bin Samsudin serve as editors of the Tropical Animal Science Journal, but have no role in the decision to publish this article. The authors also declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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