Research Article

# Effects of Different Levels of *Brachiaria Decumbens* Diets on *In Vitro* Gas Production and Ruminal Fermentation

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**Abstract** | Although *B. decumbens* is abundant in the tropics, the utilization of this grass is limited due to the presence of steroidal saponins. Therefore, the main objective of this study was to determine the effects of different levels of *B. decumbens* diets on the *in vitro* gas production and ruminal fermentation characteristics. Graded levels of *B. decumbens* were mixed with *P. purpureum*, where 10% was identified as the low-level *B. decumbens* diet (T2) and, 60% was identified as the high-level *B. decumbens* diet (T3) based on the concentration of saponins. Meanwhile, 100% *P. purpureum* was used as the basal diet, which served as control (T1). Rumen fluid was then collected from six young male Dorper cross sheep for the analyses of gas production, pH, *in vitro* organic matter digestibility, ammonia nitrogen, and volatile fatty acids (VFA). All analyses were conducted according to established methods and data collected were subjected to one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test to determine the level of significance among treatments. The net gas production and gas production kinetics demonstrated notable changes ($P < 0.05$) among treatments. Besides, only the acetic acid and total VFA showed significant differences ($P < 0.05$) for the rumen fermentation characteristics. Generally, the T3 diet consisted of 60% *B. decumbens* diet demonstrated the lowest gas production, gas production parameters, acetic acid, and total VFA concentrations as compared to the other treatments. In conclusion, 10% of *B. decumbens* mixture displayed minimal effects in the *in vitro* assessment, while 60% of *B. decumbens* mixture showed the most significant results out of all three treatments indicating the presence of saponins did influence negatively on the gas production and ruminal fermentation characteristics.

**Keywords** | *Brachiaria decumbens*, Gas production, Rumen fermentation characteristics, Saponins, Sheep.

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## INTRODUCTION

*Brachiaria* species is one of the main pastures used in ruminant production. Although the feeding of animals with *B. decumbens* has improved their overall performance, the risk of toxicity has become a limiting factor (Muniandy et al., 2020). Aside from other *Brachiaria* spp., most of the outbreaks related to poisoning in grazing ruminants are attributed by *B. decumbens*. The toxicity of *B. decumbens* is due to the presence of steroidal saponins and intoxication occurs when the grass is fed as the main source of diet for grazing animals (Chung et al., 2018). These compounds

cause the development of crystals that cause the obstruction of the bile duct and the elevation of liver enzyme. A previous study by Muniandy et al. (2021a and 2021b) showed that sheep, especially young animals under one year of age are more susceptible to *B. decumbens* toxicity compared to other ruminants.

High concentrations of saponins is known to adversely affect rumen fermentation. Saponins cause a change in fermentation products, decreased methane, hydrogen, and ammonia concentration (Aazami et al., 2013). These reactions produced similar effects on the ionophores during ruminal microbial fermentation. *B. decumbens* also shows high variability in the *in vitro* dry matter digestibility (IVDMD) and chemical composition at different growth stages. Lascano and Euclides (1996) found that the IVDMD and *in vivo* digestibility of immature (leaf) and mature (whole plant) of *B. decumbens* was comparable or higher than that of other tropical grasses. Meale et al. (2012) stated that *Brachiaria* spp. grass has higher cumulative gas, IVDMD, and propionate production in the *in vitro* study in contrast to other shrubs and grasses utilized for ruminant grazing in Ghana and Australia. Sheep grazing on *B. decumbens* pastures demonstrated a considerably lesser amount of volatile fatty acids (VFA) production because of reduced cellulolytic activity and decreasing rumen microbes owing to the presence of saponins (Faccin et al., 2014).

*B. decumbens* is an extremely prolific grass that is present abundantly in the tropics (Chung et al., 2018). Even so, the preventive reason for the optimal utilization of this grass is the presence of steroidal saponins. There were many reports of decreased productivity and deaths in different species of ruminants, and the disease is more severe in sheep leading to huge economic losses. Nonetheless, limited data is available on the effects of different amounts of *B. decumbens* intake in ruminants. *In vitro* assessment would give a clearer understanding of the impact on those animals consuming the grass. Therefore, the main objective of this study was to determine the effects of different levels of *B. decumbens* diets on the *in vitro* gas production and ruminal fermentation characteristics.

## MATERIALS AND METHODS

### Saponins extraction and measurement

Different concentrations of *B. decumbens* (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 %) were mixed with *P. purpureum* and subjected to saponins extraction and measurement according to Yuliana et al. (2014) to identify the low and high levels of *B. decumbens* diets. 10% was identified as the low *B. decumbens* level (T2) meanwhile, 60% was identified as the high *B. decumbens* level (T3) based on the concentration of saponins that peaked at 60% and remained constant at higher levels of *B. decumbens* mixture (Figure 1). Meanwhile, 100% *P. purpureum* was used as the basal diet, which served as control (T1). Table 1 illustrates the nutrient composition of each diet.

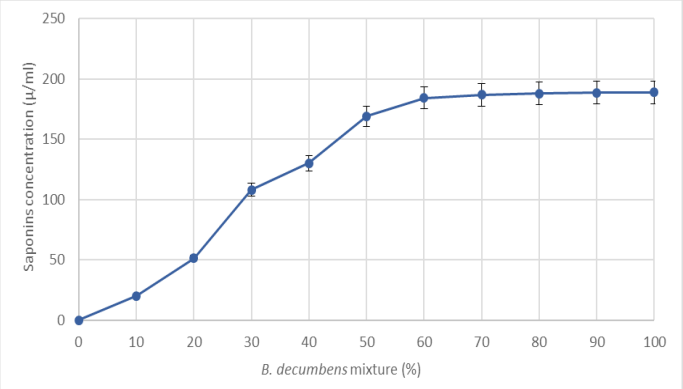


**Figure 1:** The concentrations of saponins at different percentages of *B. decumbens* and *P. purpureum* mixture.

**Table 1:** Nutritional composition of T1, T2, and T3 diets.

| Composition | T1 (Control) | T2 | T3 |
|---|---|---|---|
| Dry matter (% as fed) | 24.12 ± 0.83 | 19.12 ± 0.20 | 17.38 ± 0.46 |
| Organic matter | 95.63 ± 0.29 | 95.24 ± 0.14 | 94.90 ± 0.01 |
| Crude protein | 16.92 ± 0.12 | 16.41 ± 0.13 | 15.03 ± 0.15 |
| Ether extract | 2.22 ± 0.11 | 1.92 ± 0.10 | 1.71 ±0.03 |
| Neutral detergent fiber | 63.64 ± 0.19 | 60.71 ± 1.04 | 54.16 ± 0.90 |
| Acid detergent fiber | 42.37 ± 1.48 | 38.53 ± 0.959 | 36.15 ± 0.43 |
| Acid detergent lignin | 5.23 ± 0.25 | 4.54 ± 0.24 | 3.69 ± 0.29 |
| Gross energy (kJ/kg DM) | 15.73 ± 0.20 | 16.37 ± 0.32 | 16.69 ± 0.56 |

Note: T1: 100% *P. purpureum*, T2: 10% of *B. decumbens* + 90% *P. purpureum*, T3: 60% of *B. decumbens* + 40% of *P. purpureum*.

### Collection and preparation of rumen fluid

A total of six male Dorper cross sheep (six-month-old) weighing approximately 17.1 ± 0.52 kg fed with *P. purpureum* and commercial pellets were used in this study. After two weeks of acclimatization, 200 mL of rumen fluid was collected into a pre-warmed vacuum thermal flask via stomach tubing. The collected rumen fluid was then filtered through four layers of cheesecloth into a conical flask that was placed in the water bath (39°C). Throughout the preparation of the rumen buffer medium, the filtrate was mixed with phosphate and bicarbonate buffer at ratio 1:2 under continuous flushing of carbon dioxide ($CO_2$) to maintain the anaerobic condition.

### *In vitro* medium preparation

The *in vitro* medium was made up of 400 ml of distilled

water, 200 ml of buffer solution, 200 ml of macro solution, 0.1 ml of micro mineral solution, 1 ml of Resazurin solution, and 40 ml of reducing solution (Menke, 1988). The solution was mixed accordingly, continuously stirred, and flushed using ($CO_2$) until the bluish color of Resazurin turned pink or colorless due to the reduction process.

### *In vitro* incubation

According to Menke, (1988), 0.2 ± 0.001 g DM of samples was weighed and placed inside 100 ml calibrated glass syringes fitted with pistons with a rubber clip attached at the tip of the syringe. The standard sample weighed approximately 0.2 ± 0.001 g DM of *P. purpureum*. Blank syringes (buffered ruminal fluid only) that were both utilized as calibration and gas production correction in the fermentation of the substrate respectively were also added for incubation. A total of 30 ml of buffered rumen medium were dispensed into the syringes that contained substrates of different diets and blank syringes without substrate. The initial volume of gas was recorded before the syringes were incubated in a water bath maintained at a temperature of 39°C. Each treatment was replicated five times, while blank was replicated thrice.

### Measurement of gas production

Incubation was done for 48 hours, and the gas produced was recorded at 2, 4, 8, 12, 24, and 48 hours intervals. The net gas produced at the respective time intervals was measured and recorded, while the cumulative gas production characteristic was estimated using NEWAY computer package program, version 5.0 (Dr. X. B. Chen. IFRU, Rowett Research Institute; Aberdeen, UK). Net gas production was corrected for blank incubation. The blank incubation was used to determine net gas production. The cumulative gas production data were calculated according to the nonlinear equation model by Orskov and McDonald (1979). The rumen fluid was then used for the determination of pH, IVOMD, ammonia, and VFA at the end of the incubation.

### Rumen pH

After 48 hours incubation period ended, the rumen fluid pH was measured instantly using a Mettler-Toledo pH meter (Mettler-Toledo, Ltd, Leicester, UK). Approximately 15 ml of the fluid was collected for further ammonium nitrogen and VFA analyses.

### *In vitro* organic matter digestibility (IVOMD)

All the contents of the glass syringes for each treatment and blank were emptied and transferred into pre-weighed sintered glass after incubation. The rumen fluid residues were thoroughly rinsed with distilled water and dried in a 105°C oven until a constant weight was produced. IVOMD (%) was determined based on the calculation: 100 × (weight of organic matter of initial sample – residue – blank)/weight of organic matter of initial sample.

### Determination of ammonium nitrogen in rumen liquor

There were five solutions prepared for the determination including phenol solution, sodium nitroprusside solution, alkaline reagent solution, sodium hypochlorite solution, and oxidizing solution (mixture of 4 parts alkaline reagent contained 0.25 M sodium hydroxide and 0.775 M sodium citrate and 1 part of 5.25 % (v/v) sodium hypochlorite). For every 5 ml of rumen fluid, 0.2ml of 1 M phenol solution in 95 % ethyl alcohol was added and vortexed. The solvent was then added with 0.5 ml oxidizing solution and 0.2 ml of 0.02 M sodium nitroprusside solution before vortexed. The solutions were left for incubation at room temperature for 1 hour. A blue color will appear indicating the presence of ammonia. The standard ammonia chloride consisting of 0.2, 0.5, 1.0, and 2.0 ppm were prepared, and the absorbance was read using an SC spectrophotometer (GENESYS™ 20 Thermo Scientific™, USA) at 640 nm. The regression equation was obtained from the standard and the ammonia rumen samples were analyzed and calculated. The standard curve linear regression equation was calculated according to the standard solution, and the $NH_3$-N concentration was derived accordingly.

### Volatile fatty acids (VFA) analysis

Firstly, the filtered rumen sample was acidified with 25% meta-phosphoric acid (w/v) in the ratio of 4:1 (v/v) before centrifuged for 10 minutes at 3000 x g, followed by incubation overnight at room temperature. The supernatant was collected, filtered, and 0.5 ml of clear supernatant were mixed with 4-methyl-N-valeric acid (Sigma, St. Louis, MO), which acts as an internal standard. For peak identification, 20 mM acetic, and 10mM each of propionic, butyric, and 4-methyl-valeric acids were used as an external standard (Sigma-Aldrich, St. Louis, MO). The VFA separation was determined using a Quadrex 007 Series (Quadrex Corp., New Haven, CT 06525, USA) bonded phase fused silica capillary column (15 m, 0.250 mm ID, 0.25 µm film thickness) with a 6890 N Network GC System gas chromatograph (Agilent Technologies) equipped with a flame ionization detector. Nitrogen gas was supplied as carrier gas at the rate of 60 ml/min, with the temperature of the column was set at 200°C, while the injector and detector were both at 230°C. The identification of VFA molar concentration was determined based on a single point of internal and external standards.

### Statistical analysis

All data collected were subjected to one-way analysis of variance (ANOVA), using the General Linear Model (GLM) of Statistical Analysis System software (SAS) version 9.4 (SAS Institute, USA). Duncan´s Multiple Range

**Table 2:** *In vitro* gas production of low and high levels of *B. decumbens* diets.

| Parameter | T1 (Control) | T2 | T3 |
|---|---|---|---|
| Net gas production (ml/200 mg DM) | | | |
| Incubation time (h) | | | |
| 2 | 6.30 ± 0.30$^{a}$ | 5.20 ± 0.49$^{ab}$ | 4.40 ± 0.51$^{b}$ |
| 4 | 10.55 ± 0.66$^{a}$ | 8.35 ± 0.10$^{b}$ | 5.65 ± 0.51$^{c}$ |
| 8 | 17.80 ± 0.37$^{a}$ | 15.20 ± 0.30$^{b}$ | 9.50 ± 1.09$^{c}$ |
| 12 | 24.25 ± 0.61$^{a}$ | 19.65 ± 0.51$^{b}$ | 17.85 ± 1.32$^{b}$ |
| 24 | 37.90 ± 1.90$^{a}$ | 33.60 ± 0.60$^{a}$ | 27.40 ± 1.96$^{b}$ |
| 48 | 38.90 ± 0.10$^{a}$ | 36.00 ± 0.90$^{a}$ | 31.10 ± 1.92$^{b}$ |
| Gas production parameter | | | |
| a (ml) | -0.79 ± 0.14 | -0.71 ± 0.57 | -1.36 ± 0.67 |
| b (ml) | 42.07 ± 1.26$^{a}$ | 39.46 ± 1.31$^{ab}$ | 35.06 ± 2.98$^{b}$ |
| c (ml/h) | 0.80 ± 0.01$^{a}$ | 0.66 ± 0.01$^{b}$ | 0.06 ± 0.01$^{b}$ |
| a + b | 41.28 ± 0.48$^{a}$ | 38.75 ± 1.08$^{a}$ | 33.70 ± 2.39$^{b}$ |
| IVOMD (%) | 57.37 ± 2.39 | 56.25 ± 1.55 | 50.20 ± 2.05 |

Note: All values were expressed as mean ± SE; a, b, c values with superscript within row are significantly different at $P < 0.05$. T1: 100% *P. purpureum*, T2: 10% of *B. decumbens* + 90% *P. purpureum*, T3: 60% of *B. decumbens* + 40% of *P. purpureum*. a: volume of gas produced from immediate soluble fraction, b: volume of gas produced from insoluble fraction, c: gas production rate constant from insoluble fraction, a+b: potential extent of gas production, IVOMD: *in vitro* organic matter digestibility.

Test was used to determine the significant difference among the treatments. The means were considered significant at $P < 0.05$.

## RESULTS

### *In vitro* gas production

Table 2 displays the *in vitro* gas production of the different diets. Significant differences ($P < 0.05$) were shown in the net gas production and gas production kinetics, while no significant change ($P > 0.05$) was displayed in the IVOMD throughout the incubation period.

The gas production notably declines as the level of *B. decumbens* rises (Figure 2). The *in vitro* incubation using 60% of *B. decumbens* diet (T3) showed significantly lower net gas production compared to the control (T1) and 10% *B. decumbens* diet (T2). The gas production reduced significantly by 30.16, 46.45, 46.62, 26.39, 27.70, and 20.05% at 2, 4, 8, 12, 24, and 48 hours respectively than that of the control. Additionally, the gas production parameters of T3 such as volume of gas produced from insoluble fraction (b), gas production rate constant from insoluble fraction (c), and potential extent of gas production (a+b) were also significantly reduced by 16.67, 92.5, and 12.50% correspondingly as opposed to control.

Overall, the T3 diet consisted of 60% *B. decumbens* diet demonstrated the lowest gas production and gas production parameters. Numerically, IVOMD was the lowest despite not having a significant difference indicating a reduced digestibility of this diet.

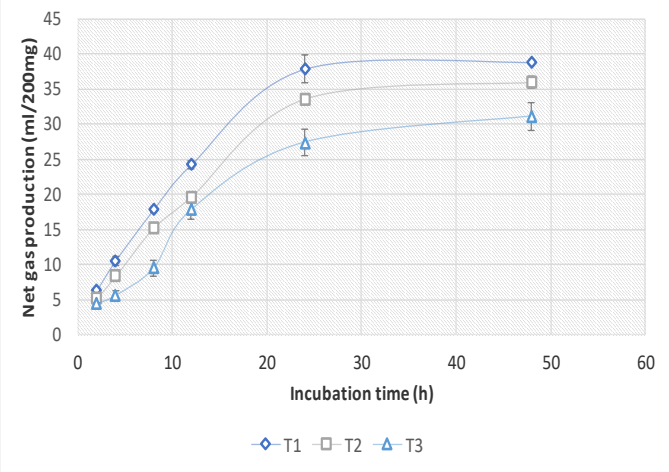


**Figure 2:** Cumulative *in vitro* gas production of different levels of *B. decumbens* diets. T1: 100% *P. purpureum*, T2: 10% of *B. decumbens* + 90% *P. purpureum*, T3: 60% of *B. decumbens* + 40% of *P. purpureum*.

### Rumen fermentation characteristic

Table 3 illustrates the influences of different levels of *B. decumbens* on the rumen fermentation characteristic post 48 hours of *in vitro* incubation.

The pH and ruminal ammonia nitrogen ($NH_3$-N) showed no evidence of being affected with the different levels of *B. decumbens* although the value of ammonia concentration declines as the level of *B. decumbens* rises. There were also no changes ($P > 0.05$) in individual VFA apart from acetic

**Table 3:** Rumen fermentation characteristics of low and high levels of *B. decumbens* diets.

| Parameter | T1 (Control) | T2 | T3 |
|---|---|---|---|
| pH | 6.10 ± 0.13 | 6.62 ± 0.23 | 6.22 ± 0.21 |
| $NH^{3}$- N (ppm) | 1.32 ± 0.78 | 1.28 ± 0.76 | 0.96 ± 0.31 |
| VFA (mM) | | | |
| Acetic acid (A) | 78.12 ± 2.85[a] | 76.55 ± 2.02[a] | 69.70 ± 3.02[b] |
| Propionic acid (P) | 32.06 ± 2.46 | 30.9 ± 2.30 | 28.8 ± 2.30 |
| iso-butyric acid | 1.29 ± 0.14 | 1.21 ± 0.12 | 1.19 ± 0.12 |
| Butyric acid | 7.59 ± 0.98 | 6.81 ± 1.01 | 6.34 ± 0.67 |
| Valeric acid | 1.154 ± 0.16 | 1.134 ± 0.07 | 1.064 ± 0.13 |
| iso-valeric acid | 2.11 ± 0.34 | 1.96 ± 0.19 | 1.83 ± 0.12 |
| Total VFA | 122.32 ± 3.26[a] | 118.56 ± 2.96[a] | 108.92 ± 2.76[b] |
| A:P | 2.33 ± 0.72 | 2.37 ± 1.65 | 2.32 ± 1.05 |

Note: All values were expressed as mean ± SE; [a,b] values with superscript within row are significantly different at $p<0.05$. T1: 100% *P. purpureum*, T2: 10% of *B. decumbens* + 90% *P. purpureum*, T3: 60% of *B. decumbens* + 40% of *P. purpureum*. $NH^{3}$- N: Ammonia nitrogen, VFA: Volatile fatty acids.

acid and total VFA ($P < 0.05$). Both acetic acid and total VFA of T3 decreased by 10.78 and 11.48% respectively when compared to T1.

Generally, the rumen fermentation characteristics were not much affected by either low or high levels of *B. decumbens* diet. Nonetheless, the alterations in the acetic acid and total VFA could influence the digestibility and growth of sheep ingesting high levels of *B. decumbens*.

## DISCUSSION

### *In vitro* gas production

Gas is primarily produced from the fermentation of carbohydrates into VFA by the rumen microbes (Singer et al., 2008). The amount of gas produced correlates to the degradation of carbohydrates in the rumen. Moreover, the type of feedstuff, their chemical composition, as well as the microbial population in the rumen also affects the total gas production. The current research indicated that higher levels of *B. decumbens* diets diminished rumen fermentation rates by decreasing the rumen gas production, rumen fermentation kinetics, and IVOMD, which could be attributed to the presence of steroidal saponins or protodioscin in *B. decumbens*. Besides, the presence of lignin in *B. decumbens* was also shown to influence the digestibility process and subsequently decrease the IVOMD (da Costa et al., 2021). This is because lignin hinders the optimal utilization of structural carbohydrates by the rumen microbes as lignin covalently bonds to them. However, Leal et al. (2020) concluded that protodioscin content has a more significant negative relationship with the degradability of *Brachiaria* cultivars due to the reduced value as well as rumen fermentation capability of the grasses. This finding is similar like previous report by da Costa et al. (2021) who observed a decline in total gas production using *Brachiaria* spp. However, Lascano and Euclides (1996) found that the IVOMD of both immature and mature *B. decumbens* leaves was higher than other tropical grasses. Likewise, Meale et al. (2012) concluded that the cumulative gas production and IVOMD of *B. decumbens* was the highest as compared to some other common grasses, leguminous, and non-leguminous shrubs.

Although optimum amounts of saponins were reported to be beneficial in maintaining *in vitro* rumen bacterial activity and fermentation, higher concentrations of saponins were found to be detrimental (Unnawong et al., 2021). This is because the large molecular weight and increased level of crude saponins can suppress cumulative gas production, indirectly limiting enzyme secretion and feed digestion via ruminal microorganisms (Makkar, 2003). For instance, Santoso et al. (2007) reported that *in vitro* gas production decreased linearly as the supplementation percentage of *Acacia mangium* to *P. purpurerum* increases due to the higher amount of saponins. A similar trend was reported by Wang et al. (2000), who found that *in vitro* gas production decreases because of higher Yucca saponins supplementation above 75mg/L concentration. In a more recent study, a higher concentration of *Sebasnia graniflora* (SES) (commonly known as "rambutan peel" in Malaysia) above 0.6% produced no significant differences between treatments in cumulative gas production after 96 and 72 hours of fermentation, respectively (Unnawong et al., 2021).

The discrepancies between the results of this study and previous works may suggest the influence of saponins on digestibility can be attributed to the dietary composition, source of saponins, and their relative amounts in the diet (Aazami et al., 2013). Nevertheless, despite the diversity of

studies conducted on saponins, especially steroidal saponins, there is a paucity of data and limited research on the effects of *B. decumbens* saponins on *in vitro* study considering the abundance of the grass in Malaysia.

### Rumen fermentation characteristic

Almost 70 to 80% of the energy requirement of ruminants is roughly provided by VFA as the primary source of energy when the ruminal microorganism ferments the fibrous component of the plants (Russell and Rychlik, 2001). In this study, high level of *B. decumbens* had a negative impact on the VFA. Both acetic acid and acetate to propionate ratio increased with increasing *B. decumbens* levels, which indicates a lower degradability. According to Baile and Pfander (1966), higher intake of *B. decumbens* increases the concentration of acetic acid, due to increasing osmolarity and acid moiety. The reduction in substrate due to higher levels of *B. decumbens* resulted in lower concentration of VFA, which is reflected as lower feed intake in the sheep. Also, in agreement with the result of this study is Faccin et al. (2014), who reported a significant difference in total VFA and acetic acid concentrations of sheep foraging on *B. decumbens* pasture. The researchers concluded that the presence of saponins decreased the rumen microbial population, as well as the cellulolytic activity.

The findings observed in this study also corroborate the results of other research on saponins. For instance, Das et al. (2012) has shown that saponins from alfalfa had negative effects on rumen fermentation. The study reported a decline in total VFA and acetate to propionate ratio in the presence of 1% saponins. The authors concluded that the detrimental results were probably due to the ability of rumen microbes to deglycosylate the saponins to release steroid moiety, which eventually affected rumen fermentation. Likewise, the decrease in the cellulolytic bacteria population can further explain the reason for lower gas production and IVOMD discussed earlier. On the contrary, Hundal et al. (2019) found that the production of total and individual VFA increased by more than 30% in goats supplemented with *Macrotyloma uniflorum*, which contained saponins. The saponins source, diet composition, and their amount of inclusion in the diet again could lead to the discrepancy among different studies.

The concentration of ammonia is another factor that can influence rumen fermentation. The lower concentration of ammonia-N was observed in this study, following high *B. decumbens* level, is most probably due to the presence of steroidal saponins, which decreased the rumen microbial population, as well as their cellulolytic activity. As a result, decreased degradation or utilization of ammonia by bacteria after feeding will cause the rumen ammonia concentration to decline (Wina et al., 2005). In accordance with a previous study, a similar result was obtained where the ammonia-N concentration decreases linearly as the level of saponins increases (Santoso et al., 2007). Conversely, Hundal et al. (2019) found the opposite result on ammonia-N concentration, while Nasri et al. (2011) reported that there was no significant associations between ammonia-N concentration and saponins concentrations. Thus, the differences in rumen fermentation characteristics might be due to the dissimilar saponins concentrations, sources, and the efficiency of ammonia degradation by rumen bacteria.

## CONCLUSION

*Brachiaria* spp is a highly productive tropical grass, with *B. decumbens* being one of the most cultivated species of the genus *Brachiaria* despite the presence of steroidal saponin. The current study investigates the *in vitro* characteristics of different levels of *B. decumbens* diets using sheep rumen fluid. 10% of *B. decumbens* mixture displayed minimal effects on the *in vitro* assessment, while 60% of *B. decumbens* mixture showed the most significant results out of all three treatments indicating the presence of saponins did influence negatively on the gas production and ruminal fermentation. These findings will indirectly convey conscientious utilization of *B. decumbens* pasture and prompt farmers to seek alternatives forages.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## NOVELTY STATEMENT

These results add to the existing body of knowledge and provide additional information to fill the research gap on the effects of different levels of *B. decumbens* diets on *in vitro* gas production and ruminal fermentation, especially in sheep.

## AUTHORS CONTRIBUTION

All authors contributed equally to study design, sampling, methodology, interpretation of results, and manuscript writing.

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