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Fermentability and total digestible nutrients of *Pennisetum purpureum* cv Mott supplemented with buffalo rumen content as probiotic and various level of readily available carbohydrate in Balibul sheep *in vitro*

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ABSTRACT

The purpose of the study was to produce probiotic origin from buffalo rumen-contents enriched with N, S, P, and cassava flour as a source of readily available carbohydrate (RAC) to increase fermentability and energy production of *Pennisetum purpureum* cv Mott grass in the rumen of Balibul sheep. Materials used in this study were *Pennisetum purpureum* cv Mott grass, buffalo rumen-contents, elements N, S and P and cassava flour. The study was conducted in vitro using a completely randomized design (CRD) consisting of 4 treatments and 4 replications. Variables measured were total volatile fatty acids (VFA), ammonia (NH_3) and total digestible nutrients (TDN). Results of the study showed that supplementation of cassava flour as a source of RAC significantly increased the production of VFA, and TDN value ($P<0.05$), but significantly decreased NH_3 production. Average VFA production were 56.67 (T0), 63.33 (T1), 73.33 (T2) and 66.67 mM. Average NH_3 production were 5.61 (T0), 5.28 (T1), 5.06 (T2) and 5.04 mM. Average TDN value were were 50.47 (T0), 5.28 (T1), 5.06 (T2) and 5.04%. It can be concluded that supplementation of cassava flour as a source of RAC increase VFA production and TDN value whith supplementation of 5% RAC and reduces NH_3 production.

Introduction

Sheep are domesticated livestock that are widely cultivated because they are easy to maintain, do not require a large space and produce main products in the form of meat which are consumed to meet protein and nutrient needs (Sutrisno *et al*., 2020). Sheep are widely raised in Indonesia, but the lack of fulfillment of nutrient needs, especially when young, causes their digestive tracts to not develop quickly so that sheep productivity is low. Low livestock productivity is influenced by the lack of nutrients that occurs due to the provision of feed that does not meet requirements, low quality and quantity of feed provided and the function of the digestive tract of young ruminant is not yet able to digest crude fiber optimally which results in poor digestive tract function and caused feed consumption and livestock productivity are also low. Increasing productivity can be done by paying attention to fulfill nutrient requirements from the beginning of life, increasing the capacity and function of digestive tract and increasing the quality and quantity of feed consumed by introducing roughage from an early age.

One of green fodders that is often given to sheep as feed is Odot grass. Odot grass (*Pennisetum purpureum* cv Mott) is a green fodder that can adapt to land conditions with low fertility and can live in the shade because Odot grass has strong roots and stems that are not hard (Araujo *et al*., 2019; Malesi *et al*., 2022; Asminaya *et al*., 2023; Nganjiand Sudarma, 2023). Providing fiber-rich feed from an early age can stimulate faster rumen development and improve feed utilization for young Balibul sheep. Adding microbes in the form of probiotic can also enhance rumen development and improve the digestibility of fiber-rich feed.

Beneficial live bacteria known as probiotics can help balance the microbes in the digestive tract, making feed easier to digest and perhaps increasing animal productivity (Yulvizar, 2013; Pamungkas and Anggraeny, 2006; Hamid *et al*., 2021). Buffalo rumen-contents can be used as a probiotic because it contains cellulolytic bacteria that can break down cellulose better than other ruminant livestock (Wijaya *et al*., 2016).

There are 102 – 180 CFU/g fungal colonies in buffalo rumen-content (Astuti *et al*., 2020); 10^{10}-10^{11}cells/ml (Matthews *et al*., 2019) total bacteria and total cellulolytic bacteria rnages from $3.74x10^7$-$10.^9x10^7$/ml (Varel and Dehority, 1989). *Ruminococcus albus*, *Bacteroides succinogenes*, *Butyrivibrio fibrisolvens, Clostridium lochheadii*, *Clostridium longisporum* and *Clostridium* spp. are among the cellulollytic bacteria found buffalo rumen-content (Sutrisno *et al*., 2020).

Fermentation process in the rumen can work optimally if the required nutrients are met with the presence of N, S, and P elements and balanced with readily available carbohydrates (RAC). RAC are easily fermented carbohydrates to increase feed fermentability which provides products in the form of α-keto acids which are useful for microbial protein synthesis (Rahayu *et al*., 2018). The use of cassava flour as RAC and N, S, P elements is needed to help the growth and development of microbes so that the fermentation process runs better and the production of VFA and NH_3 also increases. There has been little research that incorporates elements or minerals and carbohydrate sources into buffalo rumen contents. This research's novelty was the supplementation of N, S, P elements and cassava flour as RAC sources to buffalo rumen contents.

The study aimed to produce probiotic feed that can increase livestock productivity in the form of buffalo rumen microbes enriched with N, S, P, and RAC to increase fermentability in the rumen of balibul sheep. The benefits of this study are to determine the best level of RAC addition to Odot grass given probiotics and enriched with NSP elements in balibul sheep. The hypothesis of the study was the provision of probiotics from buffalo rumen-contents enriched with N, S, P, and RAC on Odot grass increased the production of VFA, NH_3 and total digestible nutrients (TDN) in balibul sheep in vitro.

Materials and methods

The study was carried out at Universitas Diponegoro's Faculty of Animal and Agriculture Sciences, Semarang. At Laboratory of Nutrition

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and Feed Science, Faculty of Animal and Agriculture Sciences, Universitas Diponegoro, Semarang, proximate analysis, VFA, NH_3 and TDN in vitro analysis were carried out.

Materials

Materials used in this study were Odot grass as a source of fiber feed, N, S and P elements, cassava flour as a source of readily available carbohydrate (RAC) (added as much as 0; 2.5; 5; and 7.5% based on DM of Odot grass), buffalo rumen-contents, rumen fluid of balibul sheep, 0.5 N NaOH, phenolphthalein indicator, 0.5 N HCl, McDougall's solution, pepsin HCl, aquadest, urea (N), ammonium sulphate (S) and sodium biphosphate (P). Previous research using rumen contents with NSP supplementation and without RAC supplementation showed that the best supplementation level was 5%. Therefore, it is necessary to try lower and higher levels to determine the effectiveness of adding cassava flour as a source of RAC.

Equipment used were blender, grinder, filter cloth, thermos, oven, porcelain crucible, analytical balance, water bath, fermenter tube and rubber cap, pH meter, cooling box, tube rack, tray, clamp, desiccator, beaker glass, Erlenmeyer flask, measuring pipette, stirrer, stove, Whatman filter paper No. 41, centrifuge, a set of VFA test distillation equipment and a set of NH_3 test equipment.

Experimental design

A completely randomized design (CRD) including four treatments and four replications was the experimental setup employed in this investigation. The following were the treatments used:

T0= Odot grass + buffalo rumen-contents probiotics +NSP+ 0% cassava flour

T1= Odot grass + buffalo rumen-contents probiotics + NSP + 2.5% cassava flour

T2= Odot grass + buffalo rumen-contents probiotics + NSP + 5% cassava flour

T3= Odot grass + buffalo rumen-contents probiotics + NSP + 7.5% cassava flour

Volatile fatty acids (VFA), ammonia (NH_3), and total digestible nutrients (TDN) of odot grass treated with buffalo rumen-contents as probiotics enhanced with NSP elements and RAC sources were among the variables measured.

Preparations

Study on the production of VFA, NH_3 and TDN of Odot grass supplemented with buffalo rumen-contents as probiotics enriched with NSP elements and RAC in balibul sheep was conducted in vitro by preparing the necessary materials including buffalo rumen-contents, Odot grass, urea, ammonium sulphate and sodium biphosphate. Odot grass and buffalo rumen-contents were dried and ground into flour, then analyzed for proximate and digestibility of organic matter in vitro. These ingredients are formulated according to the treatment, namely Odot grass added with buffalo rumen- contents of each treatment as much as 5% and enriched with N, S, P, and different levels of cassava flour (0, 2.5, 5 and 7.5%). Samples were then analyzed in vitro to determine the production of VFA, NH_3 and TDN.

In vitro fermentability analysis was conducted by preparing equipments and materials to be used. Sample was inserted into the fermenter tube as much as 0.55-0.56 g. McDougall's solution was added as much as 40 ml and rumen fluid of balibul sheep as much as 10 ml to fermenter tube and flowed with CO_2 to create anaerobic conditions and then closed tightly (Tilley and Terry, 1963). Fermenter tube was inserted into water bath with a temperature of 38-39°C.

Volatile fatty acids (VFA) analysis

Steam distillation method was used to analyze volatile fatty acids. After three hours of incubation for the VFA assay, the fermenter tube was cooled by submerging it in cold water to cease the fermentation. After centrifuging the contents of the fermenter tube for 15 minutes at 3,500 rpm, the supernatant was extracted. Five milliliters of the supernatant were transferred to a distillation tube, where they were combined with one milliliter of 15% H_2SO_4 and placed in a distillation flask with 600 milliliters of water. The flask was then connected to a reverse-cooler for the distillation process. The distillation results were collected in an Erlenmeyer flask containing 5 ml of 0.5 N NaOH until it reached a volume of 100 ml. Next, two drops of 1% phenolphthalein indicator are added to Erlenmeyer, and the mixture was titrated with 0.5 N HCl until the color shifted from pink to clear. Five milliliters of 0.5 N NaOH are used to create the blank, which is then titrated with 0.5 N HCl after two drops of 1% phenolphthalein indicator have been added. Calculation of total VFA production as follows (Fathul and Wajizah, 2010):

Total VFA = (Y – Z) x N HCl x 1000/5 mM

Explanation:

N HCl: Normality of HCl solution

Y: ml of HCl required for blank titration

Z : ml of HCl needed for titration of distillation results

Ammonia (NH_3) analysis

The Conway microdiffusion method was used to evaluate NH_3 production. Vaseline was used to smear the Conway cup's lid and edges. One milliliter of boric acid and one drop of a combination of methyl red and bromcresol green indicators were placed in the middle of the cup. One milliliter of supernatant and one milliliter of saturated sodium carbonate (Na_2CO_3) solution were placed on either side of the Conway cup. After closing the Conway cup and giving it a gentle shake to ensure that the sodium carbonate and supernatant solution were evenly combined, it was allowed to left at room temperature for 24 hours. The cup lid was opened 24 hours later, and 0.0055 N H_2SO_4 is used to titrate the solution until the color shifts from purple to pink. Calculation of NH_3 production as follows (Fathul and Wajizah, 2010):

NH_3 = (ml titration × N-H_2SO_4 × 1000) mM

Total digestible nutrients (TDN) analysis

The examination of organic matter digestibility (OMD) and ether extract digestibility (EED) yielded the TDN analysis. The in vitro digestibility assay started with setting up a water bath at 38–39°C. Next, a sample weighing 0.55–0.56 grams was placed in a fermenter tube, to which 10 milliliters of rumen fluid and 40 milliliters of McDougall solution were added. CO_2 was then pumped to make the fermenter tube anaerobic, and the tube was then securely sealed. After shaking the fermenter tube to mix the solution, it was incubated in a water bath for 48 hours, shaking every six hours. After 48 hours of incubation, the fermenter tube was cooled by submerging it in cold water for 10 to 15 minutes. The residue was then removed using a centrifuge set to 3,500 rpm for 15 minutes, and 50 milliliters of pepsin HCl was added. Fermentation tube and its contents were incubated again for 48 hours and shaken every 6 hours. After 48 hours fermentation was stopped and filtered using Whatman 41 filter paper for OMD test and qualitative filter paper for EED test.

The filtered samples were analyzed for organic matter content by ovening at 105°C for 4 hours and then ashing in a furnace at 600°C for 4 hours. Ether extract analysis was carried out by ovening samples at 105°C for 6 hours, after that samples were extracted with N-hexane solution using Soxhlet for 3 hours. Samples were taken and dried and then ovened for 2 hours at 105°C. Calculation of OMD, EED and TDN as follows (Tillman *et al*., 1998):

OMD (%)=(OM sample (g)-(OM residue-OM blank)(g))/(OM sample (g)) x 100%
EED (%)=(EE sample (g)-(EE residue-EE blank)(g))/(EE sample (g)) x 100%
OMd= (OMD × OM content) ×100%
EEd= (EED × EE content) × 100%
TDN= OMd (%) + (1,25 × EEd (%))

Results

The average of VFA, NH_3 and TDN production of Odot grass supplemented with buffalo rumen-contents and enriched with N, S, P, and various level of RAC in balibul sheep in vitro for each treatment was presented in Table 1.

Table 1. VFA, NH_3 and TDN Production

Variables	Treatments				SEM
	T0	T1	T2	T3	
VFA (mM)	56.67^{c}	63.33^{b}	73.33^{a}	66.67^{b}	2.30
NH_3 (mM)	5.61^{a}	5.28^{b}	5.06^{c}	5.04^{c}	0.07
TDN (%)	50.47^{c}	54.22^{b}	58.49^{a}	54.85^{b}	0.90

Different superscripts in the same row indicate significant differences (P<0.05)
T0 = Odot grass + buffalo rumen-contents probiotics + NSP + 0% cassava flour
T1 = Odot grass + buffalo rumen-contents probiotics + NSP + 2.5% cassava flour
T2 = Odot grass + buffalo rumen-contents probiotics + NSP + 5% cassava flour
T3 = Odot grass + buffalo rumen-contents probiotics + NSP + 7.5% cassava flour

Result of analysis of variance showed that treatment of Odot grass supplemented with buffalo rumen-contents as probiotics and enriched with N, S, P, and supplemented with cassava flour as RAC source significantly increased (P<0.05) on VFA production. The average VFA production of each treatment was 56.67 mM (T0), 63.33 mM (T1), 73.33 mM (T2), 66.67 mM (T3). Treatment applied significantly decreased (P<0.05) on NH_3 production. The average NH_3 production for each treatment was 5.61 (T0), 5.28 (T1), 5.06 (T2), and 5.04 mM (T3). Result of the analysis of variance showed that treatment applied significantly increased (P<0.05) on TDN value. The average TDN values in each treatment were 50.47% (T0), 54.22% (T1), 58.49% (T2), and 54.85% (T3).

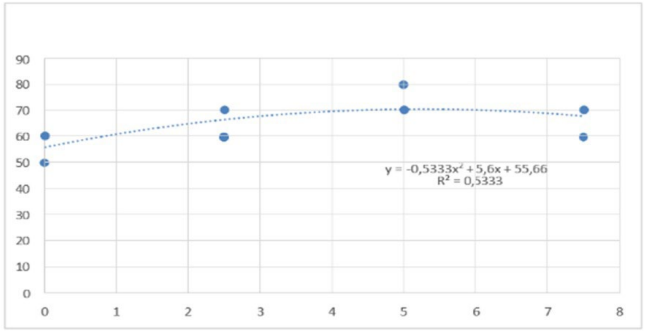

Figure 1. Orthogonal Polynomial Analysis for VFA.

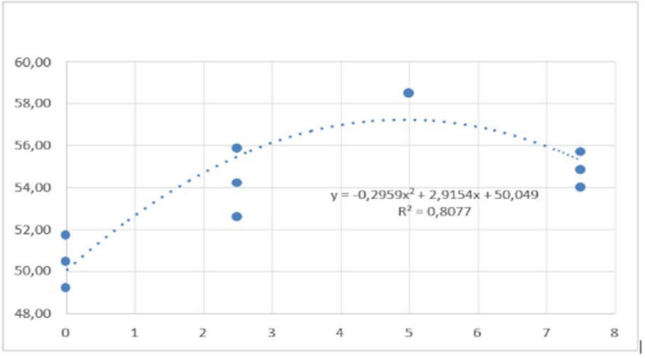

Figure 2. Orthogonal Polynomial Analysis for TDN.

Discussion

VFA production in vitro showed that the addition of cassava flor as RAC source increased the VFA of Odot grass. Increased VFA production was closely related to the increase in organic matter digestibility (OMD). High VFA production is influenced by the amount of organic matter that is easily digested by microbes in the rumen, so that the fermentability process can work optimally (Noziere *et al*., 2010). VFA produced are not only derived from carbohydrates but also from the protein of the feed provided (Ramos-Suarez *et al*., 2021). VFA are a source of energy for ruminants and play a role as a source of carbon skeletons in the formation of microbial proteins (Aluwong *et al*., 2010). Increased of VFA production indicated that rumen microbes were working optimally in utilizing feed. This occurs because the N, S, P elements and cassava flour provided support microbial growth in the rumen, thus optimizing it. Nitrogen is also required by rumen microbes for rumen microbial synthesis (Zhu *et al*., 2020). Sulfur is essential for rumen microbes to form sulfur amino acids such as cystine and methionine (Zain, 2009). Phosphorus administration functions to form microbial proteins and aids in the production of DNA, RNA, and ADP (Suharyono *et al*., 2015). Fulfilling the N, S, and P requirements for microbes leads to optimal microbial growth and development. Proteins, which are necessary for almost all cellular functions, including transport, structural support, and enzyme catalysis, are fundamentally built of nitrogen (Grzyb *et al*., 2021); nucleic acids, which carry genetic information, also rely on nitrogen for their structure. Furthermore, nitrogen is a component of ATP, the primary energy currency of the cell, and other vital molecules such as NADH and NADPH, which are crucial for cellular respiration and biosynthesis. Sulfur is essential for the formation of certain amino acids such as cysteine and methionine, which are crucial for protein structure and function. It also plays a role in enzyme cofactors and redox reactions (Wu *et al*., 2021). For microorganisms to flourish, phosphorus (P) is a macronutrient that is necessary for many cellular functions. It is a crucial part of phospholipids, DNA, and RNA, which are necessary for genetic material, energy transfer, and cell structure (Oliverio *et al*., 2020). VFA are the end result of fermentation of carbohydrates contained in feed and absorbed by the rumen walls. The end product of carbohydrate fermentation is VFA, primarily acetic, butyric, and propionic acids (Nisa *et al*., 2017), it also produces carbon dioxide (CO_2) and methane (CH_4).

VFA production in T3 treatment decreased with the addition of 7.5% cassava flour. The decreased of VFA production was influenced by the crude fiber content in the treatment feed (crude fiber 22.56% in T2 versus 21.68% in T3). The decreased VFA production is influenced by the decreased of crude fiber content as a carbohydrate source (Hikmawan *et al*., 2019). Crude fiber of feed components are cellulose, hemicellulose, starch, and lignin. Cellulose, hemicellulose, and lignin form bonds that are difficult to digest and will inhibit rumen microbes from degrading carbohydrate feed sources, resulting in low VFA production (Kusmiati *et al*., 2024). In addition to crude fiber, low VFA is also influenced by microbial protein. Low VFA production can be influenced by microbial protein, which causes abnormal microbial activity and the process of protein degradation into amino acids, ultimately producing NH_3 and α-keto acids which are not optimal (Van Soest, 1994), α-keto acids will be converted into VFA in the form of iso-valerate, iso-butyrate and 2 methyl butyrate which are used as a source of carbon skeletons for microbial protein synthesis. VFA production ranges from 56.67–73.33 mM. The optimal VFA production for microbial growth ranges from 60–120 mM (Waldron *et al*., 2002). Factors that influence VFA production are the amount of feed and the level of feed fermentability. The type of microbe, absorption and fermentability of carbohydrate source feed in the rumen are among the factors that influence VFA production (Muchlas *et al*., 2014). VFA production can also be influenced by bacteria in the rumen, especially carbohydrate-digesting bacteria. Bacteria found in the rumen include cellulolytic and proteolytic bacteria which influence the results of feed breakdown in

the rumen, including VFA (Yurleni *et al*., 2013).

NH_3 production decreased with increasing levels of cassava flour. The decrease in NH_3 occured because the addition of cassava flour as RAC source allegedly caused NH_3 to be immediately synthesized into microbial protein. Undecomposed protein will reduce NH_3 production because it does not degrade. Low NH_3 production is caused by the low solubility of feed ingredients, especially protein, because insoluble protein is more easily degraded by the rumen, resulting in low NH_3 production (Wijayanti *et al*., 2012). Protein availability must also be balanced with energy sources to ensure a balanced diet. If energy exceeds nitrogen availability, fermentation efficiency and rumen microbial growth will decline (Fitriyanto *et al*., 2021). The decreased degradability of crude protein) is due to the microbial synthesis of non-protein nitrogen (NPN) into ammonia, resulting in pure protein. This occurs because feed protein is broken down into amino acids, which are then converted to NH_3; therefore, decreased protein levels affect NH_3 production. Pure protein contained in feed will be broken down by microbes into amino acids which will be converted into ammonia and α-keto acids. Feed containing protein will be broken down by rumen microbes into ammonia and α-keto acids to be used in the formation of microbial proteins (Kusumaningrum *et al*., 2018). The amount of crude protein in diet affected how much NH_3 was produced. Crude protein in the diet affects the rumen's production of NH_3 (Prayitno *et al*., 2018). NH_3 production increases with protein concentration, and vice versa; if crude protein is low, NH_3 production is likewise low.

NH_3 is a source of nitrogen for rumen microbes. If ammonia level in rumen meets the microbial needs for growth and development, microbial activity in digesting feed will be more optimal. For rumen microbial protein production, NH_3 is the primary nitrogen supply, allowing for the best possible rumen digestion (Sairullah *et al*., 2016). In addition to protein, NH_3 production is also obtained from NPN for microbial protein synthesis (Hindratiningrum *et al*., 2011). The average NH_3 production in treatments T0 (5.61 mM), T1 (5.28 mM), T2 (5.06 mM), and T3 (5.04 mM). NH_3 concentration to support optimal rumen microbial protein ranges between 4 - 12 mM (Erwanto *et al*., 1993).

The analysis of variance result indicated that adding cassava flour as a source of carbohydrates raised the TDN value. The increased TDN value indicated that feed fermentability in the rumen also increased with the absorption of more feed nutrients by the body. The increased TDN value occured because feed digestion in the rumen works optimally due to the presence of sufficient N, S, and P elements for rumen microbial production, resulting in better digested energy. The presence of N, S, and P elements can balance the fermentation process and optimize rumen microbial activity (Fadilla *et al*., 2020). The supplementation of cassava flour as RAC source can increase rumen microbial fermentation by increasing carbohydrate-digesting bacteria. Increased digestibility of diets and nutrients results from the proliferation of bacteria in the rumen, particularly cellulolytic bacteria, which are supported by an availability of carbohydrate sources (Uhi *et al*., 2006).

The decrease in TDN values at T3 is related to the decrease in organic matter digestibility (OMD). This was because organic matter contains crude protein (CP), ether extract (EE), crude fiber (CF), and nitrogen-free extract (NFE); so if OMD decreased, TDN value will also decrease. TDN value is related to the digestibility of feed nutrients such as crude protein, ether extract, crude fiber, and nitrogen-free extract in feed that help fermentation process and support microbial growth in the rumen (Munawaroh *et al*., 2015). The high and low TDN values can be caused by OMD of each treatment. OMD of T0 was 58.51%, T1 was 63.27%, T2 was 64.86%, and T3 was 62.83%. The organic matter content in the feed can determine the high and low TDN values utilized as energy by rumen microbes (Saputro *et al*., 2016). Factors that influence the TDN value are livestock's ability to digest TDN and the quality of the feed consumed. TDN value is influenced by the balance of crude protein, energy, ether extract, and crude fiber (Faradilla *et al*., 2019).

The orthogonal polynomial analysis was used to determine which level of cassava flour supplementation as RAC source provided the most optimal response to VFA, and TDN. The orthogonal polynomial test was only used for the VFA and TDN variables because there was a decrease in NH_3 production with increasing cassava flour supplementation as RAC source.

Based on orthogonal polynomial analysis for VFA variable, a linear equation was found, namely $Y = -0.5333x2 + 5.6x + 55.667$ ($R^2 = 0.5333$), where VFA production (Y) was 70.367% and the supplementation of cassava flour as RAC source (X) was 5.2%; so it can be concluded that the optimal treatment for VFA production was T2 treatment with the supplementation of 5% cassava flour as RAC source (T2 = 73.33 mM). R^2 value of the equation obtained was 0.5333. It meant that 53.33% of the observed variation in the dependent variable could be explained by the independent variables in the model (supplementation of N, S, P elements and cassava flour), while the remainder (46.67%) was influenced by other factors not included in the model (Figure 1).

Then, based on the orthogonal polynomial analysis for TDN value, a linear equation was found, namely $Y= -0.2959x2 + 2.9154x + 50.049$ ($R^2 = 0.8077$), where TDN value (Y) was 57.23% and the addition of RAC (X) was (4.9%), so it can be concluded that the optimal treatment for the TDN value was in treatment T2 with the addition of 5% RAC (T2 = 58.49%). R^2 value of the equation obtained was 0.8077. It meant that 80.77% of the observed variation in the dependent variable could be explained by the independent variables in the model (supplementation of N, S, P elements and cassava flour), while the remainder (19.23%) was influenced by other factors not included in the model (Figure 2).

Conclusion

Odot grass supplemented with buffalo rumen contents as probiotics enriched with N, S, and P, and supplemented with different levels of cassava flour as RAC source increased VFA production and TDN values, and reduced NH_3 production. Based on VFA, NH_3 production and TDN, it is recommended to supplement cassava flour as a source of RAC at 5% in buffalo rumen contents containing N, S and P.

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Conflict of interest

The authors have no conflict of interest to declare.

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