Effect Of Dietary Concentrate And Bypass Fat Supplements On *In Vitro* Rumen Fermentation, Digestibility And Rumen Microbial Population In Buffaloes

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Abstract

Background: The effect of *Brachiaria decumbens* supplemented with concentrate and bypass fat ratio 100, 70:30, 70:15:15 (w/w) on nutritional composition, *in vitro* rumen fermentation and microbial population in Murrah cross and Swamp buffaloes were investigated. Incubation were conducted using rumen fluid obtained from the breed of each buffalo which were fed the same based diet (100% *Brachiaria decumbens*). For the *in vitro* fermentation characteristic after 48h incubation, total gas production, pH, total volatile fatty acid (TVFA), apparent rumen degradable carbohydrate (ARDC), methane (CH₄) and ammonia (NH₃) were determined. The molecular technique also was used to quantify rumen total bacteria, total protozoa, total methanogens, *Fibrobacter succinogens* and *Ruminococcus albus*.

Results: The results revealed Diet C showed significantly highest in dry matter, crude fat, metabolized energy and optimum in crude protein and carbohydrate value while lower in crude fiber as compared to Diet B and Diet A (P<0.05). In both breed were showed parameters of gas production, total volatile fatty acid (TVFA) and its proportion, and total microbial population were increased parallelly with the increase of 30% concentrate levels in Diet B, while Diet C was moderate with the presence of 4% bypass fat and 26% of concentrate (P<0.05). The methane concentration as well the total methanogens population increased significantly (P<0.05) in Diet B when compared among other diets, but no significant difference was found when comparing between buffalo species. Meanwhile, pH value was slightly decreased with the dietary supplementation in both breeds, but the population of cellulolytic bacteria was not affected.

Conclusions: This study showed that dietary concentrate and bypass fat supplementation had improved nutritional composition, *in vitro* fermentation characteristics by increasing VFA concentration, altering total microbial population, and potentially used as new diet for buffaloes in Malaysia.

Background

In Malaysia, Murrah cross and indigenous Swamp are the common buffaloes breed founded and play a significant role in producing a high value of animal protein such as meat and milk for human consumption [1]. According to Cruz [2], the buffalo industry in Malaysia contributed around 21% of protein sources and 11.5% of the buffalo meat from the total of ruminant meat. Thus, the government has set the goal of growing the population of livestock such as cattle and buffalo to reach Malaysia's target of 50 percent self-sufficiency by 2020. Unfortunately, the buffalo population in Malaysia has declined progressively with a declining rate of 1.97% per annum from 2013 until 2017 [3]. Indeed, the number of animal supplies to meet the local demand for meat were still inadequate, following the increase in a human population [4]. One of the main factors that could contribute to the dropped in buffalo production is due to inefficiency of feed management. Indeed, imbalance nutrition and inadequate availability of quality feedstuffs and their utilization are hampering the performance and productivity of the buffalo. Previous study has reported that low buffalo production in developing countries such as Indonesia, India and Philippine may be due to insufficient nutrient supply in a ration dependent on high forage as well as less supplementation given to animals [5, 6]. Therefore, new sources, strategies and technologies for improving the feeding systems are essential for buffalo production systems [7].

Recently, dairy and meat farmers worldwide are incorporating feed supplementation in animal diets to enhance the performance and health status of animals [8]. Supplementation such as concentrate, and bypass fat are common practice given by farmers to buffaloes in Brazil, India and Philippine in order to provide a high amount of nutrients to increase growth and production performance [9]. However, because of scarce information on nutritional value, rumen fermentation characteristics and rumen microbial population, the benefit of using these supplements in the basal diet, especially in the buffalo industry in Malaysia, is still limited.

Previous research suggested that the ratio and types of supplementation vary significantly in their rumen fermentation characteristics and rumen microbial population, which may be due to the variations in the physical and chemical

compositions within each supplement [11, 45]. Bypass fat and concentrate containing high fat and protein were commonly used in ruminant nutrition, especially in cattle and sheep to regulate the activity of the rumen fermentation [10] and rumen microbial population. Together with that, supplementation in buffalo diets tends to be a potential source in improving the performance and productivity of buffalo.

Therefore, the aim of this research was to determine the composition of the nutrients and to evaluate *in vitro* fermentation characteristics and rumen microbial population of three different diet supplemented with different proportions of concentrate and bypass fat sources on a different breed of buffaloes, such as Murrah cross and Swamp.

Methods

Preparation of diet

The experimental design was fully randomized with three replicates per treatment. Three total mixed rations (TMR) were prepared to contain three components namely *Brachiaria decumbens* (G), commercial concentrate (C) (corn grain (44.7%), rice bran (18.0%), soya bean meal (32.0%), calcium carbonate (1.0%), molasses (2.8%), vitamin-mineral premix (0.5%), sodium chloride (0.5%), dicalcium phosphate (0.5%)), and bypass fat (sources from fractionated palm fat without trans-fat, B) (**Table 1**). The total mixed ration was formulated and labeled as a control for Diet A consist of 100% G, for Diet B consist of 70%G:30%C and Diet C consist of 70%G:26%C:4%B.

Proximate analysis

All samples used in the current study, namely the control (Diet A) and treatment group (Diet B and Diet C), were analyzed for nutritional composition (% DM). Dry matter (DM) of the samples was calculated using a standard analytical method by 24 hours oven-drying at 105°C [13]. Organic matter (OM) was evaluated by combustion the sample at 550°C for 4 hours in a muffle furnace [13]. Crude protein (CP) determination has included three separate stages. The first stage was the sample digestion process with concentrated sulfuric acid (H₂SO₄), followed by a distillation process with sodium hydroxide (NaOH) by Kjeldahl system and finally titration against acid. The amount of nitrogen (N) found was converted to crude protein (value crude protein= nitrogen x 6.25) [14]. Crude fibre (CF) was determined by washing and boiling the sample in H₂SO₄ and NaOH using fibre bag technique. Analysis of ether extract (EE) was determined by petroleum ether extraction [14]. The value of total carbohydrate was given by: 100 - (% of ash + % of total lipid + % of protein + % of crude fiber) [13]. Gross energy (GE) of the diets were measured using a bomb calorimeter (IKA C2000 Basic, IKA-Werke, Staufen, Germany). All experiments have been carried out in triplicate.

Lignocellulose content

All the diets were weighed 1 g per fibre bag. Insoluble fibres (lignin, cellulose and hemicellulose) in the samples were calculated as neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) based on the Gerhardt application fibrebag-system protocol [15]. All experiments were done in triplicate.

Neutral detergent fiber analysis

Fiber bag was dried at 105°C for 1 hour and was allowed to cool for 30 min in desiccator. Sample with 1 g was weighed in fiber bags. Solution of neutral detergent fiber was prepared using EDTA ethylenediamine tetra acetic acid-disodium salt and disodium tetra borate-decahydrate, dodecylsulfate-sodium salt, 2-ethoxyethanol, sodium dihydrogenphosphate and heat-stable α -amylase. The NDF (%) were determined as follow using the blank value as shown in the equation below [15]:

$$\text{NDF}(\%) = \frac{(m3 - m1) - (m4 - m5)}{m3} \times 100$$

Where m1 – weight of fibre bag (g), m2 – initial sample weight (g), m3 – weight of crucible with dried fibre bag and sample residue after digestion, m4 – weight of crucible with as (g), m5 – blank value of empty fibre bag (g).

Acid detergent fibre (ADF) analysis

Acid detergent fibre solution was prepared by diluting N-cetyl-N, N, N-trimethyl-ammoniumbromide in sulphuric acid. The procedure of ADF analysis was similar to the NDF analysis [16].

Acid detergent lignin (ADL) analysis

For the ADL procedure, the ADF procedure was used as a preparatory step. However, the components of cellulose and lignin were not eluted from the feed by the acid detergent solution. The cellulose was therefore dissolved with 72% sulphuric acid in order to receive the crude lignin (ADL). Hemicellulose was calculated as NDF – ADF and cellulose as ADF – ADL [15].

***In vitro* fermentation study**

Site of study

This study was conducted in Buffalo's Breeding and Research Centre located at Telupid, Sabah, Malaysia (Coordinate 5° 30' N, 117° 7' E). The buffaloes were cared for in accordance with the animal ethics guidelines of the Animal Utilization Protocol approved by the Institution Animal Care and Use Committee (IACUC)(Approval No. UPM/IACUC/AUP-R039/2016), Universiti Putra Malaysia. The sampling from the experimented animals were strictly conducted under veterinary supervision.

Source of rumen fluid from animals and experimental design

Murrah cross and Swamp buffaloes with live weight 330 ± 4 kg, were maintained on a daily diet containing 100% dry matter (DM) fresh *Brachiaria decumbens* grass, drinking water and trace mineral blocks were provided *ad libitum* throughout the experiment. Animals were fed twice daily at 08:00 and 17:00 h on a dry matter basis. Rumen contents were collected via stomach tubing from both Murrah cross and Swamp before the morning feed at for assessment of *in vitro* fermentation characteristics [36]. Rumen samples were mixed, squeezed, and strained through a four layered cheesecloth. The ruminal fluids samples were immediately placed in prewarmed insulated vacuum flasks, sealed, maintained at 39 °C, and immediately transported to the laboratory.

***In vitro* incubation with rumen fluid and analysis.**

The *in vitro* incubation using ruminal Murrah cross and Swamp ruminal fluid was performed in syringes (volumes 100ml) according to the published method [17]. The rumen fluid was mixed for 30 seconds in a mixture (Waring Products Division, New Hartford, USA) and filtered over four layers of cheesecloths. The rumen fluid (5 mL) was mixed with 20 ml of bicarbonate and phosphate buffer in 100 mL of sterile gas-tight syringes containing 0.25 g of each sample (Diet A, Diet B and Diet C) following the modified by [18]. Air was removed from the syringes before the tip had been closed. The experimental design was a 2x2x3 factorial arrangement in a completely randomized design (CRD), with three pretreatment replications including triplicates of blank (medium only) in three incubation sequences. The dietary treatment were three samples (Diet A, Diet B and Diet C) with two different kind of rumen fluid (Murrah cross and Swamp). All treatments were done in triplicate and incubation was done at 39°C for 48 h in the oven. Syringes were

shaken carefully to ensure complete mixing of the incubated contents. Gas production was measured and recorded by reading the scale on the syringe at 0, 2, 6, 8, 12, 18, 24, and 48 h of incubation. Following 48 h incubation, the pH value of the rumen fluid were measured with a pH meter and the rumen fluid were acidified with 25% metaphosphoric acid in the water, centrifuged (10 min, 4 °C at 15,000 × g) and filtered before the filtrate was used to determine the volatile fatty acid (VFA). After that, 500ul of supernatant with 500ul methyl n-valeric acid (internal standard) were transferred into the 1.5 ml vial until further analysis.

Determination of volatile fatty acid and apparent rumen degradable carbohydrate

The sample recovered for VFA was analyzed using a gas chromatography flame ionization detector (GC FID: Hewlett Packard 6890 GC system). Acetic, propionic and butyric acids (mmol) were utilized to estimate methane (CH₄) production according to the following equations [19]: CH₄ (mM/L) = 0.45(Acetic) - 0.275(Propionic) + 0.4(Butyric). The Apparent rumen degradable carbohydrate (ARDC) was determined based on the equation below [17]: with 162 the assumed molecular weight of 1 mol fermented carbohydrates [20] and Ac: acetic acid, Pr: propionic acid and But: butyric acid expressed as net micro-molar production.

$$\text{ARDC (mg)} = \left(\frac{\text{Ac}}{2} + \frac{\text{Pr}}{2} + \text{But} \right) \times \frac{162}{1000}$$

Ammonia determination

Ammonia (NH₃) was determined using the colorimetric method. Rumen filtrated samples were centrifuged at 12,000 x g for 20 min. Five ml of supernatant was collected and kept for further determination of the ammonia content using a protocol described by [21]. A standard solution was prepared using 1.908 g of ammonium chloride dissolved in 500 ml distilled water to give 1000 mg/l ammonia- nitrogen (ammonia-N). A standard 0.2, 0.5, 1.0, and 2.0 ppm solution were prepared by dissolving 0.02, 0.05, 0.10, and 0.20 ml of the stock solution with 100 ml distilled water, respectively. Five ml of water (blank) or standard was added in an Erlenmeyer flask, and 0.2 ml of the phenol solution was added and swirled. In sequence, 0.2 ml of nitroprusside and 0.5 ml of oxidizing solution were added. The flask was then swirled, stopped and allowed to stand for one hour at room temperature. This intensity was measured at a wavelength of 640 nm by spectrophotometer (Thermo Scientific, GENESYS 20). The regression equation was determined from blank and standard samples before ammonia-N was estimated in the samples.

Determination of rumen microbial populations

Genomic DNA isolation

For microbial analysis, the rumen fluid samples were centrifuge at 10,000 x g for 15 min and the DNA was extracted from the precipitate using QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencis, CA) according to manufacturing procedures.

Quantitative PCR assay

As well, extracted DNA was subjected to a quantitative real-time polymerase chain reaction (qPCR) to quantify the population of the selected microbe's population. The primers used are expressed in **Table 2**. Real-time quantitative PCR was conducted for the extracted DNA using the BioRad CFX96 Touch (BioRad, USA). The qPCR reactions were performed in a total volume of 25 µL using the Maxima SYBR Green Mastermix (Thermo Scientific, USA). The reactions consisted of 12.5 µL SYBR Green Supermix, 1 µL of each reverse primer and forward primer, 2 µL of extracted DNA samples, and 8.5 µL of DNase-free H₂O. The qPCR conditions were applied to all samples which consisted of an initial incubation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing for 30 s, and extension at 72°C for 20 s.

The annealing temperatures for primers of total bacteria, total fungi, total protozoa, total methanogens, *Butyrivibrio fibrisolvens*, *Ruminococcus albus* and *Fibrobacter succinogenes* were 55°C [46]

Statistical Analysis

All analyses were carried out using Statistical Package for Social Science 25.0 (SPSS software for Windows, release 25.0 SPSS, Inc., Chicago, IL, USA). Since two types of the breed were used in this study, the parameter for breed were analyzed as an independent T-test. For the parameters diet, incubation period of gas production and rumen microbial population, the above three sets of parameters were statistically evaluated using a one-way analysis of variance (ANOVA) using the general linear model (GLM) procedure for a complete randomized design at a significance level of 5% between the controls and different treatment of diets [78,79]. Mean differences were determined using the Duncan multiple range test at $P < 0.05$.

Results

Nutritional composition of the total mixed rations

The nutritional compositions of Diet A, Diet B and Diet C are shown in **Table 3**. *Brachiaria decumbens* was used as the forage source in this study and contained 6.09% CP and 64.27 neutral detergent fiber (NDF). In Diet B, 30% of concentrate was formulated with forage to contain 8.08% CP, 57.96 NDF, and 12.1 MJ/kg GE. For Diet C, 26% of concentrate and 4% of bypass fat were formulated with forage to contain 6.56% CP, 49.63% NDF, and 14.59 MJ/kg GE.

The study showed CP and carbohydrates contents were significantly ($P < 0.05$) higher recorded in Diet B when compared to Diet C and Diet A. Ash content was showed highest in Diet C followed by Diet B and Diet A, but no significant ($P < 0.05$) were observed among the diets. The value of CP, carbohydrate and ash was higher in Diet B when compared with other diets due to addition of commercialize concentrate which has ingredient more on CP and carbohydrate sources such as corn grain, soybean meal, rice bran and molasses. This finding also supported by Agle et al. [25] showed the diet formulated by addition of high concentrate increase the CP and starch content due to the present corn silage, corn grain, barley grain, cottonseed, wheat and alfalfa hay. Other than that, the total ash content of by Diet C and Diet B (5.93% and 5.69%) was comparatively higher than Diet A (5.09%) due to high level of minerals such as calcium from bypass fat and concentrate. According to Ranjan et al. [37], the study was consistent with our finding in Diet C that increased total ash content of bypass fat supplement (21.78%) was relatively higher than other feeds and fodder due to a high calcium contain from bypass fat.

The content of EE and GE showed significantly ($P < 0.05$) higher in Diet C followed by Diet B when compared to control (Diet A). The high value of EE and GE value in Diet C as compared to other diets is due to the addition of bypass fat supplement which this supplement able to provide high fat and energy. Besides, it has been reported by Kumar and Thakur [38] which bypass fat is able to provide 74.36% of total fat content and predominantly have 15.06% of palmitic acid (C16:0), 12.31% of stearic acid (C18:0), 24.32% of oleic acid (C18:1c9) and 31.51% linoleic acid (C18:2).

The content of CF, NDF, ADF, hemicellulose and cellulose in all treatments (Diet B and Diet C) were decreased significantly ($P < 0.05$) as compared with control (Diet A). Meanwhile, ADL content showed lowest in Diet C and Diet B as compared with Diet A, but no significant ($P < 0.05$) were observed among the diets. Our finding has same pattern with other study by Singh et al., [39] showed that the lower forage or roughage to concentrate ratio (80:20, 50:50, 20:80) give the lower of NDF (66.10%, 57.25%, 48.40%) and ADF (40.68%, 28.95%, 17.22%) value. The lower value of CF, NDF, ADF, and ADL in Diet B and Diet C (70%) as compared with Diet A (100%) can be explained due to low of fibrous supply.

In vitro gas production pattern following 48 hours incubation

Cumulative gas production (ml) for each of the diets are presented in **Figure 1**. There was a linearly increase in the levels of gas produced by all substrates in control and treatment groups throughout the 48 hours incubation period. Total gas productions were showed significantly ($P<0.05$) difference between the diets and among the breeds. Murrah cross showed the highest gas produced as compared with Swamp breed. Indeed, the highest cumulative gas production across different breeds (Murrah cross vs Swamp) following 48 hours incubation was diet containing high concentrate ratio (Diet B) (18.07ml vs 14.50ml, respectively) followed by diet supplemented with 4% bypass fat and 26% concentrate (Diet C) (14.40ml vs 10.20ml, respectively). Meanwhile, Diet A (100% forage) was shown the lowest gas produced after 48 hours incubation (11.93ml vs 9.30ml, respectively).

***In vitro* rumen fermentation pattern following 48 hours incubation**

The pH, TVFA production, the proportion of volatile fatty acid (acetate, propionate, and butyrate), acetic to propionic ratio (C2:C3), methane, and ammonia (NH_3) production for Murrah cross and Swamp breed were shown in **Table 4**.

In Murrah cross breed study, *in vitro* fermentation parameters of TVFA, propionic, butyric, and methane production were significantly ($P<0.05$) influenced by the diets. The pH value was showed lower in treatment group (Diet B and Diet C) as compared with control group (Diet A), although not significant ($P>0.05$). Furthermore, the value of TVFA, fatty acid proportions (acetate, propionate, and butyrate), ARDC and methane levels in control group showed lower as compared with Diet B but higher when compared to Diet C. Meanwhile, the C2:C3 ratio, and NH_3 level was recorded high in treatment group as compared with control group.

For Swamp breed, the *in vitro* fermentation parameters of TVFA, propionic, butyric, and methane production were significantly ($P<0.05$) influenced by the diets. The pH value and C2:C3 ratio was revealed lesser in treatment group (Diet B and Diet C) ($P>0.05$) when contrasted with control group (Diet A). This finding was in line with Kang and Wanapat [42], who reported pH of Swamp breed was around 6.7 to 6.9 with extension time of *in vitro* fermentation incubation up to 48h in the treatment with higher level of concentrate ratio (75 roughage:15 concentrate). Moreover, the value of TVFA, acetate production, ARDC and methane levels in control group showed lower as compared with Diet B but higher when compared to Diet C. Nonetheless, propionate, butyrate, and NH_3 level was showed high in treatment group as compared with control group.

In this study also showed value of TVFA, acetic, butyric, and methane production were significantly ($P<0.05$) affected by the breeds. The level of acetate was found higher in both breeds followed by propionate and butyrate. The C2:C3 ratio were showed higher in Murrah cross treatment groups, however the C2:C3 ratio showed vice versa in Swamp treatment group. In addition, the mean pH of rumen fluid and ARDC levels pattern at 48 hours reported similar among the different breeds, while no significant ($P>0.05$) was found. Interaction among the breeds on production of CH_4 were significant ($P<0.05$) after 48 h incubation. Furthermore, the mean values of ruminal ammonia for both breeds were showed increased ($P>0.05$) by the addition of supplementation in treatment groups.

***In vitro* rumen microbial population pattern following 48 hours incubation**

The effect of supplemented diet on the *in vitro* rumen microbial population of Murrah cross and Swamp buffaloes quantified by qPCR are shown in **Table 5**. Astoundingly, result of this study showed the population of total bacteria, total protozoa, total methanogens, *Ruminococcus albus* and *Fibrobacter succinogens* was highly affected ($P<0.05$) when compared with the treatment diets. Through supplementation of 30% concentrate in Diet B was revealed significantly highest ($P<0.05$) in numbers of all microbe as compared with other diets. Meanwhile, additional of 26% concentrate with 4% bypass fat in Diet C only tended to be moderate in number of all microbes as compared to Diet A and Diet B.

The population of total bacteria and total protozoa in Swamp breed not significantly different ($P>0.05$) and being higher in Diet B as compared with Murrah cross. However, Swamp buffalo recorded significantly higher in composition of *Ruminococcus albus* as compared with the Murrah cross. The population of total methanogens was not different ($P>0.05$) and being higher in Diet B and Diet C for both breeds. In addition, *Fibrobacter succinogens* population showed slightly increased ($P>0.05$) in numbers on both breeds when tested under Diet B as compared with Diet C and Diet A.

Discussion

Nutritional composition of a total mixed ration

In the current study, Diet A (control, 100% *Brachiaria decumbens* with no supplementation) was a diet that commonly practiced by buffalo farms in Malaysia. In this group, the nutritional composition of *Brachiaria decumbens* for CP (6.29%), EE (4.03%), CF (26.38%), hemicellulose (30.3%), NDF (66.6%) and ADF (34.4%) were similar as reported earlier [27]. In addition, low crude protein content of the *Brachiaria* grass reported in this study could be due to several factors including species variety, soil, pasture management, maturity, and climate [44]. Indeed, the *Brachiaria* grass used was harvested during hot seasons and the pasture was poorly managed by the farmer with improper irrigation and inadequate fertilizer. The study also reported that animals relying on grass alone without supplementation still inadequate to fulfill energy and protein requirement to improve their growth performance [28]. According to Kahindi et al. [47], crude protein content of dry mature tropical grasses is often below the minimum 8% that can provide minimum ammonia required for optimum microbial function in the rumen. Imbalance of energy and protein supply in buffalo ration could cause negative energy balance which lead to nutritional metabolic disease such as ketosis or acidosis [43]. To address the problem, one can supplement using commercialize concentrate and bypass fat as source of protein and fat [47].

A new diet with supplementation (Diet B and Diet C) were formulated to improved levels of nutrient value parameters such as CF, EE, CP, carbohydrates, GE, hemicellulose and cellulose content ($P<0.05$). The finding of nutritional composition on new formulated diet with supplementation was in line with Hartati et al. [22] and Van Dung [7], who reported that by addition of supplementation bypass fat and concentrate in the diet were able to increase the fat and protein composition and did not give any adverse effect on animals. Moreover, supplemented diet also was proven to be as additional feedstuffs providing nutrients required for animals to support the desired levels of production [28]. According to report by Liu et al. [29], *in vitro* gas fermentation technique is one way to evaluate the quality of formulated diet. Moreover, the researcher also reported that *in vitro* gas production has a good correlation with *in vivo* fermentation of ruminant feed [19]. Therefore, the gas production method is frequently applied to assess the ruminal fermentation of feed mixtures [30].

Effect of different diets on *in vitro* rumen fermentation parameters

In the present of *in vitro* study, the similarities of rumen fermentation characteristic in Murrah cross and Swamp buffaloes could be observed in gas production and rumen fermentation parameters in different types of diets. However, this study showed Murrah cross has dominant effect on gas production ($P<0.05$) as compared with Swamp breed. The TVFA, proportion of VFA, pH, and methane are the prominent internal environmental markers of rumen fermentation.

The present study showed the production of gas, TVFA, ARDC and methane concentration was increased by the present of a high concentrate ratio in Diet B, which can have two justifications. Firstly, elevated of fermentation activity occurred in syringes containing high ratio concentrate, which led to greater gas production, ARDC and methane produced. Serment et al. [12] also reported that a high ratio of concentrate in the basal diet increases the cumulative gas production, total volatile fatty acid as well as methane levels at 24 hours incubation of *in vitro* fermentation. Other studies also agreed on an increase in cumulative gas volume as the ratio of concentrate to hay increased in the donor animal diet [31]. Concentrate digestibility is faster than forage digestibility, which explains the higher total gas production observed at

high concentrate proportion [36]. Secondly, the pH in the syringes did not drop enough to have an impact on methanogenesis. Indeed, this finding also agreed with Serment et al. [12], which revealed that inhibitory effects of pH on methanogenesis occurs with a pH less than 6.0. In Diet B, the increased of butyrate concentration compared to other diets suggests that acetate was a precursor to butyrate [65]. The high production of VFA in high concentrate level and low production of VFA in low proportion of concentrate in this study in line with Lana et al. [57] finding when they fed steers with high ratio of concentrates, the VFA level increased significantly. Meanwhile, the presence of high carbohydrates (starch and sugars) such as concentrates usually process of fermentation occur faster than forage and causing in increased levels of propionate. This research explained the higher of VFA level, especially propionate, observed in high proportion of concentrate as compared with other diets.

Meanwhile, the supplementation of 26% concentrate and 4% bypass fat in the basal diet of buffaloes also give significies toward both quantity of gas and fermentation parameters during 48h incubation. Indeed, differences between the diets were significant for gas production parameters. It has been reported by Shawaluddin et al. [32], the degradability rate of bypass fat supplement in the basal diet is 6.42% compared with the hay only 42.56%. It showed that supplemented bypass fat has potentially function as a fat supplement where it as not fully digested in the rumen. In rumen fermentation process, sugars and the glycerol released from glycolipids are fermented to volatile fatty acids. The lower TVFA and proportion of VFA production in the diet with bypass fat have little or no effect on the rumen fermentation process. It was due to a high melting point of bypass fat, resulting in low solubility of fat in the rumen and easily digested when pH in range 2.5 that present in abomasum [33]. The lower gas production produced in Diet C was in line showed by Shawaluddin et al. [32] and it was reported that the lower gas production produced in diet supplement with bypass fat indicates that less methane was released by fat sample. The methane production across different breeds showed in Diet C produced in Murrah cross 14.06 mM/L and Swamp 19.72 mM/L of methane which 40% to 60% lower compared to Diet B (34.46 mM/L and 30.59 mM/L, respectively), and 77% to 86% lower compared to control treatment (18.08 mM/L and 22.50 mM/L, respectively).

During rumen fermentation, pH is thought to be a significant factor affecting rumen fermentation, rumen microbial population, methane production and VFA concentration [36]. Furthermore, ruminant animals are dependent on cellulolytic ruminal bacteria for digestion cellulose; hence, the higher pH observed in absence or low concentrate ratio may be due to the concentrate diet supplied to the buffalo. If rumen pH was stable in between 6.2 to 7.0, the environment of ecological rumen microorganisms could be relatively stable and could ensure a healthy fermentation of rumen [34]. In this study, pH values after 48 hours of incubation for all diets in both breeds indicate that the buffering capacity of the medium has always been adequate to maintain the pH within the 6.2 to 6.8, which is necessary to ensure a favourable environment for cellulolytic bacteria activity [23]. Although, the treatment group recorded lower in pH levels compared to control groups, the cellulolytic bacteria (*Fibrobacter succionogen* and *Ruminococcus albus*) are still capable of survival because fiber digestion enzymes required to breakdown the fiber and do not function effectively only at pH < 6.0, and also the growth rate of fibrolytic activity declines markedly at low pH (Russel and Wilson, 1996). Moreover, these findings also indicated that the increased ratio of concentrate as well as adding up bypass fat did not induce acidity in rumen environment, which is usually defined as decrease in rumen pH to below the threshold value of 6.0 [35]. In fact, this study also in agreement with Liu et al., [35] reported that type and proportion of supplements introduced into the basal diet is one of the factors affecting the pH value in rumen environment. In this study, additional high ratio of concentrates on Diet B tended to decrease the ruminal pH. However, there was no significant difference ($P < 0.05$) that could be due to carbohydrate content. This study was in line with study by Kim et al. [36], whereby the higher pH recorded in low concentrate ratio could be due to a low or no concentrate diet given to cattle. Other than that, Diet C showed a slightly decrease from Diet B in pH value due to lower concentrate proportion in the basal diet. Indeed, the presence of bypass fat in Diet C did not involve the digestion during the fermentation process, which bypass fat insolubility at range pH 6 to 7 [22].

The concentration of ammonia was altered with the supplementation of feed among the treatment groups, although not significant ($P>0.05$). The additional 30% concentrate (Diet B) in the basal diet resulted in increment of ammonia concentration as compared with the control group. This result is consistent with the study by Agle et al. [25] where inclusion of 30 to 52% concentrate on DM may slightly increase the ammonia value due to the presence of concentrate with high ratio proportion in the diet. In the meantime, the inclusion of 26% concentrate and 4% of bypass fat resulted in decrement of ammonia in both breeds. At the other hand, it was found that beef cattle were supplemented with RPF, which increased in the ammonia levels [48]. However, similar finding has been observed in the research carried out by Atikah et al. [24] which recorded a decrease in the level of rumen ammonia during fermentation in the fat enriched ruminant diet. In addition, the rumen bypass fat also has protective layer which capsulate the fat and allowing it to pass through the rumen without interfere the rumen fermentation [26]. Furthermore, the digestion of bypass fat occurs only in the abomasum and then absorbed efficiently at the small intestine.

Ruminants depend primarily on microbes for efficient feed digestion; therefore, understanding the function of rumen microbial population as well as fermentation process would have far-reaching implications. The current study found that TVFA as well as the proportion of VFA levels in Swamp were higher than those in Murrah cross when tested with Diet A and Diet C, while not much different found when tested with Diet B; indication that the pattern of rumen fermentation was different between both breeds. These results are consistent with findings of Iqbal et al. [40] and Chanthakhoun et al. [41], which also indicated different in levels of TVFA, short chain fatty acids percentage and C2:C3 ratio in *in vitro* fermentation study in Swamp and Murrah cross buffaloes that were fed with different proportion diet of forage and supplement.

Effect of different diets on *in vitro* rumen microbial population

Diet is one of the factors that influence rumen microbial communities [71,54]. A wide variety of rumen microorganisms play an important role in the digestion of fiber and other nutrients present in feed components including cellulolytic, proteolytic, amylolytic bacteria, and protozoa [46,72]. Real time PCR results showed a major shift in number of total bacteria, total methanogens, total protozoa, *Fibrobacter succinogen*, and *Ruminococcus albus* under the change of diet ration.

The disparity in the VFA concentration may be due to the presence of rumen microbes of the animal and the types and ration of diet used. Fermentation by rumen microbes resulting in production of VFA and microbial mass is indeed a potential source of protein which the host may digest and absorb for growth [69]. However, the proportions of bacteria and protozoa in the rumen are influenced by dietary composition and pH levels and also set the limits for biomass production and efficiency of feed used [70]. Our research has shown that ruminal pH is negatively correlated with the concentration of VFA. Thus, if level of VFA is greater than absorption, the pH in the rumen will decrease and leading to disturbance of the rumen microbial population [64]. Surprisingly, with the little decrement of pH in treatments group, our finding showed did not significantly difference in total bacteria numbers as compared with control. This could be linked to the concentrate diets ratio in all the treatment groups and is confirmed by the rumen fermentation results. In addition, different breed and species of animals also could be potential factor in different results of fermentation characteristic as well rumen microbial population. According to Alves et al. [66], buffaloes have a good buffering capacity to maintain high pH level and to adapt of rumen microorganisms to different energy and protein conditions in the diet compared to cattle.

Apart from archae, methanogens were known to be the main source of methane, but with the shift in dietary proportion in this analysis, the numbers of total methanogen were constant. The total methanogens population was comparable between Diet A (control) and Diet C and is consistent with the findings stated by Beauchemin et al. [54] who confirmed that bypass had no effect on methanogenesis and nutrient digestibility. Indeed, total methanogens population

significantly gives relation on total methane production in this study. It has shown that there is relationship between the number of total methanogens and methane production, which is comparable to the research of Kim et al. [36] study. Analysis of the total methanogen revealed that after 48 H incubation from different diets had little effect on methanogen population. However, the high concentrate level did not significantly influence the methanogen density of the rumen, but significantly affected the diversity of methanogens [59]. The researcher also reported that the dominant methanogens in rumen fluid were correlated with the genus *Methanobrevibacter* under a high ratio of concentrate diet, which was consistent with the findings of other studies [58,60]. The other factors that influence the survival of methanogens are their strong association with protozoa, which enables methanogens to live extra an intracellularly in these eukaryotic organisms and to gain some protection from lower pH [61]. Concentrate feed contains lower cell wall components than forage; thus, increased concentrate diets for methane mitigation have been proposed. However, industrially sold concentrates differ slightly in nutrient content and therefore differ in production of methane [56].

Many of the parameters measured in this research showed minor variations, although not statistically significant in all cases, collectively reveal a shift fermentation trends and rumen microbial population response to concentrate and bypass fat supplementation as the study progressed. The rumen C2:C3 ratio was numerically decreased in Swamp buffalo that received supplementation as compared to control. In addition, different behaviors of methanogenesis were reported positively associated with the C2:C3 ratio [56]. Thus, the lower methane production observed in Diet C is associated with the lower C2:C3 ratio relative to Diet B. Similarly reported by Kim et al. [36] where the methane production also observed in lower in medium proportion of concentrate. However, the results can vary as the incubation period has increased or as a result of the substrate used during *in vitro* fermentation [36]. Furthermore, the C2:C3 ratio also might be differed in pattern between breeds depending on the types and purpose of breed animal. Previous research was suggested that due to interaction of genotype microbiota, different cattle breeds may carry different microbial species composition [67] and potentially offer various fermentation characteristics in the rumen.

The significant difference ($P < 0.05$) in rumen protozoan population between treatment diets was the highest increase seen in the Diet B and Diet C groups. Similar to the results of this research, concentrate and bypass fat supplementation increased protozoan population in ewes [74]. Conversely, reported by Li et al. [45], showed number of total bacteria, methanogens and protozoa were decrease with the in increasing of concentrate ratio in the diet. This finding also might be differing among study because of the variation mixture in the commercialize concentrate provided to the animals. For instance, previous study was showed used the same substrate produce different level of methane [63,64]. Thus, these results suggest that the source of rumen fluid inoculum for *in vitro* fermentation varies depending on diet given to the animals.

The *Ruminococcus albus* and *Fibrobacter succinogenes* are the most prevalent cellulolytic species in the rumen, which obtain nutrients by breaking down cellulose by the host organism's digestive system and, as such, changes in their relative amounts may have an effect on the ruminal fibers metabolisms and the volatile fatty acids concentrations [73,77]. There was a significant increase showed in both breed in terms of the *F. succinogenes* and *R. albus* population under high ratio of concentrate supplementation as compared to the diet without or less supplement. This study reported an increase in cellulolytic bacteria with the increasing fiber content in the diets.

Ruminococcus albus were cellulolytic and starch degrading bacteria capable of producing acetic acid and succinic acid during starch degradation [75]. Succinic acid was gradually converted into propionic acid [76] to provide energy for microbial protein synthesis in rumen. The increase in these two cellulolytic bacteria in treatment groups may be due to the impact of the of ingredient contains in concentrate such as corn grain, soybean meal and rice brain, which are known to have enhancer effect on cellulolytic bacteria and eventually improve fiber digestibility. With a rise in dietary starch content, the number of cellulolytic bacteria also increased significantly in this experiment, suggesting a shift in the carbohydrate fermentation substrate from a non-structural to structural carbohydrate [45]. The lower population of

protozoa in this study, with a significant increase in *Ruminococcus albus* is described by the decline of protozoa in the rumen often results in greater proliferation of bacteria and greater passage of bacterial nitrogen N out of the rumen [68]. This study also in line with previous research who also reported the increment population of *Ruminococcus albus* in Dorper sheep that fed with 5% rumen protected fat diet [11].

Methanogens and rumen bacteria interact jointly via hydrogen transfer due to the incorporation of methanogens into bacterial biofilms on feed particles themselves represent a form of interaction, and most fermentative ruminal bacteria produce carbon dioxide and hydrogen as a substrate for methanogens [50]. Such interspecies hydrogen transfer has been shown in methanogens cocultures with *Ruminiococcus albus* and *Ruminococcus flavefaciens* [51,52]. The interaction between rumen bacteria and methanogens affects energy conservation, VFA profiles, and methane production by the rumen microbiome. However, more studies are needed in metagenomic and metatranscriptomic analysis to examine microbial interaction at the microbiome level.

Conclusion

Based on this finding, it could be concluded that changes in diet from non-supplemented to supplemented diet potentially improved the nutritional value by decreasing the lignin contents and increase the crude protein and energy (e.g. crude fat, GE, and carbohydrates) levels. The rumen fermentation in both breeds (e.g. Murrah cross and Swamp) were affected by the type and ratio of supplement added to the main diet. The rumen pH in both breeds was not affected by changes in diet. As a result, the newly formulated diets for buffaloes feed tested on *in vitro* rumen fermentation gives a definite effect on TVFA concentration and proportion of VFA, CH₄ production, ARDC and NH₃ as well as rumen microbial population pattern. This research can contribute to enhancing models aimed at forecasting rumen functioning across the different breed of buffaloes. Future analysis of microbial communities using pyrosequencing is needed to understand beyond the effect of supplemented diet on rumen fluid across different breeds of buffaloes in *in vitro* technique studies.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Availability of data and equipment used and analyzed during this study is available from the correspondence author on reasonable request.

Competing of interest

The authors declare that they have no competing interests.

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Authors' contributions

AH conceived the manuscript purpose and design and critically revised the manuscript. MA conducted the study, data interpretation, wrote the initial version of the manuscript and revised according to AH's suggestions. All authors read and approved the final manuscript submitted.

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Tables

Table 1
Nutritional composition of feedstuff (% DM basis)

Nutrient composition (% DM)	Diet components		
	Grass (G)	Concentrate (C)	Bypass fat (B)
DM	9.66	9.64	0.25
Ash	5.09	5.44	1.00
CF	26.03	7.49	0.23
EE	2.03	5.46	100
CP	6.09	18.15	9.54
NDF	64.27	56.87	-
ADF	33.86	17.38	-
ADL	3.55	2.96	-
Carbohydrates	59.43	63.46	-
GE	11.79	15.74	37.65
Data shown are the mean of triplicate analyses of each component			

Table 2
The PCR primers used for quantification of rumen microorganisms

Microorganism	Primer sequence 5' – 3'	Amplicon (base pair)	Reference
Total bacteria	F ¹ -CGGCAACGAGCGCAACCC R ² -CCATTGTAGCACGTGTGTAGCC	145	[80]
<i>Ruminococcus albus</i>	F-CCCTAA AAGCAGTCTTAGTTCG R-CCTCCTTGCGGTTAGAACA	175	[80]
<i>Fibrobacter succinogenes</i>	F-GTTCGGAATTACTGGGCGTAAA R-CGCCTGCCCCTGAACTATC	122	[81]
Total methanogen	F-CCGGAGATGGAACCTGAGAC R-CGGTCTTGCCCAGCTCTTATTC	160	[60]
Total protozoa	F- CTTGCCCTCYAATCGTWCT R-GCTTTCGWTGGTAGTGTATT	223	[82]
Note: ¹ F: forward; ² R: reverse; bp: base pair (product size)			

Table 3
Nutritional composition of Diet A, Diet B and Diet C on a dry matter basis (% mean $\pm$ SE).

Ingredient	% Inclusion				
	Diet A	Diet B	Diet C		
<i>Brachiaria decumbens</i> (G)	100	70	70		
Concentrate (C)	-	30	26		
Bypass fat (B)	-	-	4		
Nutrient	Estimated content			SEM ¹⁾	P-value
DM ²⁾	9.66 ^a	9.70 ^a	8.40 ^b	0.24	0.000
Ash	5.09 ^a	5.69 ^a	5.93 ^a	0.33	0.916
CF ³⁾	26.03 ^a	23.73 ^b	21.65 ^c	0.66	0.000
EE ⁴⁾	2.03 ^a	2.92 ^b	16.66 ^c	2.87	0.000
CP ⁵⁾	6.09 ^a	8.08 ^b	6.56 ^a	0.36	0.012
NDF ⁶⁾	64.27 ^a	57.96 ^b	49.63 ^c	2.17	0.000
ADF ⁷⁾	33.86 ^a	28.7 ^b	26.65 ^c	1.10	0.000
ADL ⁸⁾	3.55 ^a	3.32 ^a	2.96 ^a	0.18	0.442
Carbohydrates	59.43 ^a	61.53 ^a	48.65 ^b	2.29	0.001
GE (MJ/kg) ⁹⁾	11.07 ^a	12.1 ^a	14.59 ^b	0.56	0.000
Hemicellulose	30.41 ^a	29.25 ^a	22.98 ^b	1.24	0.002
Cellulose	30.32 ^a	25.38 ^b	23.69 ^b	1.04	0.001
¹⁾ SEM, standard error of the mean; ²⁾ DM, dry matter; ³⁾ CF, crude fiber; ⁴⁾ EE, ether extract; ⁵⁾ CP, crude protein; ⁶⁾ NDF, neutral detergent fiber; ⁷⁾ ADF, acid detergent fiber; ⁸⁾ ADL, acid detergent lignin; ⁹⁾ GE, gross energy.					
Diet A (control): 100% <i>Brachiaria decumbens</i> ; Diet B: 70 <i>Brachiaria decumbens</i> + 30% Concentrate; Diet C: 70% <i>Brachiaria decumbens</i> + 26% Concentrate + 4% Bypass fat. Data are the mean of triplicate analyses of each diet. ^{a,b,c} Means in the same row with different superscript are significantly different (P < 0.05)					

Table 4

Effect of diet supplemented with concentrate and bypass fat on pH, total volatile fatty acid and methane production from in vitro incubation of Murrah cross and Swamp buffalo rumen fluid

Diets	Breed	pH	TVFA ¹⁾	C2 ²⁾	C3 ³⁾	C4 ⁴⁾	C2:C3 ¹¹⁾	ARDC ¹²⁾	CH ₄ ⁵⁾	NH ₃ ⁶⁾
			(mmol/L incubation)					(mg)	(mM/L)	(mg/l)
A ⁷⁾	Murrah	6.69 ^a	111.08 ^{ab}	52.93 ^a	36.37 ^{ab}	10.42 ^a	1.69 ^a	8.92 ^a	18.08 ^a	69.56 ^a
B ⁸⁾		6.52 ^a	172.03 ^c	83.92 ^b	49.10 ^{bc}	24.59 ^b	1.9 ^a	14.76 ^b	34.46 ^c	93.96 ^a
C ⁹⁾		6.55 ^a	81.27 ^a	39.73 ^a	25.09 ^a	8.49 ^a	1.82 ^a	6.63 ^a	14.06 ^a	74.22 ^a
A ⁷⁾	Swamp	6.67 ^a	142.36 ^{bc}	55.35 ^a	32.49 ^a	8.55 ^{ab}	1.73 ^a	8.5 ^a	22.50 ^{ab}	68.54 ^a
B ⁸⁾		6.53 ^a	172.17 ^c	75.67 ^b	52.06 ^c	27.96 ^b	1.45 ^a	14.88 ^b	30.59 ^{bc}	95.30 ^a
C ⁹⁾		6.55 ^a	109.76 ^{ab}	51.45 ^a	35.53 ^{ab}	14.99 ^{ab}	1.44 ^a	6.75 ^a	19.72 ^{ab}	84.28 ^a
SEM ¹⁰⁾		0.04	7.90	3.53	2.21	1.88	0.04 ^a	0.74	1.73	4.71
P-value										
Breed		0.362	0.000	0.000	0.000	0.064	0.095	0.123	0.044	0.716
Diet		0.135	0.022	0.057	0.048	0.008	0.961	0.419	0.035	0.118
¹⁾ TVFA: Total volatile fatty acids; ²⁾ C2: Acetate; ³⁾ C3: Propionate; ⁴⁾ C4: Butyrate; ⁵⁾ CH ₄ : methane production(mM/L); ⁶⁾ NH ₃ : Ammonia; ⁷⁾ Diet A CON – (100% <i>Brachiaria decumbens</i>); ⁸⁾ Diet B – (70% <i>Brachiaria decumbens</i> + 30% Concentrate); ⁹⁾ Diet C – (70% <i>Brachiaria decumbens</i> + 26% Concentrate + 4% Bypass fat); ¹⁰⁾ SEM: standard error of means. ¹¹⁾ C2:C3: Acetate:Propionate ratio; ¹²⁾ ARDC: Apparent rumen degradable carbohydrate; ^{a,b,c,d} Means with different superscript letters in the same column are significantly different at P < 0.05.										

Table 5

The effects of different level supplementation of concentrate and bypass fat on rumen microbial population (copies/mL) in Murrah cross and Swamp buffalo.

Breed	Murrah cross			Swamp			P-value		
SEM	Diet	Breed	Diet	A	B	C	A	B	C
Microorganisms (Log ¹⁰ Cells/mL)									
TB ¹⁾	11.35 ^a	11.74 ^{ab}	11.75 ^{ab}	11.40 ^{ab}	11.90 ^b	11.25 ^a	0.11	0.004	0.475
TP ²⁾	2.54 ^a	3.25 ^b	3.38 ^b	2.70 ^{ab}	4.78 ^c	2.77 ^{ab}	0.34	< 0.001	0.290
TM ³⁾	4.85 ^a	5.45 ^c	5.41 ^c	5.11 ^b	5.60 ^d	5.40 ^c	0.11	< 0.001	0.250
FS ⁴⁾	4.02 ^a	4.72 ^b	4.54 ^b	4.77 ^b	4.92 ^b	4.13 ^a	0.15	< 0.001	0.258
RA ⁵⁾	8.21 ^a	8.35 ^b	8.31 ^b	10.95 ^d	12.33 ^e	9.62 ^c	0.70	< 0.001	< 0.001
Note: A: (control) 100% <i>Brachiaria decumbens</i> ; B: 70 <i>Brachiaria decumbens</i> + 30% Concentrate; C: 70% <i>Brachiaria decumbens</i> + 26% Concentrate + 4% Bypass fat; TB ¹⁾ : Total bacteria; TP ²⁾ : Total protozoa; TM ³⁾ : Total methanogen; FS ⁴⁾ : <i>Fibrobacter succinogens</i> ; RA ⁵⁾ : <i>Ruminococcus albus</i>									
a,b,c,d,e Different superscript lowercase letters in each row within a treatment variable indicate significant difference at P < 0.05, SEM ⁶⁾ = standard error of mean. All data are the mean of triplicate analyses of each diet and the result expressed in Mean ± SE; n = 6.									

Figures

Figure 1

In vitro gas measurement (ml) of different formulated diet on Murrah cross vs Swamp buffalo. Notes: Diet A (100% *Brachiaria decumbens*); Diet B (70% *Brachiaria decumbens* + 30% Concentrate); Diet C (70% *Brachiaria decumbens* + 26% Concentrate + 4% Bypass fat)