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Research article

Effect of the fermented total mixed ration on *in-vitro* gas production and digestibility in cattle

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A B S T R A C T

In this study, Experiments were conducted to evaluate the fermented total mixed ration (FTMR) and total mixed ration (TMR) by rumen in *in vitro* fermentation technique and their effects on methane (CH4) emission and digestibility measurement. Ruminal samples were collected from ruminal digesta and grind TMR feed used as a substrate. There were four diets, one was without molasses-yeast mixture (control), another was in addition of molasses at 0.1% inclusion rate (T_1) and the other two were in addition of molasses-yeast mixture at 0.1% (T_2) and 0.3% (T_3) inclusion rate. The present study indicated that there was significant ($p<0.05$) difference in pH among different treatment groups and decreasing pattern of gas production in treatment group than control group. In this study lowest total gas produced in T_3 (33.8 ml) group than C (40.4ml) group and CH_4 production considerably lowest in fermented group (26.2 ml in T_2 and 27.6 ml T_3) than C (31.6 ml) at 24 h of incubation period. The *in vitro* organic matter digestibility (IVOMD) was tended to higher in T3 (92.93%) diet than C (91.66) at 72 h intervals. It can be concluded from the present study that the FTMR at 0.3% (T_3) inclusion rate has better methane reducing capacity and higher digestibility than TMR.

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1. INTRODUCTION

Ruminants are an essential part of livestock sector, because ruminant is an expert in converting cellulose and other fibrous materials into high quality milk & meat. Besides they also have great role in green-house gas (carbon dioxide, methane, nitrous oxide) production (Henry and Eckard, 2009). Another important problem facing ruminant production is the losing of energy and high biological value proteins as a result of ruminal fermentation. This may cause a limited productive performance (Ahmed et al., 2016; Kholif et al., 2014) release of pollutants to the environment (Calsamiglia et al., 2007). Many factors influence methane emissions from cattle and include the following: level of feed intake, type of carbohydrate in the diet, feed processing, addition of lipids or ionophores to the diet, and alterations in the ruminal microflora. Cattle industry has become one of the most important economic activities all over the world. But to get maximum production, a perfectly balanced nutrition supplement to the animal is inevitable. Regarding this situation, total mixed rations (TMR) can be an alternative solution to support

the dairy cows for achieving maximum production by stall feeding without grazing indoor-housed system like dairy producing countries of the world. To ensure that, a total mixed ration (TMR) can be supplied to the animals, which will avoid selective feeding. TMR feeding enhances feed intake, improves the ecology of the rumen leading to stimulated microbial activity to digest more feed, and then finally increases productivity of the cows. The benefits of a TMR include increased feed intake, enhanced use of low-cost alternative feed ingredients, ability to control the forage concentrate ratio, lower incidence of metabolic and digestive disorders and reduced labor input for feeding (Owen, 1984). TMR is a proper type of feed especially when agricultural by-products with high moisture are to be included (Li et al., 2003). Wachirapakorn et al. (1997) compared two feeding regimes (separate and TMR feeding) and found that TMR feeding increased dry matter intake (DMI) and milk production compared to separate feeding. It has also been experimentally confirmed in other studies that fibroid materials assorted feed is advantageous in maintaining the homeostasis of ruminant stomach pH, reducing the incidence of metabolic disease, and improving milk production (Nocek et al., 1986; Harrison et al., 1989; Kellems et al., 1991). Fattening of cattle has become a very common practice all over the world. But most of time, steroidal hormones androgen and such as estrogens are used which may have human health consequences by consuming the beef and when released through excreta into the environment, it might pose chronic risk to wildlife. Farmers raising homebred fattening cattle are showing increased interest in fibroid material assorted feed, such as the TMR allowance over concentrates because homebred fattening cattle (rapid growing) require more feed intake for rapid body weight gain (Kim et al., 2003). Again fermenting of total mixed ration (FTMR) is a simple method to potentially improve nutrient utilization and extend the shelf life of the feed. FTMR is made by mixing roughage with concentrate and then fermenting them in incubator for 72 h. In dairy cows (Yuangklang et al., 2004) showed that FTMR increased feed intake and improved nutrient digestion. Vasupen et al. (2005) confirmed that FTMR improved the digestibility of dry matter (DM), organic matter (OM), fiber, and non- structural carbohydrate. If the coarse forage that is not suitable for feeding separately can be fermented and incorporated in TMR, it will reduce the wastage and improve feed quality. Including fermented feed in TMR may change its digestibility as well as feed efficiency and may be used widely in fattening. However Yeast, as a natural feed additive, has the ability to stabilize rumen fermentation and prevents rumen flora disorders and disturbances (Pinloche et al., 2013) with increasing the numbers of viable bacterial cells. In case of fermented mixed feed, supplementation of probiotic yeast maintained a healthy fermentation in the rumen of cattle with higher rumen pH. Yeast products formulated with *Saccharomyces cerevisiae* have good effects on the dynamics of gas production, *in vitro* digestibility and there was no interaction with forage quality. Therefore, This study aimed to evaluate the *in vitro* gas production, DM and OM digestibility of fermented TMR over simple TMR.

2. MATERIALS AND METHODS

The study was conducted in postgraduate laboratory under the Department of Animal Science and Nutrition, Chittagong Veterinary and Animal Sciences University (CVASU) Khulshi, Chattogram. The chemicals and most of the instruments were provided by Animal Science & Nutrition department laboratory and most of the experiments were performed in Department of Physiology, Biochemistry and Pharmacology and PRTC laboratories of CVASU.

Study period

The overall research was conducted from July, 2018 to January, 2019.

Collection of Feed

The concentrate and roughage type feed materials of the cattle were collected from Chittagong Veterinary and Animal Science University (CVASU) Bangladesh. Feed powder of less than 1mm (<1mm) was prepared using mortar.

Chemical composition of feed

The chemical composition of the Total Mixed Ration and Fermented Total Mixed Ration are presented in Table 1 which were analyzed in the Animal Nutrition Laboratory, Chattogram Veterinary and Animal Sciences University, Khulshi, Chattogram.

Table 1: Chemical composition of the experimental fermented and non-fermented feeds.

Parameter	Dietary treatment			
	C	T_1	T_2	T_3
DM	95.3	94.3	94.3	94.2
Ash	7.9	7.8	7.9	7.8
OM	92.0	92.2	92.0	92.1
CP	12.9	13.5	13.6	13.7
CF	16.8	17.2	17.2	17.1

Optimization of yeast concentration

The adequate amount of sugar molasses solution was taken in fermentation flask and the pH and temperature were maintained at 4.0 and 35^{o}C and kept in a constant temperature shaker. The quantities of yeast like 2.0 gm were added. An anaerobic condition was maintained for four days and during this period, the strain converts sugar into bio-ethanol with the evolution of CO_2 and the fermented solution was analyzed at every 48 h and 72 h intervals (Periyasamy et al., 2009). After 72 h of incubation period, were count the yeast cells was 4.4×10^{8} cells/ml in Neubauer chamber at direct 1: 10 fold dilution method.

Rumen Fluid Collection

Rumen fluid was collected from a freshly slaughtered indigenous cow from slaughter house. The rumen fluid was collected early in the morning, whereas the required buffers were made the day before for time constraint. On an important note, it is essential to preserve the rumen fluid temperature for the *in vitro* test. Thereby, immediate collection of rumen fluid is vital after slaughtering of the cow. The collected ruminal content were squeezed to obtain the rumen fluid. Thereby, 1L of rumen fluid was filtered with four folded cheesecloth and poured in an airtight flask. The usual temperature for rumen fluidis 39°C. It was maintained since immediately after filtering the rumen fluid in flask, the flask was sealed and kept in ice box. Afterwards, it was immediate transfer to laboratory of department of Animal science & Nutrition for a balanced temperature management. The rumen fluid was immediately dispensed with Nitrogen gas for maintaining an anaerobic condition that is vital for rumen fermentation. The rumen fluid was collected from a cow which was fed rice straw and commercial feed composition twice in a day. The cow feed, times of feed and the cow breed were recognized after consultation with the workers and owner of the slaughter house.

Buffer for Rumen Fluid

The buffer medium was prepared according to the method described by (Asanuma et al., 1999) with the following composition in mg/L: dipotassium phosphate (K_2HPO4), 450; monopotassium phosphate (KH_2PO4), 450; magnesium sulfate heptahydrate ($MgSO_4.7H_2O$),190; calcium chloride dehydrate ($CaCl_2.2H_2O$), 120; Sodium chloride (NaCl), 900; cysteine hydrochloride ($C_3H_7NO_2S.HCl$), 600; ammonium sulfate ($(NH_4)_2SO_4$), 900; Trypticase peptone (BBL; Becton Dickinson, Cockeysville MD), 1000; and, Yeast extract (Difco Laboratories, Detroit, MI), 1000. The chemicals were poured in distilled water of one liter. Firstly, all the chemicals were poured and a very small amount of distilled water was put for the solution to mix evenly. Yeast extract and trypticase peptone were dissolved by hands since they clump immediately when these come in contact with air. Thereby, immediate mixture of these chemicals was needed. In this process, a certain pH is required for the efficient function of the in vitro test the required and desired pH is 6.9. The pH was balanced by adding one to two drops of Sodim Hydroxide (NaOH) (Base) or Hydrochloric Acid (HCl) (Acid). Afterwards, the buffer was dispensed with 100 % Nitrogen (N_2) gas for creating anaerobic condition. Lastly, the buffer was autoclaved at 121°C for 15 minutes. Finally, the buffer was collected after almost one hour when the buffer was cooled after autoclaving and preserved till the next day for mixing with freshly slaughtered

rumen fluid. However the rumen fluid was mixed with the buffer the next day after collection of freshly slaughtered cow rumen fluid. The upper residue of the rumen fluid was removed while the middle portion was collected and used in the experiment. The pooled and particle-free rumen fluid was transferred to a buffer medium bearing pH 6.9 (Hino et al., 1992) in a 1:3 rumen fluid:buffer ratio. A 4500 ml of total liquid was prepared.

Serum Bottles Preparation

Fifty ml of buffered rumen fluid was an aerobically transferred under a constant flow of N_2 gas atmosphere in order to make it oxygen free as per suggested by (Asanuma et al., 1999) to 100 ml serum bottles containing the 0.5g TMR feed added with molasses and molasses containing yeast at different concentrations. Finally, the rumen fluid buffer was poured in 80 different serum bottles for the ultimate *in-vitro* experiment. Sealing with rubber septum stopper and aluminum cap (Asanuma et al., 1999) of the bottles containing the mixed substrate and buffered rumen fluid will follow which will then be incubated subsequently at 39°C for 6, 24, 48, and 72 h in a shaking incubator with 120 rpm (Hattori and Matsui, 2008).

Serum Bottle Setup

The final bottle setup was made according to the following treatments were: non addition, 0.1% Molasses, 0.1% and 0.3% Yeast culture and, hereafter referred to as control, treatment 1 (T_1), treatment 2 (T_2), treatment 3 (T_3) and keeping five replication of each treatment. Thereby, the incubation times were 6, 24, 48 and 72 hour. As for bottles, four types of bottles were made, where 20 bottles for each control and treatments. There were 5 bottles fixed for every 6 hour, 24 hour, 48 hour, 72 hour at both control and treatments group. Finally, all the bottles of both control and treatments group were put into shaking incubator at 39°C temperature for *in vitro* gas production with 120 rpm (Hattori and Matsui, 2008).

Collection of Total Gas

Calibrated gas syringe made of glass was used to collect the gas produced in the *in vitro* test. Fermentation parameters were monitored at the end of each incubation time set. A needle channel connected to the syringe was extended into the sealed fermentation bottle to measure the positive pressure created by the gas build up in the headspace of the syringe at room temperature and allowing the gas to flow inside a syringe barrel. The plunger was pulled gradually until the pressure the volume of gas trapped inside the barrel was recorded as the total gas (TG) produced in ml.

pH Measurement

The pH meter used to determine the pH value after opening each serum bottles.

CO_2 and CH_4 measurement

Lime-water were prepared for the measurement of CH_4 and CO_2. The TG contained gas syringe sink into the lime-water jar and backward pressure of syringe take the lime water into the syringe tube where the CO_2 itself reacts with the lime and disappear. The rest of the gas in the syringe tube indicates the amount CH4 production in ml. Rest of this CH4 amount subtracted from measured TG and this result indicates CO_2 production in ml(Mel et al., 2014).

Determination of in vitro dry matter and organic matter digestibility

Earlier to the *in vitro* rumen fermentation, the DM and organic matter (OM) of concentrate feed was determined by drying at 105°C for 16 h and ashing at 550°C for 12 h, respectively. The resulting percent DM and percent OM was used to compute the initial DM (DMi) and initial OM (Omi) of the substrate in grams. Fermenta samples from each serum bottle after the specified incubation period were drained in dried, pre-weighed nylon bags and knotted using nylon thread, then splashed with flowing water for 15 minutes or until the turbidity of water resulting from washing disappeared. The final DM (DMf) and OM (Omf) of the feed were determined using the same conditions applied when determining the initial values (Dmi and Omi). The DM and OM digestibility (%) were calculated as ([DmiDMf]/Dmi) X 100 and ([Omi=Omf]/Omi) X 100, respectively.

3. RESULTS

In vitro fermentation parameters

pH

Decreasing tendency of pH value with increasing incubation period where significant difference was noticed in TMR and FTMR feed at 24 h, 48 h and 72 h, respectively ($p<0.05$).

Total Gas

In case of total gas, significant ($p<0.05$) difference was observed after 24 h of incubation period. Though there was no significant difference was observed at 6, 48 and 72 h respectively ($p>0.05$). But tended to lowest total gas was in T3 (45.1 ml) after 72 h than the C (48.4 ml). Significantly lowest total gas was observed in T3 (33.8 ml) ingroup and highest was in C (40.4 ml) group at 24 h of incubation period (Table 4.3).

Table 2. pH from *in vitro* rumen fermentation of the experimental fermented and non-fermented feeds.

Incubation period	Treatment				P- value
	C	T1	T2	T3	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
6 h	5.64 ± 0.05	5.57 ± 0.02	5.61 ± 0.01	5.61 ± 0	0.07
24 h	5.46 ± 0.01	5.41 ± 0.01	5.45 ± 0.03	5.43 ± 0.01	0.02
48 h	5.33 ± 0.01	5.27 ± 0.01	5.31 ± 0.01	5.32 ± 0.01	0.00
72 h	5.29 ± 0.01	5.22 ± 0.03	5.27 ± 0.01	5.24 ± 0.04	0.03

†C = Diet without molasses-yeast mixture, T1 = Diet containing molasses at 0.1% of TMR DM, T2 = Diet containing molasses-yeast mixture at 0.1% of TMR DM and T3 = Diet containing molasses-yeast mixture at 0.3% of TMR DM.

Table 3. Total gas (ml) production from *in vitro* rumen fermentation of the experimental fermented and non-fermented feeds.

Incubation period	Treatment				P- value
	C	T1	T2	T3	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
6 h	12.2 ± 1.1	12.8 ±0.8	13.2 ± 1.3	11.6 ± 0.5	0.09
24 h	40.4 ± 2.5	37.8 ± 4.2	34.8 ± 4.0	33.8 ± 2.0	0.02
48 h	45.2 ± 3.0	39.2 ± 5.2	42.6 ± 3.2	42.6 ± 2.4	0.11
72 h	48.4 ± 1.1	46.2 ± 5.2	46.4 ± 4.3	45.1 ± 1.4	0.50

†C = Diet without molasses-yeast mixture, T1 = Diet containing molasses at 0.1% of TMR DM, T2 = Diet containing molasses-yeast mixture at 0.1% of TMR DM and T3 = Diet containing molasses-yeast mixture at 0.3% of TMR DM.

Methane (CH_4) production

There was no significant ($p>0.05$) difference observed after 6, 24, 48 and 72 h of incubation period. But Methane production decreased at 24 h of incubation period in T3 (26.2 ml) group than C (31.6 ml) group (fig 1).

CO_2 production

In case of CO_2 production there was significant ($p<0.05$) difference noticed at 48 h of incubation period. Whereas no significant difference was observed at 6, 24 and 72 h, respectively. Highest CO_2 production was observed in C (11.2 ml) group and lowest was in T3 (9.6 ml) group at 72 h of incubation period (fig 2).

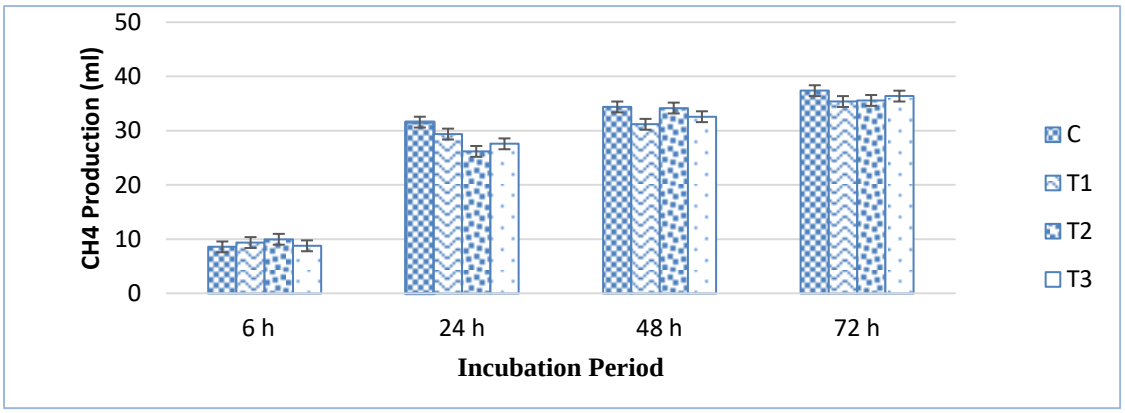

Figure 1. CH_4 production (ml/0.5 g DM) from *in vitro* rumen fermentation from fermented and non-fermented feeds.

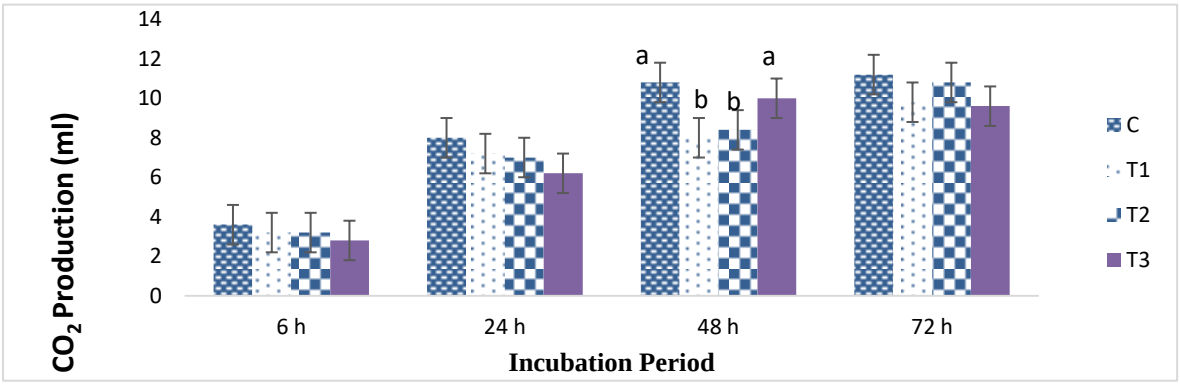

Figure 2. CO_2 production (ml/.5 g DM) from *in vitro* rumen fermentation from fermented and non-fermented feeds.

Dry Matter and Organic Matter digestibility

There was no significant ($p<0.05$) difference observed at 6 h, 48 h, and 72 h of incubation period. Significantly highest DM digestibility was observed in T3 (37.45%) group and lowest DM digestibility was observed in C (33.23%) after 24 h incubation (fig 3).

On the other hand, there was no significant difference on OM digestibility after 6 h, 24 h and 48 h of incubation period. Significantly highest OM digestibility was observed in T3 (92.93 %) group after 72 h of incubation than the C (91.66 %) group (fig 4).

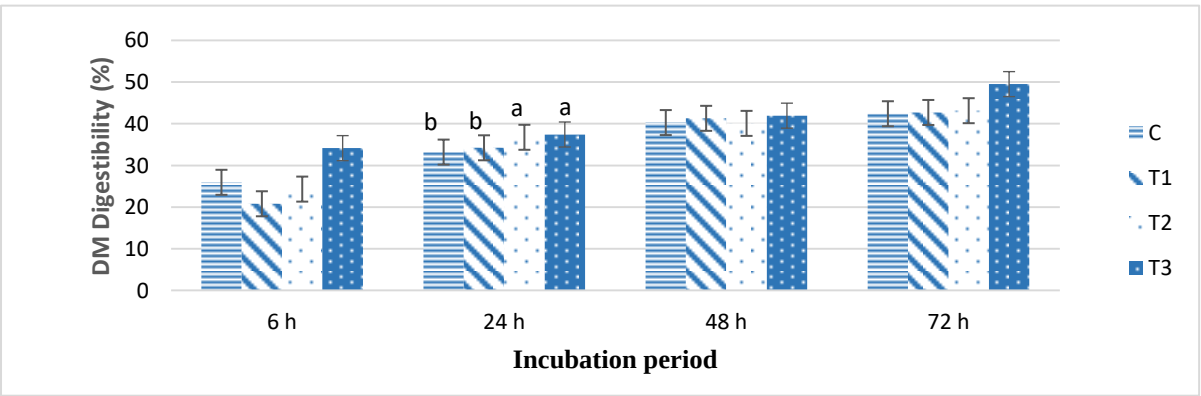

Figure 3. Dry matter (DM) digestibility of different fermented and non-fermented feeds

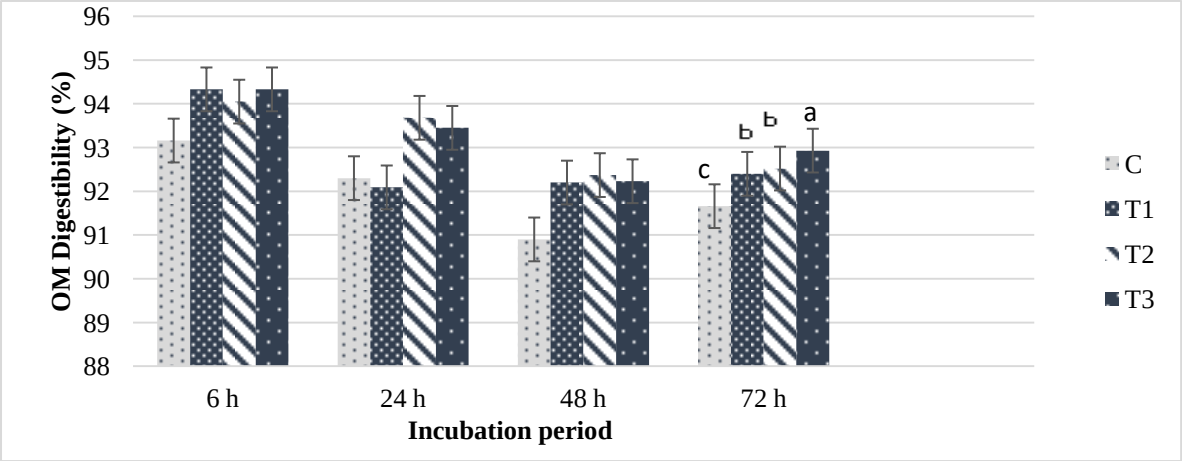

Figure 4. Organic matter (OM) digestibility of different fermented and non-fermented feeds.

4. DISCUSSION

This experiment was designed to analyze the effect of TMR and Fermented TMR on *in vitro* rumen fermentation. The current *in vitro* experiment indicated that better digestibility and less gas production with FTMR feed & decreasing tendency of pH at each 6 h, 24 h, 48 h and 72 h incubation period. The results of the experiment confirmed that gas production increased with the advancing incubation period. But fermented ration feed produced significantly less gas production then total mixed ration in each incubation period. Ruminant animals solely depend on cellulolytic ruminal microorganisms to digest cellulose. In the rumen fermentation process, pH is considered a leading factor affecting rumen microbiome, fermentation and CH_4 production. It has also been experimentally confirmed in other studies that fibroid materials assorted feed is advantageous in maintaining the homeostasis of ruminant stomach pH, reducing the incidence of metabolic disease, and improving milk production (Nock et al., 1986; Harrison et al., 1989; Kellems et al., 1991). The DM digestibility decreased with pH declining, this may relate to a negative effect of acid condition on microbial activity, particularly that of fibrolytic bacteria (Russell et al., 1996). In this study, significant difference was noticed in TMR and FTMR feed at 24 h, 48 h and 72 h respectively ($p<0.05$) which is supported by Kim et al. (2012). Ruminal pH affects ruminal bacteria that is neutral pH contribute to ruminal bacteria to digest feed sample and produce high total VFA. The low pH seems to contribute to the conversion of lactic to propionic acid in the rumen. The addition of probiotics stimulates bacteria of the rumen which affects the increase of lactic acid resulting in the stabilization of rumen pH. In additions yeast increases the production of organic acid. These organic acids reduce the pH of the rumen. This illustrates the similarity between the present and previous study.

The higher total gas production observed in high proportion of non-fermented TMR than fermented TMR feed. The results of the experiment confirmed that gas production increased with the advancing incubation period. But fermented TMR feed produced significantly less gas production than non-fermented feeds in each incubation period. Significantly lowest total gas was observed in T3 (33.8 ml) in group and highest was in C (40.4 ml) group at 24 h of incubation period. Less gas production occurred with fermented feed supported by different reports such as Arangsri et al. (2017); Cao et al. (2010); Kim et al. (2012) and Chao et al. (2016).

Methane production per animal is affected by dry matter intake (DMI), feed composition, feed quality, and production level besides individual animal variation (Ramin et al., 2013). Feeding a fermented total mixed ration (FTMR) to sheep was reported to reduce methane emission and increase digestibility (Cao et al., 2010). There was no significance difference observed between FTMR and TMR feed in present study but tended to reduce methane production among treatment group is supported by Chao et al.

(2016). Rumen methane production is generally higher when more fibrous feed is applied to cattle (Dehority et al., 2003). A remarkable decrease in methane generation in response to ensiling TMR was reported in a previous in vitrostudy (Cao et al., 2012). Yeast has the ability to shift H_2 utilization from methanogenesis to reductive acetogenesis through the homoacetogenic bacteria that can produce acetate from CO_2 and H_2 (Mwenya et al., 2004). *In vitro* studies have shown beneficial effects of feeding live yeast strain on growth and H_2 utilization and acetate production by acetogenic bacteria isolated from a rumen of lambs, even in the presence of methanogens (Chaucheyras-Durand et al., 1997). Martin et al. (2010) reported a 20% reduction in CH_4 production after a 48 hours incubation of alfalfa supplemented with a live yeast product. CH_4 production is significantly decreased in case of fermented feed than non- fermented mixed feed. Less methane production occurred with fermented feed also supported by Cao et al. (2010) and Kim et al. (2012). In this study there were significant difference was observed in CO_2 production. CO_2 production consistently decreased in treatment group that supported the results of Kim et al., (2012) where they observed the effect on CO_2 production as a result of yeast addition at different doses in treatment group.

In present study, there was significant difference was observed in DM digestibility at 24h of incubation period at 0.1% fermented TMR diet treatment, which indicated that fermented TMR diet could improve nutrient digestibility. Cao et al. (2012) reported that FTMR supported higher *in vitro* DM digestibility which is revealed with the present study. The OM digestibility resulted for the C and T diet were not different and consistent over time, whereas OM digestibility was reduced by both the diet from 6 hours onwards. Tended to highest OM digestibility was observed in T3 (92.93 %) group after 72 h of incubation than the C (91.66 %) group. Cao et al. (2012) reported increased OM digestibility of fermented ration compared with fresh ration which agrees with the present study.

5. CONCLUSION

Total mixed rations (TMR) can help to achieve maximum performance and can be the most adopted method for feeding high producing animals in almost all over the world. The result of this *in vitro* study stated that increased amount of Fermented TMR feed decreases the total gas production. In addition, significantly highest DM digestibility was observed in FTMR feed. Not only did the FTMR increase *in vitro* DM digestibility, it also reduced methane production. Regardless of the TMR used, DM digestibility and methane decreased with pH declining. Based on the findings of this study, it may be concluded that, FTMR has significant effect to decrease CH_4 production and increase digestibility.

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