Master's Thesis of Science in Agriculture

Effects of TMR and Separate Feeding System on Ruminal Methane Production, Total Digestibility, Rumen Metabolic and Microbial Profile

February 2017

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Abstract

Limited researches are available on effect of feeding total mixed ration (TMR) and roughage and concentrate separately (SF) on ruminant methane production. Two experiments were performed to study the difference in methane production and ruminal characteristics between feeding methods at two different feed intake levels. Each experiment was conducted using six male Holsteins with BW ranging from 230–570 kg. Feed intakes for experimental animals were adjusted to achieve average daily gains of 1.4 (experiment 1) and 0.7 kg (experiment 2), and methane production was investigated by using a triplicated 2 × 2 Latin square design. Animals were provided either SF or TMR containing 73% concentrate and 27% forage, with the same ratio of same ingredients and grasses, twice a day. No significant differences in methane emissions were observed in animals fed diets at 2.4% of BW in experiment 1. Animals fed TMR at 2% BW in experiment 2 emitted significantly more methane (138.5 vs. 118.2 L/d; $P < 0.05$) and lost more methane energy (3.98 vs. 3.49 %; $P = 0.005$) compared to those fed SF. In experiment 2, ruminal fermentation characteristics were largely affected by the feeding method. Cattle those received SF exhibited significantly lower total VFA and NH_3-N concentration, and propionate percentage at 1.5h whereas higher after 4.5hr compared to those fed TMR. A significantly ($P < 0.05$) lower acetate: propionate ratio at 4.5 h in those fed SF depicts the H_2 sink towards propionate synthesis when compared to TMR. Significantly higher levels of isobutyrate and isovalerate ($P < 0.05$) were observed in those fed SF compared to those fed TMR might increase microbial protein synthesis and net consumption of H_2. The ruminal pH and total tract digestibility of CP, NDF, and OM were not affected by two different feeding

systems. Overall, these results indicate that, compared to TMR, SF significantly reduces methane emission from ruminants and increases VFA production without affecting the total tract digestion.

Keywords: methane, rumen, separate feeding, TMR
Student Number: 2015-22097

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List of abbreviations and formulas

ADF- Acid detergent fiber

ATP- Adenosine triphosphate

BW- Body weight

CF- Crude fiber

CP- Crude protein

DGGE- Denaturing gradient gel electrophoresis

DM- Dry matter

DMD- Dry matter digestibility

DMI- Dry matter intake

DNA- Deoxy ribonucleic acid

DOM- Digestible organic matter

FCE- Feed conversion efficiency

FCM- Fat corrected milk

FPCM- Fat protein corrected milk

GEI- Gross energy intake

GHG- Greenhouse gases

GWP- Global warming potential

IACUC- Institutional Animal Care and Use Committee

NADH- Nicotinamide Adenine Dinucleotide Hydrate

NDF- Neutral detergent fiber

NDFD- Neutral detergent fiber digestibility

NGS- Next generation sequencing

OM- Organic matter

OMI- Organic matter intake

OTU- Operational taxonomic units

PSPSS- Penn State Particle Size Separator

QIIME- Quantitative Insights into Microbial Ecology

RNA- Ribonucleic acid

SAS- Statistical analysis software

SCFA- Short chain fatty acid

SF- Separate feeding

TMR- Total mixed ration

TRFLP- Terminal restriction length polymorphism

VFA- Volatile fatty acids

CH_4- Methane

CO_2- Carbon di oxide

H_2 - Hydrogen

NH_3- Ammonia

$(NH_4)\ HCO_3$- Ammonium bi carbonate

N_2O- Nitrous oxide

1. Introduction

1.1. Study Background

The fermentation of feed macromolecules by microorganisms in the rumen contributes to the production of CH_4. Methanogenic archaea in the rumen predominantly use H_2 as energy source to reduce CO_2 to CH_4, which is the last step of fermentation process in rumen. The methanogenesis process, besides its negative impact on the environment, represents a loss of 2-15% of gross energy intake of the animal and leads to an unproductive use of dietary energy (Johnson et al., 2002). Also, greenhouse gas (GHG) emissions from livestock production systems are expected to rise over the coming decades due to the projected increase in demand for livestock products (FAO, 2009). Hence increasing the productivity of cattle to reduce CH_4 emission is a key area of interest (Mills et al., 2009) because reducing the ruminant population being farmed is not an option.

Advances in understanding the ruminant nutrition and rumen microbiology have opened novel insights into the function of rumen ecosystem relevant to methane formation. This has led several strategies to reduce CH_4 emission from rumen, which are of interest, only when they have no negative impacts on animal and environment. Those strategies include the use of chemical inhibitors like bromochloromethane (Immig et al., 1996; Lila et al., 2004; Mitsumori et al., 2011; Knight et al., 2011), electron receptors like fumarate, nitrates, sulfates, nitro ethane (Gutierrez-Banuelos et al., 2007; Brown et al., 2011), Ionophores (Sauer et al., 1998; Van Vugt et al., 2005; Waghorn et al., 2008a; Grainger et al., 2010) and plant bioactive compounds like tannin and saponin (Woodward et al., 2001; Sliwinski et al., 2002; Zhou et al., 2011; Staerfl et al., 2012). However, the use of these compounds as feed

additives was not promising due to several adverse effects, such as reduction in fiber digestibility and feed intake, toxic to microbiome in the rumen, and question of persistence of the effect (Johnson et al., 1972; Immig et al., 1996; Leng et al., 2008; Waghorn et al., 2008; Grainger et al., 2009).

Alternatively, increased feed availability is expected to improve animal production and it causes to decrease CH_4 per unit animal products. The nutritional advantages of feeding the animal with a total mixed ration (TMR) is one of the strategies because this feeding system increases feed intake and digestibility, minimizes choice feeding among individual feeds, maintains sufficient fiber intake for supporting rumen health, such as stable ruminal pH, lower A/P ratio when compared to animal fed the roughage and concentrates components of the diet separately (Maekawa et al., 2002). Conversely, there are also reports that feeding a TMR had no effect on animal performance or carcass traits of steers (Caplis et al., 2005) and milk production and milk composition (Renna et al., 2014). Based on the literatures, it might be postulated that feeding a TMR would lead to not only more absolute amount of CH_4 production because of increased DM intake and ruminal pH but also less amount of CH_4 per unit animal production. However, very little research (Holter et al., 1977) is available on comparisons of separate and TMR feeding on CH4 production from ruminant.

1.2. Aims of research

Reducing enteric CH_4 production from livestock is one of the main challenges in lowering the environmental impact from the agricultural sector. The overall aim of this thesis was to evaluate the effect of TMR and Separate Feeding (SF) system in ruminal CH_4 emission, fermentation characteristics, total digestibility and ruminal microbial profile.

2. Literature review

2.1. Impact of ruminal CH_4 on environment and animal productivity

In recent decades, the rising global temperatures and climate change have been fueled by the increasing concentrations of greenhouse gases (GHG) in the atmosphere. This climate change is most likely due to the human activities (anthropogenic) (IPCC, 2013). In 2010, the total anthropogenic global GHG emissions were recorded as 49 ± 4.5 Gt CO_2-eq (IPCC, 2014), including the 7.1 Gt CO_2-eq per year from livestock sector (Gerber et al., 2013). Within the livestock sector, enteric fermentation from ruminants is the largest source of GHG (40%), followed by manure management. Livestock sector is estimated to contribute to anthropogenic GHG emissions as 5% of CO_2 emissions, 44% of CH_4 emissions and 53% of N2O emissions (IPCC, 2007). According to the global warming potential (GWP) of these GHG, CH_4 and N2O have 28 and 265 times higher GWP than CO_2 (IPCC, 2013).

Apart from these environmental issues, CH_4 emission has a serious effect on the productivity of animals. According to Johnson and Johnson (1995), cattle can produce 250–500 L of CH_4 per day per animal and typically lose 2–15% of their ingested energy as eructated CH_4, which could be otherwise used for milk production or other productivity mechanism. An increase in productivity by reduction of CH_4 production per kg products such as milk or meat, has been a goal in animal agriculture sector. Gerber et al. (2011) analyzed the relationship between productivity of dairy cows and emission intensity (emission amount per unit of productivity) on a global scale (Fig 1) and pointed that the most effective mitigation strategies are subjected to productivity level and CH_4 emission reduction in low productivity systems.

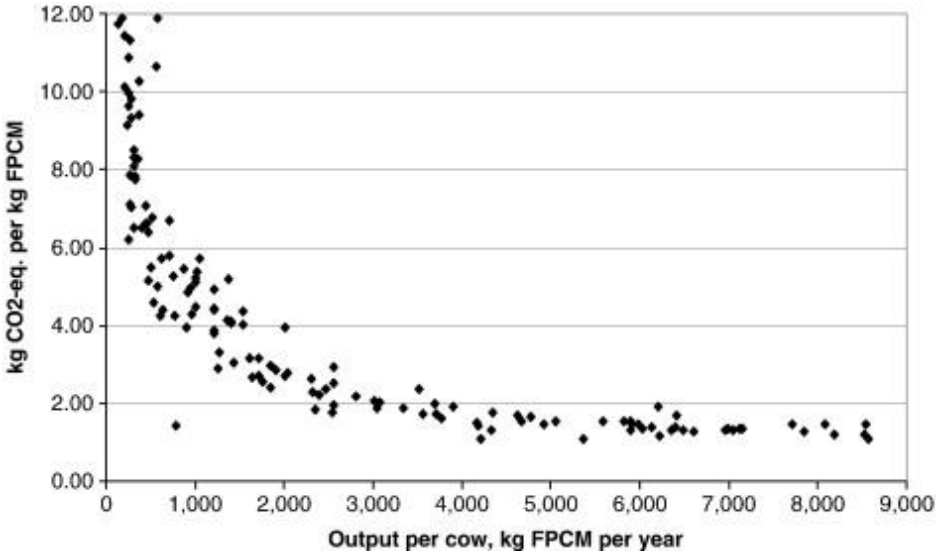


Fig 1. Relationship between total greenhouse gas emissions and output per cow. Each dot represents a country in the database. **(Gerber et al., 2011)**

In addition, Bannink et al. (2011) also explained in his work that feed conversion efficiency (FCE) was increased by decreasing the total CH4 production. Thus, mitigating CH4 losses from cattle has two important benefits. Firstly, less CH4 means a lower concentration of greenhouse gases (GHGs) in the atmosphere. Secondly, less CH4 means increased efficiency of livestock production and increased income for farmers.

The estimated increase of population from 7.4 billion in 2015 to 9.7 billion in 2050 (UNPD, 2015) is expected to increase the demand of livestock products such as milk and meat in the world. According to FAO (2011), demand for milk and meat is expected to increase by 73 and 58 %, respectively, from 2010 to 2050. This increase in demand of livestock products will increase the cattle population proportionally and increase the GHG emission. As an initiative step to control CH_4 emission in Korea, the government in 2009

announced the reduction of GHG emissions up to 30% nationwide and 5.2% in livestock agriculture with active application of reduction methods.

2.2. Dietary strategies to mitigate ruminal CH4 emission:

2.2.1. Feeding level, passage rate and digestibility

As the feed additives are found to have lot of demerits and adverse effects on animal productivity (Johnson et al., 1972; Immig et al., 1996; Leng et al., 2008; Waghorn et al., 2008; Grainger et al., 2009), greater opportunities exist in reducing enteric CH_4 emissions from ruminants through nutrition, feeding management and improvements in herd health and productivity than with the use of feed additives as rumen modifiers (Waghorn and Hegarty, 2011).

A strong relationship of DMI with ruminal CH_4 production has been reported by Shibata et al. (2010) and Charmley et al. (2016). It is obvious that total CH_4 production (L/d or g/d) increases with increasing DMI because there is more feed to be fermented. However, CH_4 conversion rate (Ym value; CH_4 energy as % of GEI) usually decreases as DMI increases above maintenance since the GEI is very high (Blaxter and Clapperton, 1965; Moe and Tyrrell, 1979; Pinares-Patiño et al., 2009). Johnson and Johnson (1995) also stated that the Ym decreased by about 1.6% per each increase in level of feed intake above maintenance. The reason for decrease in Ym was related to decrease in DM digestibility, majorly NDF (Huhtanen et al., 2009) and the associated increase in passage rates at higher intakes (NRC, 2001; Boadi et al., 2004). There were also reports that higher the digestibility, higher the CH_4 produced (Blaxter and Clapperton, 1965; Czerkawski et al., 1969; Moss et al., 1995; Kirkpatrick et al., 1997; Pelchen and Peters, 1998; Hart et al., 2014). However, total tract NDF digestibility has been studied more in terms of CH_4 production, since the impact

on NDF digestibility is expected to be 2 to 5 times higher than hemicellulose (Moe and Tyrrell, 1979). The relationships between digestibility, passage, and DMI are complex and have a major impact on ruminal production of CH_4.

2.2.2. Inclusion of concentrate

It has been reported that increase in the proportion of concentrate in the diet decreases CH_4 emissions linearly in lactating dairy cows and beef cattle (Aguerre et al., 2011; McGeough et al., 2010). Sauvant and Giger-Reverdin (2007) reported a curvilinear relationship between CH_4 production and proportion of concentrate in the diet; CH_4 losses of 6-7% of GEI at 30–40% concentrate levels and 2-3% at 80–90% concentrate levels. IPCC (2006) also reported that dairy cows fed mixed ration showed emission factor 6.5%, compared to 3% for cattle in feedlots fed commonly > 90% concentrate. Replacing structural carbohydrates (cellulose, hemicellulose) from forage in the diet with non-structural carbohydrates (starch and sugars) increased very rapidly the amount of VFA production and shifted the profile of VFA from acetate towards propionate, consequently decreasing the rumen pH, since digestion of starch is faster than digestion of forage (Krizsan et al., 2010; Cannas and Van Soest, 2000). However, negative effect on fiber digestibility by such strategy had also been reported (Firkins, 1997; Nousiainen et al., 2009; Agle et al., 2010; Ferraretto et al., 2013).

2.2.3. Particle size distribution of forage

Reducing the particle size distribution of the feed by feed processing is another strategy to decrease CH_4 emission since it alters the rates of fermentation and passage rate of the particles (Maulfair et al., 2011). Passage rate was found to be a major factor affecting the extent of digestion and patterns of VFA formation as well as microbial growth rates and has been shown to

explain 28% of the variation in CH_4 emissions (Okine et al., 1989). In a recent study, Huhtanen et al. (2016) observed an inverse relationship between passage rate of the feed and CH_4 production. Particles that are too small was found to pass the rumen undigested thereby decreasing the amount fermented (Russell and Hespell, 1981). Processing forages by grinding, chopping, or pelleting was also found to decrease rumen NDF digestibility and CH_4 emissions as a result of increased passage rate (Johnson et al., 1996; Moss et al., 2000) coupled with decreased acetate: propionate ratios (Van Nevel and Demeyer, 1996). In addition, it seems that the average size of forage necessary is 3 mm because of maintaining the pH of rumen, the chewing activity, as well as the fat percentage of milk (Shaver and Nylt, 1986). Therefore, the feeds which contained forage with particles less than 3mm has been attributed to reduction of the fat percentage of milk, the pH of rumen, and the chewing activity duration (Mertens, 1997). Studies by Kononoff and Heinrichs (2003a) and Yang and Beauchemin (2005) observed that increase in dry matter digestibility was related to the increase in ration particle size. This depicted that increase in ration particle size increases the retention time in rumen, but possibly increasing CH_4. One possible explanation was given by the study of Maulfair et al. (2010) that chewing minutes per kilogram of DMI was increased for larger particle size possibly (Owens and Isaacson, 1977) and NDFD was increase with increasing ration particle size (Yang and Beauchemin, 2005). However, There are conversial studies that ration particle size had no effect on NDFD (Krause et al., 2002; Kononoff and Heinrichs, 2003a; Yang and Beauchemin, 2007). Furthermore, fine grinding of forages has also been proven to be uneconomical due to the greater risk of acidosis and decreased milk fat concentration (Boadi et al., 2004). Overall, feed processing might be found to have mixed effects. Processing forages alters rumen fermentation and decrease rumen NDF

digestibility, but if NDF is digested in the hindgut or in manure storage, no net change in total CH_4 emissions occur (Hinrichsen et al., 2005).

2.2.4. TMR vs separate feeding of concentrate and forage

The effect of feeding systems (Total mixed ration (TMR) vs. Separate (SF) of concentrate and forage) on rumen CH_4 production is the major unexplored area (Hristov et al., 2013). TMR feeding system does not imply the use of different feed ingredients from SF system but it normally includes some extra processing of the diet ingredients, like chopping of forage, to enable the ingredient to be fully incorporated into a uniform blended mixture that seemed to give rise to an increase in daily DM intake in many studies. TMR has been known to give benefits by increased meal frequency and feed intake, enhanced fiber digestion and nitrogen utilization, and increased milk yield and milk fat production (Owen, 1984). In a study on comparisons of different feeding systems, Bargo et al. (2002) observed maximized total DMI and 19% increased milk production coupled with increased body weight when the cattle were fed TMR. In addition, Moseley et al. (1976), McGilliard et al. (1983) and Nock et al. (1985) reported that TMR system helped to maintain rumen pH and improve A/P ratio because TMR could provide more balanced ration with a uniform rate of roughage and concentrate and increased DM intake. There were also studies that showed feeding TMR increases digestion (Kim et al., 2012; Maskalova et al., 2015). Comparing TMR and SF system in a recent study, Liu et al. (2016) observed an increase in animal performance and dietary N utilization when cattle received TMR rather than SF. It is well recognized that at low levels of forage: concentrate ratio (below 40:60), mixing is considered advantageous so as to spread the intake of forage and concentrates as evenly as possible. Also, simplicity of management and full mechanization, coupled with economy of

feeding space, safety and flexibility for inclusion of a wide range of ingredients are considered as the advantages of TMR.

However, Maekawa et al. (2002) did not report any differences in feed intake or milk production and composition of dairy cows fed ingredients as a TMR. Furthermore, earlier studies by Baxter et al. (1972) reported an increase in milk production by Separate feeding system. Nocek et al. (1986) also explained higher fat-corrected milk (FCM) feed efficiency with the separate feeding system due to lower feed intake. This lower feed intake was observed due to the increased particle size of the roughage. According to the research by Manteca et al. (2008) investigating the effect of separate feeding on animal welfare, when two or more feeds are offered separately at the same time, it was observed that ruminants are able to develop recognition mechanisms for favorable or adverse feed constituents and accordingly seemed to select diets which meet their requirements (Görgülü et al., 1996) and a favorable rumen environment (Cooper et al., 1995). Separate feeding system is also believed to allow cattle select diet, corresponding to their physiological status, and consume high amount of concentrate by balancing fiber intake in a synchronized manner (Gorgulu et al., 1996; Fedele et al., 2002; Gorgulu et al., 2003; Yurtseven and Gorgulu, 2004; Gorgulu et al., 2008). Furthermore, it has been said that TMR preparation is tedious because mechanization include high capital cost and risks of mechanical breakdown (Hironaka et al., 1996).

Irrespecive of the nutritional benefits of the TMR and SF, research on their effects on CH_4 production are too limited. Vander Nagel et al. (2003) in the research on Lifecycle assessment (LCA) for TMR feeding and pasture feeding cattle, showed that TMR exhibited high emission (1.53) relative to pasture (0.84). Further, Yurtseven et al. (2009) observed Separate feeding

system produce less CH_4 than TMR system. O'Neill et al. (2011) observed TMR emit considerably high CH_4 than pasture. However, in these experiments the proportion of the feed ingredients and DMI differed considerably and no reason was attributed towards the variation in CH_4 emission. The only report that compared the effect of TMR and SF system of same feed ingredients was by Holter et al. (1977), which observed no change in CH_4 production between different feeding systems. Furthermore, through the literature, it is clear that the particle size, passage rate and digestibility are thought to play an important role in CH_4 mitigation process by these different feeding systems since TMR were found to have shorter forage length and SF found to have longer particle length. In a study by Li et al. (2003), the TMR feeding system was found to exhibit higher digestibility of NDF due to shorter particle length which could also be related to CH_4 production. However, more studies are needed to be conducted to evaluate the effect of feeding system on ruminal CH_4 production.

2.3. Understanding rumen microbiology

The rumen microorganisms with major population of bacteria (95% of the total microbiota) play a vital role in the conversion of plant materials into digestible compounds such as volatile fatty acids and bacterial proteins which defines the quality and composition of milk and meat and their production yields (Welkie et al., 2009; Sundset et al., 2009). The active bacterial population mainly depends on different parameters such as animal, breed, type of feed, composition of feed and many other factors (Agarwal et al., 2015). Furthermore, each microbial species has special substrate preferences based on which they are classified into fibrolytic (e.g., *Ruminococcus albus, Ruminococcus flavefaciens, Fibrobacter succinogenes,* and *Butyrivibrio fibrisolvens*),

amylolytic (e.g., *Selenomonas ruminantium, Streptococcus bovis*), proteolytic (e.g., *Prevotella spp*.), lipolytic (e.g., *Anaerovibrio lipolytica*), lactate producers (e.g., *Streptococcus bovis and Selenomonas ruminantium*) and lactate consumers (e.g., *Megasphaera elsdenii*) (Zhou et al., 2015). Compared with bacteria, the diversity of rumen archaea which play major role in direct CH_4 production is much less (Henderson et al., 2015) with seven known orders of methanogens: *Methanobacteriales, Methanomicrobiales, Methanococcales, Methanocellales, Methanopyrales, Methanosarcinales* and the recently discovered order *Methanomassiliicoccales* (Oren and Garrity, 2014). So far, 120 species and 33 genera of methanogens have been identified in the rumen (Cersosimo and Wright, 2015). However, the genus *Methanobrevibacter* of order Methanobacteriales which is believed to be the major producer of CH_4, is the most abundant methanogen in the rumen (Jeyanathan et al., 2011; King et al., 2011; St Pierre and Wright, 2012). Additionally, these microbes and their symbiotic relationship are believed to play an important role in maintaining host health, improving animal productivity and reducing environmental pollution. However, there is still a lack of understanding of the mechanisms governing microbe-microbe interactions and their colonization patterns based on their feed type.

Methanogenesis, the CH_4 production step, is where methanogens generate their energy in the form of ATP (Ferry & Kaestad, 2007). Methane in the rumen is produced by microorganisms called methanogens as a by-product of anaerobic fermentation by the reduction of CO_2 and H_2 . Glucose equivalents like cellulose, hemicellulose, pectin, starch, sucrose, fructans and pentosans from animal feed stuffs are broken down into pyruvate by the ruminal microorganisms. This pyruvate under anaerobic conditions undergoes oxidation reaction to produce reduced co-factors such as NADH which is then re-oxidized

to NAD to complete the synthesis of volatile fatty acids (VFAs). Depending on the amount and proportions of different VFAs produced, different amounts of CH_4 and CO_2 are also produced. When acetate is produced, re-oxidation of NADH is occurred by production of H_2 that can be further used by methanogenic archaea (methanogens) to reduce CO_2 to CH_4. According to the electron donors used, three methanogenesis pathways have been described. They are hydrogenotropic, that utilizes H_2 , methylotropic, that utilizes methanol and methylamines (Lang et al., 2015) and aceticlastic, that utilizes acetate (Janssen and Kirs, 2008). Hydrogenotropic is the most common pathway used by methanogens found in the ruminant livestock since majority of known methanogens grow when H_2 is used as the electron donor (Kim and Gadd, 2008). Carbon dioxide is the major electron acceptor combines with H_2 and reduces to form CH_4. This eradication of H_2 by methanogenesis helps in reducing the partial pressure of H_2 in the rumen favoring effective fermentation (Moss et al., 2000). Further, it also helps in keeping the partial hydrogenase activity favoring hydrogen production, thus avoiding the formation of lactate or ethanol as major end products and allowing more acetate to be produced in the rumen (Wolin and Miller, 1988). However, this CH_4 emission has a serious effect on environment and animal.

As a positive step towards understanding rumen microbiome, advances in genomics technologies have provided new opportunities to analyze entire microbial communities. Previously, culture-based methods to study rumen microbiology (Hungate, 1966; Hespell et al., 1997) provided deep knowledge on role of individual microbes and rumen dynamics (Whitford et al., 1998; Tajima et al., 1999; Weimer et al., 1999; Tajima et al. 2000; Kocherginskaya et al., 2001; Tajima et al., 2001; Whitford et al., 2001; Klieve et al., 2003; Larue et al., 2005; McEwan et al., 2005; Michelland et al., 2009; Hook et al., 2009;

Welkie et al., 2009) which helped to understand the microbial diversity in the rumen. However, these approaches were found to describe only fraction of rumen microbial diversity (Amann et al., 1995; Pace, 1997). This limitation led to emergence of advanced technologies like microarray technologies, electrophoretic sizing techniques like DGGE and T-RFLP and a targeted sequencing of amplicons that includes 454 pyrosequencing and Illumina sequencing (Next Generation sequencing, NGS) (Deng et al., 2008). The latter technologies are the current hotspot in studying microbial diversity in various ecosystem. The invention of NGS has allowed a breakthrough in sequencing of whole genomes, metagenomes and analysis due to their increased throughput and decrease in costs of sequencing. This metagenomic approach analyses total microbial DNA extracted directly from the environment and hence the microbiome could be immediately investigated to yield large amount of raw data. In addition, NGS technologies have allowed exploration of complex communities like termite hindgut (Warnecke et al., 2007), human intestinal tract (Turnbaugh et al., 2007), human saliva (Willner et al., 2011) and cow rumen (Brulc et al., 2009) at higher throughput than Sanger based sequencing. Furthermore, compared with 16S rRNA gene analysis techniques, metagenomic sequencing by NGS has been shown to achieve high coverage that accurately reflect the structure of the gut microbiome (Bhatt et al., 2013). To be precise, in NGS, Illumina sequencing is considered as the best sequencing tool nowadays than the 454 pyrosequencing due to the number of reads obtained that makes this technology the most well suited to deep-coverage sequencing and also it is less cost associated.

Many studies on the use of NGS technology in rumen studies have reported fibre-associated rumen metagenome (Brulc et al., 2009) and on 16S rRNA gene amplicon profiles (Callaway et al., 2010; Pitta et al., 2010). There

were also studies that compared the rumen microbial diversities across dairy cows fed pasture or TMR (De Menezes et al., 2011), composition and similarity of bovine rumen microbiota across individual animals (Jami and Mizrahi, 2012), steers fed different dietary fiber (Thoetkiattikul et al., 2013), steers fed forage and concentrate (Petri et al., 2013), different age groups of goats (Han et al., 2015), steers differing in feed efficiency (Myer et al., 2015), feed restricted cattle (McCabe et al., 2015), between omasum and reticulum (Peng et al., 2015), high grain diet with dicarboxylic acids or polyphenols (De Nardi et al., 2016), isoflavone enriched feed on dairy cows (Kasparovska et al., 2016), across dairy cows (Indugu et al., 2016). There were also reports on methanogen diversities in rumen related to CH_4 formation evaluated by using NGS (Snelling et al., 2014; Danielsson et al., 2014; Wallace et al., 2015). Though the effect of feeding systems on total bacteria, fungi and protozoa have been reported by Li et al. (2003), variation in microbial diversity related to animal performance and CH_4 formation at genera level has never been reported using NGS technology. It was believed that countless nutritional strategies potentially create an equal number of unique microbiomes that will enable a greater understanding of the host–microbe relationship and its impact on animal performance and CH_4 emissions. Lack of sufficient understanding of the ruminal microbiome will hinder effective enhancement of animal productivity and CH_4 mitigation. Therefore, many studies on feeding systems on rumen microbial populations has to be carried out.

3. Materials and methods

Two experiments were conducted at Seoul National University, Pyeongchang, Republic of Korea from October to December, 2015. All animal-related procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Seoul National University, Republic of Korea. Animal feeds were processed and purchased from a domestic feed mill company and all experimental chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO).

3.1. Animal and experimental design

Six Holstein steers were used for two consicutive experiments, and were divided into two group by similar BW and allocated into two pen equipped with calan doors for 30 days' adaption. A triplicated 2×2 Latin square design was used with two different feeding system of same diet over two consecutive periods of 17 days in experiment 1 including, 11 days for diet adaption in the pen and 6 days for CH_4 measurement in the indirect respiratory chamber. Experiment 2 consisted of two consecutive periods of 22 days including 11 days for diet adaption in the pen and 6 days for CH_4 measurement in the indirect respiratory chambers and 5 days for sample collection.

3.2. Experimental diet and feeding

Total mixed ration was prepared using 73% concentrates including water, yeast culture, limestone, salt and molasses and 27% roughage on a fed-basis (Table 1). The concentrates and TMR were packed in a 20 kg polypropylene bags with one-way channels to emanate gas produced during fermentation. TMR and concentrate exhibited weak fermentation on storage,

where the pH was found to be 5.49 and 5.29 and lactic acid concentrations were observed to be 1.8 and 1.4% DM, respectively. Animals were fed experimental diets at 2.4 (± 0.3) and 2.0 (± 0.4) % BW to achieve average daily gains of 1.4 kg and 0.7 kg in experiment 1 and 2, respectively (Table 2), twice a day in equal amount at 0900 and 1800 h. The animals were weighed on the last day of each experiment to determine their body weight for adjusting their feeding levels. In TMR feeding system, the roughages were chopped and mixed thoroughly with concentrate, whereas in SF the roughages were fed first without chopping and then the concentrate was fed separately to prevent unnecessary drop of the pH in initial ruminal fermentation. The animals were given full access to water and mineral block for 24 hrs in a pen as well as in respiratory chamber. The samples of the feed offered were collected and stored in a dry location to measure the dry matter content and to perform other chemical analysis.

Table 1. Ingredients and chemical composition of basal diet

Ingredients composition (% DM)		Chemical composition	
Concentrate			
Corn gluten feed	23.0	DM %	
Wheat bran	1.0	TMR	90.0
Coconut meal	13.5	Concentrate	83.0
Broken corn	5.6	OM %	79.2
Corn flake	2.4	CP, % DM	16.6
Cotton hull pellet	11.8	CF, % DM	4.4
Water	2.0	NDF, % DM	28.7
Cotton seed	4.0	ADF, % DM	12.6
Yeast culture	3.0	GE, Mcal/Kg DM	3.9
Limestone	1.0		
Salt	0.2		
Molasses	5.0		
Mineral-vitamin mixture	0.5		
Roughages			
Alfalfa hay	5.0		
Perennial rye grass	10.0		
Annual rye grass	10.0		
Klein grass	0.2		

DM- Dry matter; TMR- Total mixed ration; OM- Organic matter; CP- Crude protein; CF- Crude fiber, NDF- Neutral detergent fiber; ADF- Acid detergent fiber; GE- Gross energy

Table 2. Average body weight and nutrient intake of animals

Feeding level (% BW)	Group	Mean BW[1] (Kg)		ADG (Kg)	Nutrient intake (Kg/d)			GEI (Mcal/d)
		Initial	Final		DM	CP	NDF	
2.4 %	1	517	571	1.67	11.0	1.8	3.2	42.7
	2	280	319	1.20	7.5	1.2	2.2	29.1
2.0 %	1	571	592	0.64	9.6	1.6	2.8	37.2
	2	319	352	0.67	7.3	1.2	2.1	28.2

[1] n=6 among two experiments; DM- Dry matter; CP- Crude protein;

NDF- Neutral detergent fiber; GE- Gross energy

3.3. Methane emission measurement

On days 12 - 14, first group of three animals were placed in three chambers and CH_4 productions were recorded, followed by 2nd group on days 15 - 17. To avoid uncertainty in data, animals were placed in the same chamber while measuring CH_4 in period 2. Gas sampling for measuring CH_4 was performed using three Indirect open circuit respiratory chamber made of steel frame and polycarbonate sheet (Miller and Koes, 1988). Each chamber (137cm wide × 256cm deep × 200cm tall) was equipped with feeder, waterer, air conditioner (Busung Co. Ltd., India, model ALFFIZ-WBCAI-015H) and dehumidifier (Dryer Korea, model DK-C-150E) to maintain the temperature and humidity, respectively. The gas analysis system consisted of gas sampling pump (B.S Technolab INC., Korea), tunable diode LASER CH_4 gas analyzer (KINSCO Technology. Co. Ltd., Korea, model Airwell+7), data acquisition and analysis unit. The respiration chamber was maintained at a controlled temperature and humidity of 25°C and 50%, respectively. A flow meter (Teledyne Technologies Inc., USA, model LS-3D) was used to record and maintain the flow rate of air flowing out of the respiratory chamber to the analyzer, which was 600 L/min throughout the experiment. The gas analyzer was calibrated and recovery rate of each chamber was measured at the beginning of each experiment using a standard calibration CH_4 gas mixture (Air Korea, 25% mol/mol). The temperature, humidity and aeration flow rate for the experiment were decided by optimizing them for maximum recovery of standard CH_4 gas. Concentrations of CH_4 in the inlet and outlet air of each chamber were measured every 10 min. Some intrusions occurred every day while cleaning the chamber floor and feeding and these intrusions had little effect on measurement. However, these fluctuations were considered and

summed to derive the 24-h emission value. Finally, these emission values were recalculated based on the recovery rate of each chamber.

3.4. Digestion trial and rumen sampling

In experiment 2, the effect of feeding system on total tract digestibility of nutrients was studied using chromic oxide (Cr_2O_3) as an external marker. Chromic Oxide was top-dressed twice daily onto the feed at 0.2% of daily feed amount for TMR, whereas mixed with the concentrate for SF system. Fecal samples (100 g of wet weight) were collected from the rectum of each animal on days 18 - 21 of each period. Samples were taken at various times throughout the day, to minimize the fluctuations in Cr concentration and these samples were frozen at -20°C for further analysis.

Samples of ruminal fluid were collected 1.5, 3 and 4.5 h after feeding on day 22 of each period of experiment 2 using a stomach tube as explained by Beauchemin et al. (2014). Saliva contamination of the rumen fluid was checked and the whole ruminal contents were squeezed through four layers of cheese cloth and the pH was immediately measured using a pH meter (model AG 8603; Seven Easy pH, Mettler-Toledo, Schwerzenbach, Switzerland). The ruminal fluid was centrifuged at 8,000 rpm for 10 min, and the supernatant was transferred to a 50 ml centrifuge tube and stored at -20°C for further determination of Ammonia-N, volatile fatty acid concentrations and microbial diversity.

3.5. Feed particle size measurement

The particle size of the feed in both TMR and SF was determined using the Penn State Particle Size Separator (PSPSS) with the technique explained by

Kononoff et al., 2003. Approximately 200 g of dried feed samples were placed on the top sieve of pore size 19 mm stacked over a series of sieves of varying pore size. This set up was shook horizontally in one direction five times. The screens were then rotated a one quarter turn and shook another five times for a total of 8 sets leading to 40 shakes. The material in each of the sieves and the bottom pan were then weighed and percentage of the feed particles belonged to a range of size was calculated.

3.6. Chemical analyses

The samples of feed and feces were dried in hot air oven at 65°C for 72 hours and ground to pass through a 1 mm screen (Thomas Scientific Model 4, New Jersey, USA) and then assayed for dry matter (DM), crude protein (CP), ash and Cr by the method explained in AOAC (1990). Neutral detergent fiber (NDF) content was estimated by the method of Van Soest (1991). The gross energy (GE) of both feed and fecal samples were estimated using the bomb calorimeter (Shimadzu CA-3, shimadzu corporation, Japan).

A 5.0 mL aliquot of rumen fluid was mixed with 0.05 mL saturated $HgCl_2$, 1.0 mL 25% HPO_3, and 0.2 mL 2% pivalic acid to measure volatile fatty acids (Erwin et al. 1961), and the mixture was subjected to gas chromatography equipped with flame ionization detector and a FFAP CB column (25 m × 0.32 mm, 0.3 μm, Agilent Technologies, Santa Clara, CA, USA). Ammonia-N concentration was determined using a modified colorimetric method (Chaney and Marbach, 1962).

3.7. Genomic DNA extraction and Quantification

Genomic DNA was extracted from thawed rumen fluid samples using the NucleoSpin soil kit (Macherey-Nagel, Düren, Germany), with minor modifications. Briefly, 5 ml of thawed rumen fluid was centrifuged at 12,000 rpm using Centrifuge Smart 15 (Hanil Science Industrial, South Korea) and supernatant was discarded. Three hundred and fifty µl of Lysis buffer and 75 µl of enhancer was added to the pellet, and vortexed horizontally for 2 mins. The liquid was transferred to the NucleoSpin® Bead Tube Type A containing the ceramic beads and was vortexed using the taco™ Prep bead beater (GeneReach Biotechnology Corp., Taiwan). The rest of the procedure was followed according to the manufacturer's instructions. Finally, the quantity and quality of 100 µl of eluted DNA were determined by absorption spectroscopy, using a Nanodrop spectrophotometer ND-1000 (Thermo Scientific Inc.). A 1.5 µl blank of the elution buffer used for the extraction of the DNA sample was initially used to zero the spectrophotometer before determining the absorbance at 260 nm (A260). The purity of the nucleic acid sample was estimated from the A260/A280 ratio. The DNA sample was stored at -20°C until further use.

3.8. PCR amplification and library construction

In the present study, V4 domain of the bacterial 16S rRNA was selected as target for interrogating the bacterial communities since the genus-level coverage of this region was found to be high (Mizrahi-Man et al., 2013). For amplification of this V4 hypervariable region, the primers ArBaF515 (5′-CACGGTCGKCGGCGCCATT-3′) and ArBaR806 (5′-GGACTACHVGGGTWTCTAAT-3′) was used, as this primer set was found to be used extensively in studying rumen bacterial diversity (Webster et al., 2016;

Meale et al., 2016). This primer set targets ~312 bp of the V4 hypervariable regions can be fully covered by the Illumina MiSeq. For interrogating the methanogen diversity, primer set Ar915aF (5′-AGGAATTGGCGGGGGAGCAC-3′) and Ar1386R (5′-GCGGTGTGTG AAGGAGC-3′) targeting the ~500 bp of the V6-V8 domain of archaeal 16S rRNA was used since it mainly targets the methanogens (Skillman et al., 2004). This primer set has been reported to study rumen methanogen diversity (Henderson et al., 2015; Seedorf et al., 2015). Both the primer sets were modified to contain an Illumina adapter and linker region for sequencing on the Illumina MiSeq platform and, on the reverse primer, a 12-base barcode to enable sample multiplexing. The list of barcode used for sample multiplexing in both the primer sets is presented in Table 3 and 4. Briefly, the PCR reaction was prepared using genomic DNA (5 ng), reaction buffer with 25 mM Mg2+, dNTP (200 mM each), Ex Taq polymerase (0.75 units; Takara Bio, Shiga, Japan), and 5 pmol each of the barcoded primers. The PCR reaction for both bacterial V4 region and V6-V8 archaeal domain was carried out at 94°C for 3 min for initial denaturation, 30 cycles of 45 s at 94°C, 1 min at 55°C, 90 s at 72°C for amplification, and 72°C for 10 min for final extension. After amplification, the correct sizes of PCR products were verified by 1.5% agarose gel electrophoresis (Fig 2 and 3). Then, the PCR products of both the primer pair targeting bacteria and archaea were quantified using the Quant-iT™ dsDNA Assay Kit, high sensitivity. After quantification, all amplicons of two different target region from the 24 DNA samples were pooled into two separate pools and loaded onto a 1.5%-agarose gel. Bands were visualized and the target band was excised and extracted using QIAquick Gel Extraction Kit (Qiagen, CA, USA). The extracted DNA was used to construct the bacterial V4 and archaeal V6-V8 sequencing library with the NEBNext Ultra DNA Library Prep Kit (cat. E7370S; New

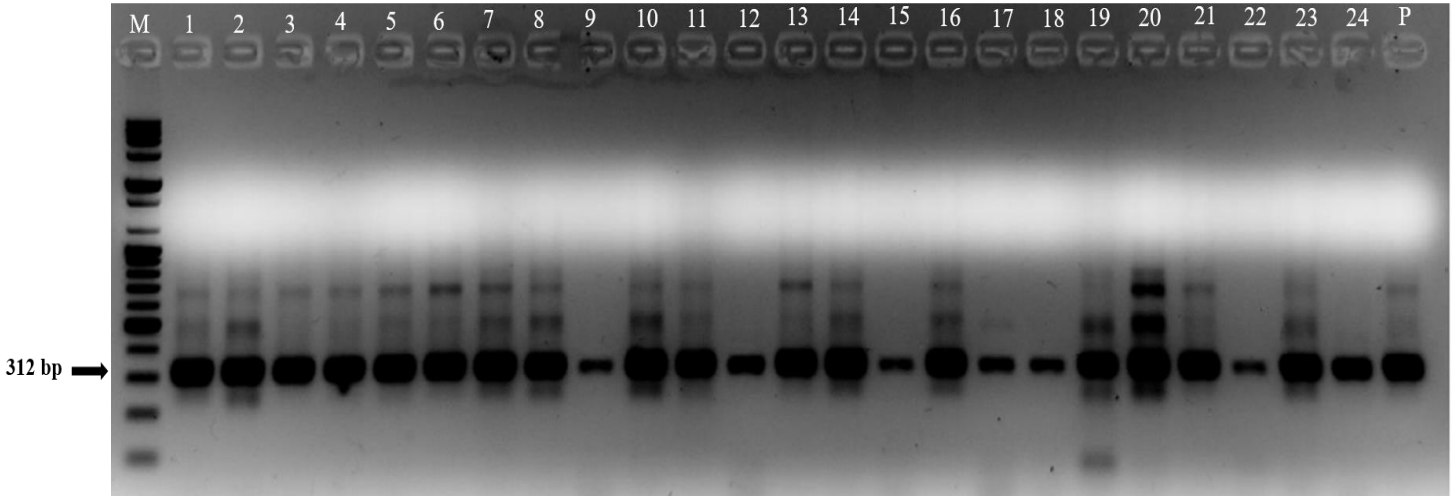
England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. Both the library was sequenced separately for paired-end 250-bp reads in the Illumina MiSeq.

Table 3. Barcode sequence used in bacterial V4 primer for multiplexing

Sample ID	Barcode Sequence	Sample details
1	CCAAAGCTACTG	TMR.1.5hr.1
2	CCAATACAGTCG	TMR.3.0hr.1
3	CCAAGTACAGTC	TMR.4.5hr.1
4	CCAACGCAGTTA	SF.1.5hr.1
5	CCAATGATAGCC	SF.3.0hr.1
6	CCAATACGGCTA	SF.4.5hr.1
7	CCAAGCCATTGA	TMR.1.5hr.2
8	CCAACAGACTTG	TMR.3.0hr.2
9	CCAACTGACAGT	TMR.4.5hr.2
10	CCAACGACTTGA	SF.1.5hr.2
11	CCAATAGTCCGA	SF.3.0hr.2
12	CCAAGACGATTC	SF.4.5hr.2
13	CCAATTACAGGC	TMR.1.5hr.3
14	CCAAGCTGACTA	TMR.3.0hr.3
15	CCAAGCACTATG	TMR.4.5hr.3
16	CCAACAATCGGT	SF.1.5hr.3
17	CCAATGCATGAC	SF.3.0hr.3
18	CCAAGATCCTAG	SF.4.5hr.3
19	CCAATGTCACGA	TMR.1.5hr.4
20	CCAACTTGGCAA	TMR.3.0hr.4
21	CCAAAAGGTCTC	TMR.4.5hr.4
22	CCAATAAGCCTG	SF.1.5hr.4
23	CCAAAATGCTCG	SF.3.0hr.4
24	CCAACAGTTGAC	SF.4.5hr.4

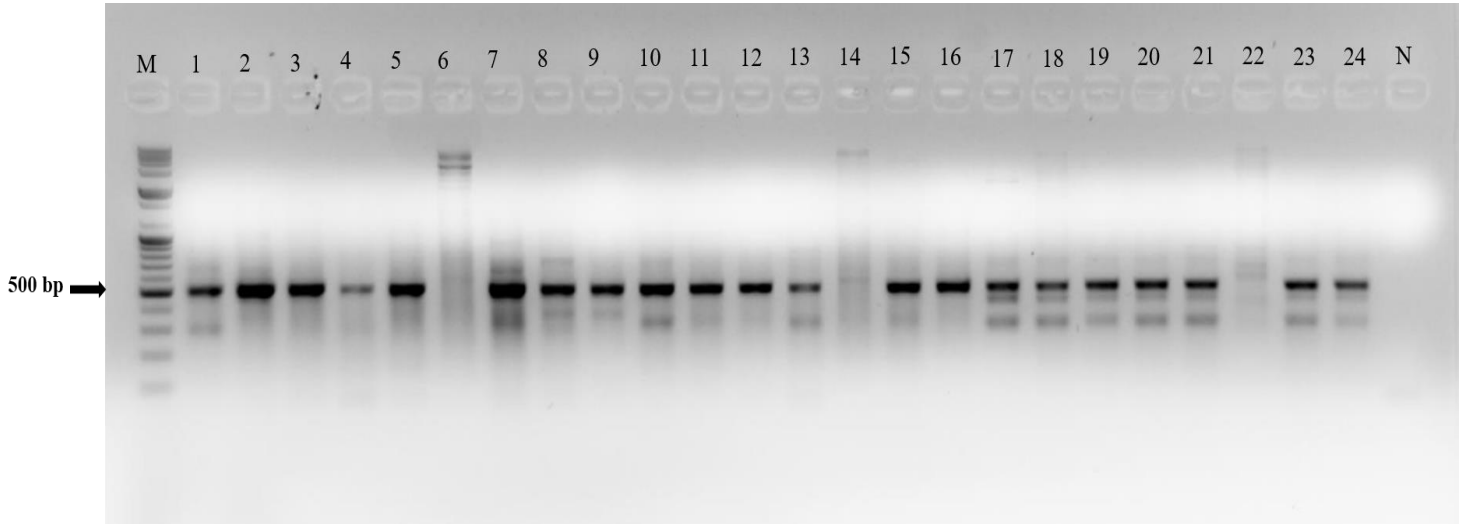
Table 4. Barcode sequence used in archaeal V6-V8 region

Sample ID	Barcode Sequence	Sample details
1	TTGGCTTAAGGC	TMR.1.5h.1
2	TTGGATCAGCTG	TMR.3h.1
3	TTGGTGATCGAC	TMR.4.5h.1
4	TTGGGTAAGCTC	SF.1.5h.1
5	TTGGGATGCATC	SF.3h.1
6	AACCCTTAAGGC	SF.4.5h.1
7	TTGGGTACCGTA	TMR.1.5h.2
8	TTGGGTCAATGC	TMR.3h.2
9	GGTTCTTAAGGC	TMR.4.5h.2
10	GGTTATCAGCTG	SF.1.5h.2
11	GGTTTGATCGAC	SF.3h.2
12	GGTTGTAAGCTC	SF.4.5h.2
13	GGTTGATGCATC	TMR.1.5h.3
14	AACCATCAGCTG	TMR.3h.3
15	GGTTGTACCGTA	TMR.4.5h.3
16	GGTTGTCAATGC	SF.1.5h.3
17	CCAACTTAAGGC	SF.3h.3
18	CCAAATCAGCTG	SF.4.5h.3
19	CCAATGATCGAC	TMR.1.5h.4
20	CCAAGTAAGCTC	TMR.3h.4
21	CCAAGATGCATC	TMR.4.5h.4
22	AACCTGATCGAC	SF.1.5h.4
23	CCAAGTACCGTA	SF.3h.4
24	CCAAGTCAATGC	SF.4.5h.4



Input PCR Products: 15ul+3µl (loading dye); M: DNA ladder 100bp Plus (Bioneer, South Korea.); 1-24: Sample ID (Table 3); P- Positive

Fig 2. PCR amplification of bacterial V4 region of 16s rRNA



Input PCR Products: 15ul+3µl (loading dye); M: DNA ladder 100bp Plus (Bioneer, South Korea.); 1-24: Sample ID (Table 4); N- Negative

Fig 3. PCR amplification of archaeal V6-V8 region of 16s rRNA

3.9. Microbial community diversity analysis

The raw Illumina MiSeq reads were demultiplexed according to the barcodes and the sequences were quality-filtered (>= Q20). The processed paired reads were concatenated into a single read, and each single read was screened for operational taxonomic unit (OTU) picking using the UCLUST embedded within the QIIME 1.9.0 with the greengenes database (gg_otus-13_8-release, 97% nucleotide identity). Alpha diversity was estimated using the QIIME pipeline, and rarefaction curves were generated based on mean values of 10 iterations with 10,000 reads per sample. Principal Component Analysis (PCA) was performed using several genera with significantly different abundance.

3.10. Statistical analysis

Data were analyzed as a Latin square using the general linear models procedure (Version 9.1, SAS 2002); animal, period and treatment were the effects in the model. Differences were considered to be significant when $P < 0.05$.

4. Results and Discussions

4.1. Effect of Feeding system on CH4 emission

There were no differences in DM and nutrient intakes between feeding systems in experiment 1 and experiment 2 (Table 5). It seems likely to have restricted their level of intake with observed mean DM intake in experiment 1 and experiment 2. When diets were fed at 2.4% BW in experiment 1, there were no significant differences between feeding systems in CH_4 production per day and CH_4 emission related to the amount of organic matter intake (OMI), and CH_4 as proportion of gross energy intake (GEI). The absence of treatments effect on CH_4 emission at feeding level of 2.4% BW have also been reported by Holter et al. (1977) who compared the effect of mixed and separate feeding of concentrate and silage fed at 2.5% BW. However, when feeding level decreased from 2.4% to 2.0% BW, significantly higher CH_4 production for TMR ($P < 0.05$) compared to SF was noted in experiment 2 (Table 6). The diurnal variation of CH4 emission between the feeding system after evening feeding clearly shows the higher CH_4 emission level for TMR (Fig.4)

Numerous advantages like choice minimization among individual feeds, reduction in labor and time required for feeding, similarity between consumed and formulated diet, sufficient fiber intake to prevent milk fat depression, easier to manage and reduced incidence of subclinical ruminal acidosis of using a TMR have been reported (Maekawa et al., 2002). However, very few studies, such as the present experiment and by Holter et al. (1977), have compared the CH_4 emissions of cattle fed same ingredients of forage and concentrates as a

TMR or separately. Though the CH_4 production per unit animal production was not determined in the present experiment it was expected that TMR feeding system stimulate greater feed efficiency and reduce CH_4 production per unit animal production than separate feeding. This must be one of the important plausible explanation for the advantage of using a TMR. In previous studies, dairy cow fed TMR (O'Neill et al., 2011,2012) produced higher enteric CH4 per cow and higher milk due to higher DMI have also been observed. However, the increase in feed efficiency was not large enough to offset the substantially great quantity of enteric CH_4 produced.

Table 5. Mean nutrient intake of animals in Exp 1 and Exp 2

	Exp 1		Exp 2	
Intake level	Mean	SEM	Mean	SEM
DM, kg/d	9.26	1.91	8.43	1.32
OM, kg/d	8.14	1.68	7.42	1.16
CP, kg/d	1.54	0.32	1.40	0.22
NDF, kg/d	2.66	0.55	2.42	0.38
GE, Mcal/d	35.91	7.42	32.71	5.15

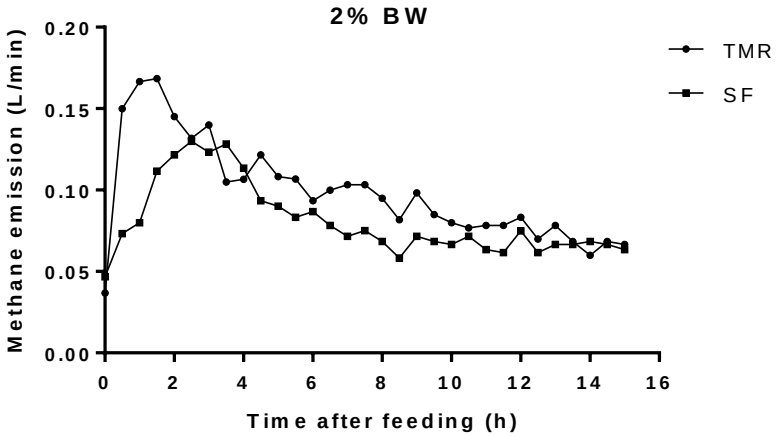
DM- Dry matter; OM- Organic matter; CP- Crude protein; NDF- Neutral detergent fiber; GE- Gross energy

Feeding Level (% BW)		TMR[1]	SF[1]	SEM	P - value
2.4 %	Methane emissions				
(Exp 1)	L/day	149.44	145.59	21.04	0.862
	g/day	106.74	103.99	15.02	0.862
	g/kg of DMI	12.24	11.60	1.37	0.660
	g/kg of OMI	13.96	13.21	1.56	0.654
	CH_4 E, % GEI	4.20	3.99	0.47	0.656
2.0 %	Methane emissions				
(Exp 2)	L/day	138.48	118.20	3.17	0.029
	g/day	96.06	84.42	2.26	0.029
	g/kg of DMI	11.28	10.29	0.11	0.011
	g/kg of OMI	13.38	11.65	0.53	0.054
	g/kg of DOM	20.67	20.09	0.46	0.338
	CH_4 E , % GEI	3.98	3.49	0.04	0.005

Table 6. Methane production of feeding systems at different feeding levels

[1] n=6 for each treatment group; DMI- Dry matter intake; OMI- Organic matter intake; DOM- Digestible organic matter; GEI- Gross energy intake

Fig 4. **Diurnal variation of CH_4 upon time after feeding**



4.2. Effect of Feeding level on CH_4 emission

Methane gas emissions per day and those per % GE intake are a critical factor determining the relationship between the dairy productivity and global warming (Gerber et al., 2011). In the present study, irrespective of the feeding system, CH_4 emission was found to be higher for animals fed at 2.4% BW in experiment 1 compared to animal fed at only 2.0% BW in experiment 2 (Table 4, Fig 5). It has been reported that there is a strong relationship between DMI and enteric CH_4 production per animal (Shibata et al., 2010; Charmley et al., 2016). It indicated that increasing feeding level resulted in increased dry matter intake (DMI) and greater intake of fermentable substrate, including both structural and nonstructural carbohydrates (Moe and Tyrrell, 1980). However, increasing feed intake, decreased CH4 emission rate (CH_4/ Kg DMI and % GEI) which was consistent with the observed results (Chaokaur et al., 2015; Hammond et al., 2013, 2014). This effect on emission rate was attributed to the decrease in digestibility of the feed at higher level of feeding which is explained by accelerated turnover rate of feed particles out of the rumen leading to a less residence time in rumen (Thornton and Minson, 1972; Owens and Goetsch, 1986). ARC (1980) also suggested that the decrease in emission rate was due to the decrease in GE availability for digestion. In addition, Nicholson and Sutton (1969) observed the same effect of decrease in digestibility of the feed coupled with the decrease in emission rate at higher levels of feeding.

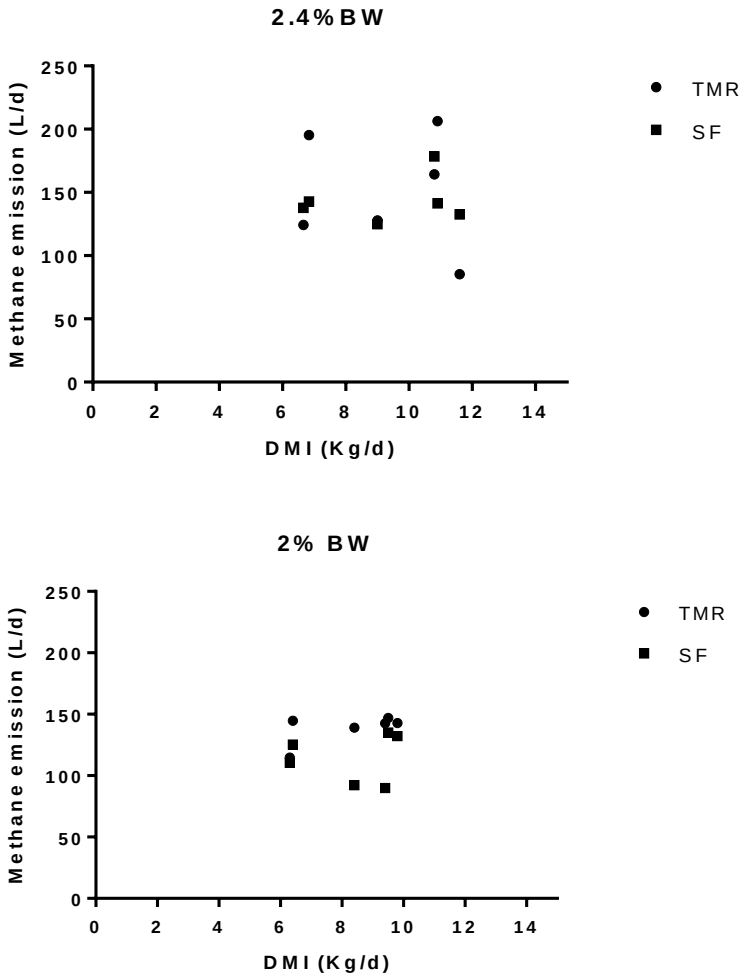


Fig 5. Effect of DMI on methane production

4.3. Effect of Particle size of roughage on CH_4 emission

The process of feed mixing for TMR can reduce particle size of forages that may increase DMI and decrease digestibility by decreasing rumen solid retention time. In the current study, as expected with TMR, the process of mixing increased the percentage of particles of size < 1.18-mm and decreased

the percentages of particles of size > 19-mm. According to the survey by Heinrichs et al., (1999), particle size greater than 19 mm corresponded typically only 7.1% for TMR but 16 to 18% for various forages, which was consistent with our observed result representing 5.4 and 18.1%, respectively (Table 7). However, there was no difference ($P > 0.05$) in the total digestibility of DM, OM, CP, NDF and intake energy between the feeding system influenced by the different particle size (Table 8). Numerous previous studies have also shown no significant differences in the nutrient digestibility between the two methods of feeding (Holter et al., 1977; Yan et al., 1998; Huuskonen et al., 2014). But, based on the reported advantages of TMR, positive ruminal fermentation characteristics and ruminal nutrient digestibility favoring CH_4 production might be postulated. In a recent study on comparison of ruminal digestibility of TMR and SF feeding system, Liu et al. (2016) reported that TMR feeding system had more proportion of ruminal contents with particle size < 1.18 mm, which is the critical size for the particle to pass the rumen (Maulfair et al., 2011). This showed that TMR exhibited higher ruminal digestibility which was also supported by the work of Kim et al. (2012), who observed increased digestibility in fermented TMR. Furthermore, in a study comparing TMR and SF system, Li et al. (2003) observed a significant increase in DM and NDF digestibility in animals fed TMR which plays a key role in ruminal CH_4 production (Blaxter and Clapperton, 1965; Takahashi , 2001) which could be related to the difference in CH4 emission between feeding system at feeding level of 2% BW.

Table 7. Feed particle size distribution (%) between the feeding system

Particle size (mm)	TMR	SF	P - value
> 19	5.43 ± 1.29	18.12 ± 3.91	0.012
19 - 8.0	29.41 ± 0.76	23.37 ± 0.84	0.002
8.0 - 1.18	35.45 ± 0.41	35.65 ± 1.87	0.89
< 1.18	29.71 ± 2.08	22.86 ± 1.91	0.026

Table 8. Apparent digestibility of nutrients between the feeding systems

	TMR[1,2]	SF[1,2]	SEM	P - value
DM, %	59.57	59.95	1.54	0.809
OM, %	61.82	62.53	0.36	0.107
CP, %	63.87	65.32	1.54	0.369
NDF, %	35.17	33.66	2.49	0.560
Energy, %	57.05	57.75	1.90	0.720

[1]n = 6 for each treatment groups;

[2] Values are LSM means with standard error (SEM);

DM- Dry matter; OM- Organic matter; CP- Crude protein;

NDF- Neutral detergent fiber

4.4. Effect of feeding system on ruminal pH, VFA and NH_3

Clear differences in the ruminal pH, volatile fatty acids (VFA) and NH_3-N production between two different feeding system were observed (Table 9). Ruminal pH for TMR feeding system was lower (P = 0.067) than that of SF system at 1.5 hr, but it was higher (P = 0.06) in TMR than in SF at 4.5 hr (Fig.6). The overall pattern of variations was similar between the feeding systems for the other variables (total VFA, individual VFA and NH_3-N) but there were significant differences observed between them at 1.5 and 4.5 hr after feeding. These changes in ruminal fermentation characteristics suggested that there was a difference in the nature of rumen fermentation between TMR and SF system. The consistent decrease in pH (Table 7) after 1.5 hr in SF can be explained by the rapid consumption of concentrate that was fed 40 minutes after feeding roughage that could have led to vigorous fermentation. Earlier reports also suggests that high digestible feeds lead to low ruminal pH due to high ruminal fermentation (Plaizier et al., 2008) and high VFA production. Also Lana et al (1998) reported that rapidly fermentable grain sources lead to low ruminal pH, C2/C3 and methane.

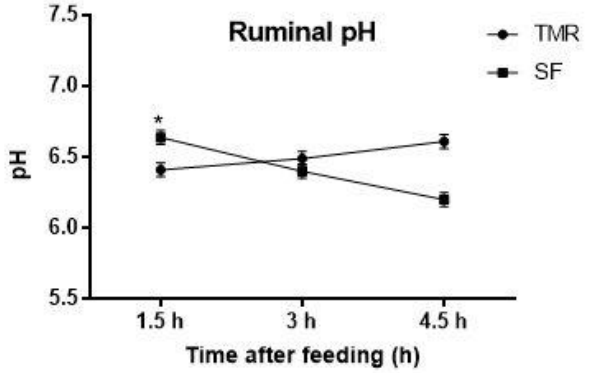


Fig 6. Effect of TMR and SF on ruminal pH

The proportion of acetate in total VFA in SF system was higher ($P < 0.05$) than in TMR system at 1.5 hr but it was found to be low ($P < 0.05$) in SF than TMR at 4.5hr. In contrast, the proportion of propionate was found to be lower ($P < 0.05$) in SF than in TMR at 1.5hr but it was observed to be high ($P < 0.05$ in SF than in TMR at 4.5 hr. Therefore, there was a significant ($P < 0.05$) decrease in A: P ratio observed after 3 hr in the SF feeding system compared with TMR feeding system (Table 9, Fig 7). However, the butyrate concentration remained at lower levels for SF at 1.5 hr and was not found to vary between feeding systems upon time. Synthesis of these short chain fatty acids (SCFA) in the rumen influence the production of CH_4. Moss et al., 2000 reported that production of acetate from pyruvate is accompanied with production of H_2 whereas butyrate and propionate production utilizes H_2 which is the major substrate for methanogenesis. The decrease in CH_4 by SF system might be due to a shift in metabolic H_2 sink towards the production of propionate, whereas the increase in CH_4 in TMR system might be due to increase in acetate from cellulose digestion (Fuller, 2004). The diurnal variation of CH_4 reflects this inter relation between VFA and CH_4 at different time points. The observed increased level of acetate and propionate at 4.5 hrs in TMR and SF respectively, correlates with their respective increase and decrease in CH_4 emission at 4.5 hr (Fig 4). This was also supported by the work of Li et al. (2003), who observed increase in xylanase activity in TMR feeding system, which is the most active fibrolytic enzyme, as well as increase in protozoal population which is also involved in fiber digestion (Bonhomme,1990; Williams and Coleman, 1991) and CH_4 production (Morgavi et al., 2012). These results suggested that TMR feeding system might have created a favorable rumen environment for CH_4 production. However, increased VFA production and decreased A: P for TMR in their study contradicted with our report. Whereas, Liu et al. (2016) observed no difference

in VFA and A: P between TMR and SF system.

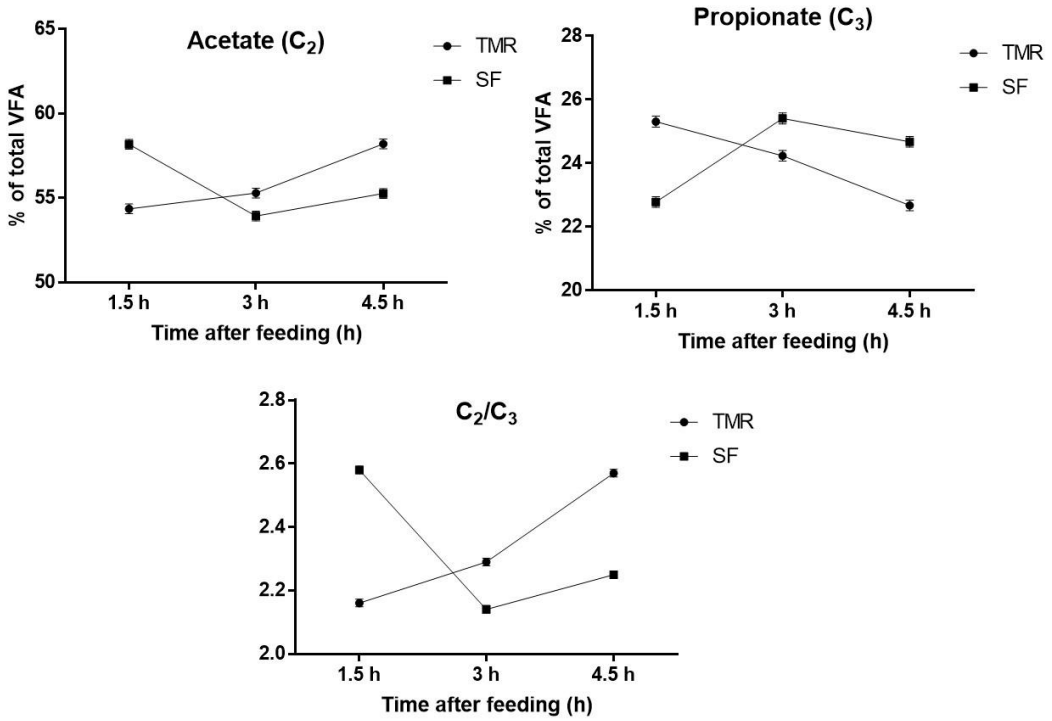


Fig 7. Effect of feeding system on AP ratio

It is interesting that, in the present experiment, the proportion of isobutyrate and isovalerate in SF was significantly higher ($P < 0.001$) than that of TMR at all the time points (Table 9, Fig 8). Isofatty acids (Isobutyrate and Isovalerate) are potential marker of ruminal protein degradation which are produced by deamination and fermentation of the released peptides (Tamminga, 1979), which occurs when optimal ruminal degradable proteins are present in rumen (Armentano et al., 1993; Yang et al., 2004). Allison (1962, 1963) reported that these isofatty acids undergo carboxylation and amination to form

peptides that are incorporated into microbes. In addition, production of valine via reductive carboxylation of isobutyrate by rumen bacteria was also observed by Allison (1971) and Russell et al. (1992). They explained that amylolytic bacteria use ammonia, peptides, and amino acids as N sources for their high maintenance requirements. Rapid growth of these bacteria might have increased the starch fermentation that lead to increased propionate production (Russel, 1998), which was observed for SF system in the present study. Hungate (1966) and Czerkawski (1986) found that the process of incorporation of peptide into microbial cells also resulted in net consumption of H_2. Therefore, in the present study, it can be postulated that, the increase in isofatty acids might have increased the microbial protein with net consumption of CO_2 and H_2, that might have lead to a decrease in CH_4 in SF system.

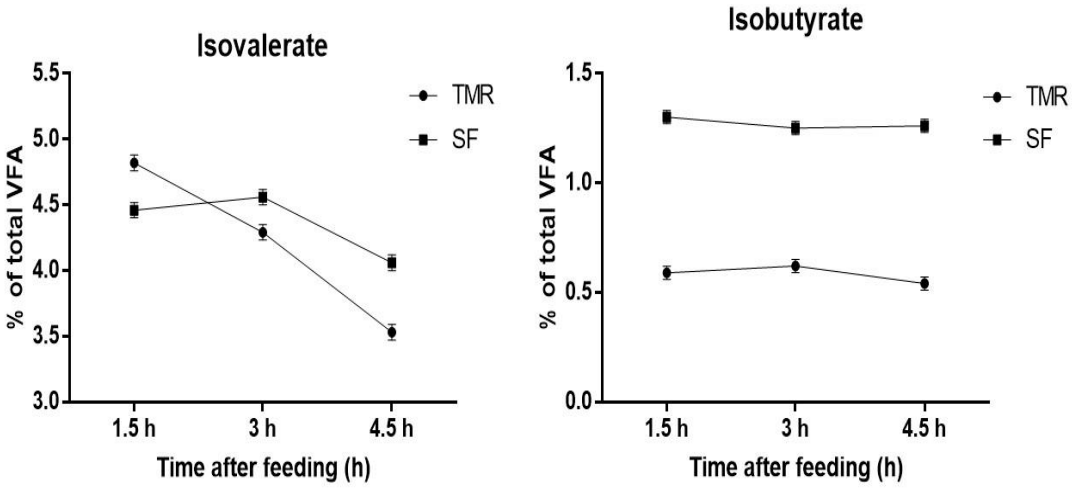


Fig 8. Effect of feeding system on isofatty acid proportion

The concentration of NH_3-N was found to gradually increase in SF system and was significantly (P = 0.005) higher than TMR at 4.5 hrs after feeding (Table 9, Fig 9). The production of NH_3-N in rumen is also another potential indicator of feed protein degradation which favors microbial protein synthesis (Bach et al., 2005). The observed increase in NH_3-N in SF system might be related to the increase in digestibility of concentrate rich in protein (Erdman et al., 1986). Liu et al. (2016) observed a similar pattern of increased NH_3-N in SF system, whereas Li et al. (2003) observed a contrasting result. However, these contradictions might be due to the variation in protein content of the feed. There were also reports suggesting that increase in ruminal crude protein digestibility decreases CH_4 production (Demeyer and Van Nevel, 1979; Kirchgessner et al., 1994; Cone and van Gelder, 1999). From the work of Patra et al. (2013), the reason for decrease in CH_4 could be attributed to the formation of (NH_4) HCO_3, where, NH_3 as the result of rumen protein degradation could be expected to combine with CO_2 and H_2, the substrate for CH_4 production, resulting in its lower production. Further, Mitsumori and Sun (2008) stated that formation of NH_3 in rumen was a result of nitrate reduction that consumed 4[2H] per mole of nitrate. As mentioned above, the increasing trend of NH_3 production in SF after feeding suggested an increase in consumption of H_2 leading to decrease in CH_4. However, it is recognized that no further explanation can be given since the researches on effect of mixed and separate feeding system on rumen fermentation are limited.

Table 9. Effect of feeding system on ruminal fermentation characteristics

Time interval[1,2]	1.5 h		3 h		4.5 h		SEM	P - value (Feed type)	P - value (Time)		
Item/Feed type	TMR	SF	TMR	SF	TMR	SF			1.5 hrs	3 hrs	4.5 hrs
Volatile fatty acids											
Total VFA (mM)	122.86	95.46	114.46	118.93	104.75	126.81	9.72	0.962	0.009	0.650	0.032
Acetate, %	55.05	58.46	56.01	54.02	58.64	55.30	1.17	0.337	0.007	0.103	0.008
Propionate, %	25.04	22.50	24.01	25.27	22.52	24.07	0.87	0.876	0.008	0.160	0.088
Butyrate, %	12.71	11.81	13.17	13.02	13.13	13.16	0.91	0.649	0.332	0.877	0.977
Isobutyrate, %	0.59	1.32	0.64	1.25	0.53	1.28	0.07	<.0001	<.0001	<.0001	<.0001
Valerate, %	1.87	1.41	2.04	1.82	1.74	1.95	0.11	0.048	<.0001	0.064	0.072
Isovalerate,%	4.74	4.49	4.16	4.60	3.43	4.23	0.26	0.027	0.352	0.110	0.006
Acetate: Propionate	2.21	2.62	2.34	2.16	2.57	2.32	0.11	0.921	0.001	0.124	0.038
NH_3-N, mg/L	18.97	9.25	14.98	14.81	9.43	15.07	1.82	0.124	<.0001	0.925	0.005
pH	6.46	6.73	6.53	6.50	6.63	6.35	0.14	0.899	0.067	0.808	0.060

[1] Sampling time after morning feeding; TMR- Total Mixed Ration; SF- Separate Feeding; SEM- Standard Error Mean

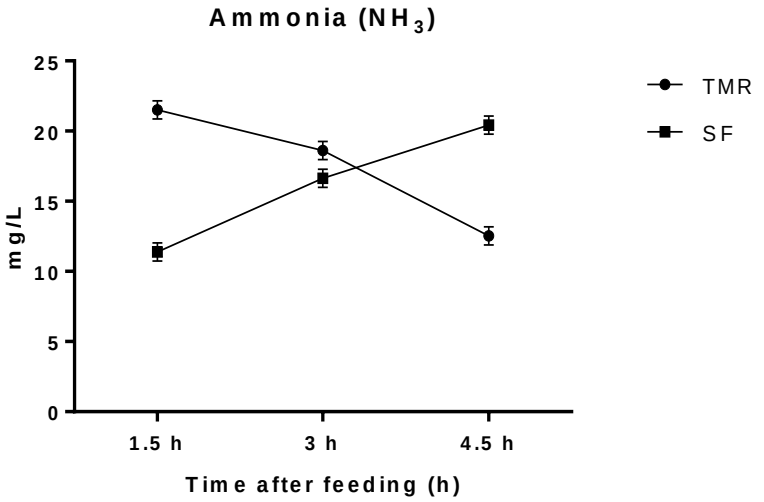


Fig 9. Effect of feeding system on ruminal ammonia production

4.5. Effect of feeding system on rumen microbial diversity

Illumina sequencing produced a total of good quality 1,231,081 bacterial and 323,775 archaeal sequences from 24 samples from 4 Holstein steers. These sequences included an average of 51,295 bacterial reads ranging from 28,357 to 176,175 reads and 15,418 archaeal reads ranging from 6,910 to 27,395 reads per rumen sample. The feeding system was found to have no significant ($P > 0.1$) effect on the total reads generated in bacteria and archaea (Table 10). All the cleaned reads of bacterial V4 and archaeal V6-V8 domain were classified into 31 phyla, 66 classes, 111 orders, 154 families, and 374 genera in total. We were able to classify the majority (more than 98 %) of the sequences below the domain level, and most OTUs could be resolved to family-level, and many to genus. However, taxonomic identifications to species level was not possible by mapping the Illumina Metagenome to 16S rRNA gene

references.

Table 10. Total number of reads generated between feeding system

	Bacteria			Archaea		
	SF	TMR	P value	SF	TMR	P value
Reads	549,988	681,093	0.365	143,759	180,016	0.291

This taxonomical classification was used to visualize the effects of feeding system on bacterial and archaeal community structure at different time intervals. At the phylum level, for all samples, around 90% of the sequences could be classified. Only the predominant sequence tags (> 0.1% of total observed tags) in both phylum and genera level classification are presented in the Table 11. Among the 30 phylum detected in bacteria, *Bacteroidetes* (40-50%) and *Firmicutes* (35-40%) were typically together representing around 80-85% of the total sequences in all samples (Table 11 and Fig 9.). Among other phyla, *Verrucomicrobia* (4-5%), *Actinobacteria* (1-3%), *Tenericutes* (1.8-2.6%), *Proteobacteria* (1.5-2.5%), *Cyanobacteria* (1 -1.9%), *Lentisphaerae* (0.7-1.4%) and *Spirochaetes* (0.8-1%) were considerably more prevalent. *Fibrobacteres, Chloroflexi, Planctomycetes* and *TM7* were found to be in low abundance (0.1-1%). Comparing individual phyla between SF and TMR feeding system, there were no significant ($P > 0.1$) difference at any times. Interestingly, *Planctomycetes* and *Actinobacteria* were significantly high ($P < 0.1$) in SF system at all times and after 4.5 hrs of feeding respectively. Whereas, phylum *Cyanobacteria* and *Lentisphaerae* were found to be significantly high ($P < 0.1$) in TMR after 1.5 hrs of feeding (Table 11). The taxonomical classification of archaeal reads assigned all OTUs to methanogen phylum *Euryarchaeota* (Table 11).

At genus level, the more predominant population (> 0.5%) in the *Bacteroidetes* phylum belonged to *Prevotella* (18-27%), *Bacteroides* (0.9-1.5%), *CF231* (0.6-0.9%) and *YRC22* (0.3-0.9%). A number of taxa were not classified to the genus level, but were present in great abundance. That includes Family *BS11* (2.7-4.7%), Family *S24-7* (1.9-3.5%) and order *Bacteroidales* (9.5-13.7%) (Fig 11a). Among the *Bacteroidetes* phylum, *Parabacteroides* was found to be significantly high ($P < 0.1$) in SF system (Table 11). Among the Firmicutes, *Ruminococcus* (2.4-3.9%), *Butyrivibrio* (1.15-3.38%), *Lactobacillus* (0.97-2.19%), *Oscillospira* (0.51-0.68%), *Streptococcus* (0.38-1.03%), *Succiniclasticum* (0.23-0.95%) and *Leuconostoc* (0.28-0.57%) were observed to be in high abundance (> 0.5%). Several abundances within the order of *Clostridiales* (7.95-10.45%), Family *Ruminococcaceae* (7.56-9.38%), *Lachnospiraceae* (3.17-4.16%) and *Christensenellaceae* (0.86-1.63%) were also prevalent (Fig 11a). More interestingly, *Leuconostoc* and *RFN-20* were found to be significantly high ($P < 0.1$) in TMR feeding system. On the other hand, *Coprococcus* was found to be significantly high ($P < 0.005$) in SF. *SMB-53* and *Anaerovibrio* were found to be high ($P < 0.5$) in TMR at 1.5h and 4.5h respectively after feeding (Table 11). In addition, *Desulfovibrio* (*Proteobacteria*) (0.54-0.75%) and *Treponema (Spirochaetes)* (0.8-1%) were also found to be predominantly high in both the feeding system. In archaea, the phylum Euryarchaeota majorly constituted of genera *Methanobrevibacter* (85.77-90.96%), *Methanosphaera* (5.59-10.63%) and *VadinCA11* (0.3-5.11%) (Fig 11b). The methanogen population was not found to vary significantly ($P > 0.1$) between the feeding system.

As witnessed by the change in ruminal ferementation characteristics between the feeding system, the results clearly suggested that there was a microbial shift in the rumen influenced by different feeding system that was

further studied through the rumen microbiome analysis between the feeding system. The concept of the microbiome (microbes, their genes and interactions with the host/habitat) is currently being evaluated in many aspects of biological science, and studies over the past decade have been dramatically advanced by Next Generation Sequencing (NGS) technology (Morgavi et al., 2013; Chaucheyras-Durand and Ossa, 2014). *Bacteroidetes* and *Firmicutes* were the most abundant phylum in the present study, irrespective of the feeding system and the results were found to be similar to that of several other studies (Jami and Mizrahi, 2012; Petri et al., 2013; Kim and Yu, 2014). Although there were no significant ($P > 0.1$) differences noted in the population of these phylum between the feeding system, the ratio of abundances between *Bacteroidetes* and *Firmicutes* were different between the feeding system. In SF system, the ratio was found to be the same at different time intervals. On the other hand, the ratio was found to vary at all observed times for cattle fed TMR (Table 11, Fig 10) . This ratio has been shown to affect energy harvesting and were correlated with increase of muscle fat (Jami et al., 2014) and alter productivity, which might have an impact on CH_4 production. It has also been reported that cattle with naturally high and low feed conversion efficiency vary considerably in the amounts of CH_4 produced (Nkrumah et al., 2006). It is clear that the feeding system plays an important role in the ratio of *Firmicutes* to *Bacteroidetes*. While we do not know the implications of the ratio shift, the change in ratio of *Firmicutes* to *Bacteroidetes* in stool samples has been associated with increases in weight gain in humans (Ley et al., 2006). Further, Erbilgin et al. (2014) provided evidence by a metabolic activity screen that Bacterial Microcompartments (BMCs) present in *Planctomycetes* are involved in the degradation of a number of plant cell wall sugars, namely L-fucose and L-rhamnose. So, it is reasonable to find this phylum in rumen. Earlier study by

Jewell et al. (2015) also observed increased population of *Pirellulaceae (Planctomycetes)* in lumen liquor in lactating cows with higher feed efficiency. It should be noted in our study that the population of the bacteria belonging to this family was found to be significantly high in population in SF system.

The most abundant genera in the *Bacteroidetes* phylum was *Prevotella* which comprise a well-known xylan degrading group (Flynt and Bayer 2008; Dodd et al. 2010) and was found to be the most predominant in rumen microbiome regardless the animal species, host diet and geographical location (Stevenson and Weimer 2007; Callaway et al., 2010; Pitta et al., 2010; Purushe et al., 2010; Lee et al., 2012; Li et al., 2012; Kim et al., 2016). This genus was also widely noted in animals fed high concentrate diets (Pitta et al., 2014) which is in accordance with the present study, where animals were fed 73% concentrate diet. Some of the species in *Prevotella* are also efficient hemicellulose, cellulose, pectin, long-chain carbohydrate, and protein digesters (Dehority, 1969; Owens et al., 1998; Nagaraja and Titgemeyer, 2007), which implies their important role in digestion. This bacterial family contributes to fumarate reductase activity, which reduces fumarate to succinate, consuming H_2, the major substrate for methanogenesis, leading to decrease in CH_4 production (Pope et al., 2011). Contrastingly, the propionate production was negatively correlated with the *Prevotella* in this experiment. In SF system, the *Prevotella* population was found to decrease numerically over time (Table 11, Fig 11a), whereas propionate production was found to increase relatively (Table 9, Fig 7). The decrease in population of *Prevotella* may be related to decrease in pH observed in SF system. This was supported by Fernando et al. (2010), who observed a decrease in *Prevotella brevis* population when pH dropped by high concentrate feeding. Our results implied that there was an another source for propionate production besides the abundance of *Prevotella.* This coincided with

the significantly high ($P < 0.005$) population of *Coprococcus* of phylum *Firmicutes* in SF system which was reported to have genome encoding the acrylate pathway that utilizes lactate to produce propionate (Reichardt et al., 2014). *Coprococcus* was also independently found to be enriched in the efficient animals' microbiomes (Shabat et al., 2016), which use H_2 for the production of propionate thereby diverting them from reducing CO_2 to CH_4 (Ungerfeld, 2015).

The next abundant genus *Bacteroides* was not found to vary significantly but the *Parabactreoides* population was found to be significantly high ($P < 0.1$) in SF system after 3 hrs of feeding. There are reports suggesting that *Parabactreoides* are able to produce bacteriocins that inhibits the RNA synthesis of other bacteria (Brook, 1999). Furthermore, these bacteriocins are believed to have direct effect on methanogens (Klieve and Hegarty, 1999) which could be related to the decrease in CH_4 observed in SF system. The other observed dominant population family BS11, that was reported to be favored by low starch diet and high pH (Zened et al., 2013) was found to be abundant in both feeding systems. Genus *CF231 (Paraprevotellaceae)* was observed to be numerically high in population in SF system. Bacteria belonging to *Paraprevotellaceae* were believed to have same function as that of *Prevotellaceae,* diverting the H_2 to propionate formation. In addition, *CF231* was reported to increase in population upon addition of nitrate (Zhao et al., 2015) and polyphenols (De Nardi et al., 2016) in feed which were reported as a potential CH4 inhibitor (Newbold et al., 2014; Jayanegara et al., 2015). Hence, there might be an unstudied relationship between *CF231* and CH4 production which is need to be focused in future.

The unclassified *Clostridiales*, *Bacteroidales* and *Ruminococcaceae* alone corresponded to almost 30% of total population which were observed to be the core microbiome in rumen across the world (Henderson et al., 2015). This implies that there are a lot of microbe in rumen that are needed to be characterized. Further, these unclassified orders were reported to play an important role in biohydrogenation (Huws et al., 2011). Irrespective of the feeding system, *Ruminococcaceae* was found most abundant bacterial family in phyla *Firmicutes* which was dominated by the *Ruminococcus* genus that includes well-known cellulolytic bacteria (Garret et al., 2011; Vodovnik et al., 2013) that are capable of producing H_2. The higher abundance of *Lactobacillus* as observed in our study is consistent with other studies where the population was observed to be high for high concentrate diets (Brown et al., 2006). Furthermore, wide functions of this genera have been discussed related to efficiency of ruminants (Jensen et al., 1956). *Oscillospira*, considerably prevalent in both feeding system has not been studied well, but were associated with feed quality (Mackie et al., 2003) and were reported to have symbiotic relationship with ciliates (Kulkerni et al., 1971). *Lachnospiraceae* was found to be the second most abundant family observed. Many members of this family have cellulolytic activity and are closely associated with other cellulose-degrading bacteria (Flint et al., 2008; Nyonyo et al., 2014). The most abundant genus of this family, *Butyrivibrio*, numerically high in population in TMR feeding system had been reported to involve in decomposition of hemicellulose and cellulose thereby producing huge amount of butyrate (Paillard et al., 2007), majorly contributing to ruminal CH_4 production (Moss et al., 2000). This could be the major reason for the observed increase in CH_4 in TMR system in the current experiment. But, contrastingly, the butyrate level in the rumen was not found to vary significantly between the feeding system after 1.5 hrs of feeding

(Table 9). *Succiniclasticum* and unclassified family *Veillonellaceae* were detected at considerable abundance in both feeding system and were numerically high in SF system and were reported to be specialized in fermenting succinate and converting it to propionate as a major fermentation product (Van Gylswyk, 1995; Kishimoto et al., 2006). In addition, *Anaerovibrio* of the same family, associated with succinate and propionate production, as well as lipid hydrolysis (Prive et al., 2013) was found to be high in TMR system at 4.5 hrs after feeding.

RFN20 (Erysipelotrichaceae) was found to be significantly high ($P < 0.1$) in population in TMR system and it was positively correlated to the weekly average milk production (Lima et al., 2015) and CH_4 production (Wang et al., 2016) in earlier studies. Similarly, the *Victivallaceae* family of phylum *Lentisphaerae* was found to be high in TMR system after 1.5 hrs of feeding. Interestingly, Myer et al. (2015) correlated *Veillonellaceae, Succiniclasticum, RFN20 (Erysipelotrichaceae)* and *Victivallaceae* to increased weight gain in steers. In addition, the family *RFP12* of *Verrucomicrobia* phylum was found to be high in abundance (Table 11 & Fig 11a) in both the feeding system. This is inconsistent with many earlier reported data, where, *RFP12* abundance was observed to be < 1%. Due to its high abundance it is thought to play an important role in rumen but the function of the bacteria belonging to this family is not studied well. All of the spirochetes strains isolated from the rumen have been assigned to the genus *Treponema* and it has been reported that bacteria from the *Treponema* strains in the rumen are able to degrade plant polysaccharides from hay or from a concentrated diet (Ziołecki, 1979; Avguštin et al., 1997) therefore, it is reasonable to find this group of bacteria in the rumen. But, the population of *Fibrobacter* was found to be too low despite the forage diet which was consistent with the studies by Pitta et al. (2010) and Callaway et

al. (2010). Further it has been reported that the *Fibrobacter* DNA was known to be hard to amplify (Tajima et al., 2001). *SMB53* genus (*Clostridiaceae*) was also observed to be significantly high in TMR system and were reported to consume mucus and plant-derived saccharides such as glucose in the gut (Wust et al., 2011). *Leuconostoc*, grouped as lactic acid bacteria that ferments structural carbohydrates to lactic acid, CO_2 and acetate was observed to be significantly high ($P < 0.1$) in population in TMR feeding system. They are also found living in association with plant material and dairy products and several studies have reported leuconostocs as the dominant microbial population on forage crops (Cai et al., 1994). Ruser (1989) also reported that heterofermentative leuconostocs were present in the highest numbers in ground maize. Further, Table 12 and Fig 12 clearly depicts the bacterial population that are significantly different ($P < 0.1$) between the feeding system.

The relationship between numbers of methanogens and amount of CH_4 produced has been a topic of debate and it has been suggested that the amount of CH_4 produced relates to the species that are present, rather than the total number of methanogens (Zhou et al., 2011; Shi et al., 2014). However, in our study the feeding system had no clear effect on population structure. These results corroborate previous studies that also showed that densities of methanogens were not significantly different between two groups of feedlot bulls (Popova et al., 2011) and two groups of lambs (Popova et al., 2013) that produced significantly different amounts of CH_4. The archaeal taxa observed in our study, 90% related to *Methanobrevibacter* species. This dominance of methanogens belonging to the genus *Methanobrevibacter* confirms previous findings (Hook et al., 2009; King et al., 2011; St-Pierre et al., 2013; Seedorf et al., 2015). Furthermore, species within the *Methanobrevibacter* genus are assumed to represent core members of the microbiome in the rumen (Jami et al.,

2012; Henderson et al., 2015). Besides many H_2 sinks explained above in SF system by various bacteria, the hydrogenotropic *Methanobrevibacter* was found to be high in abundance. The next major genera *Methanosphaera* predominantly uses methylamines as the source for CH_4 production and were usually found in low abundance (5-10%) (Mao et al., 2014; Singh et al., 2015) which is consistent with the current study. The observation in current study concludes that, despite no direct effect on methanogens, the variation in bacterial abundance that developed with the two feeding system probably vary in their metabolic potential, resulting in different proportion of metabolites becoming available for downstream methanogenic activity thereby altering the CH_4 production.

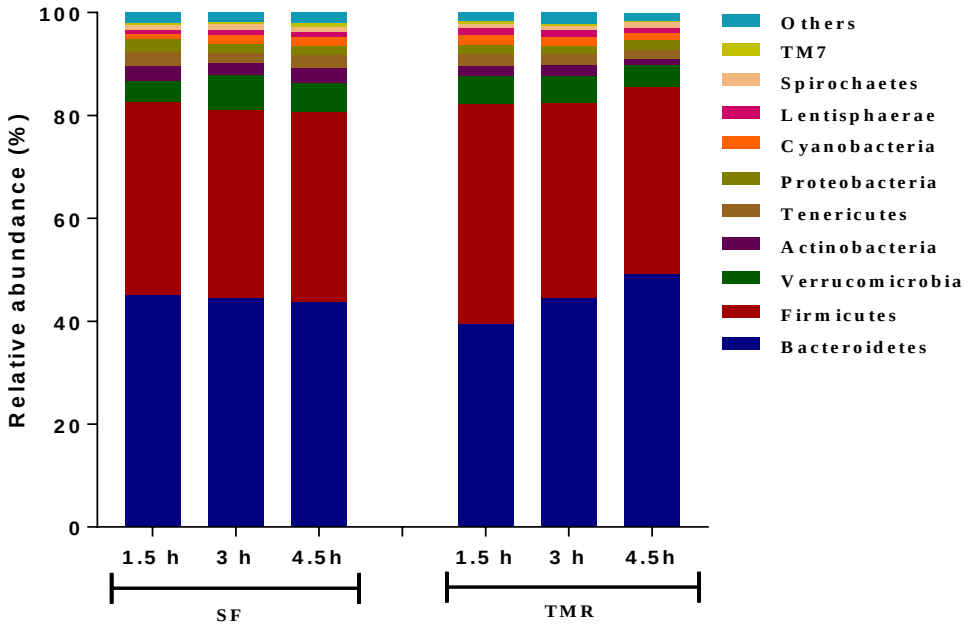


Fig 10. The taxonomic profiles for the relative phylum-level abundance of bacteria in each group classified by representation at > 0.5% of total sequences

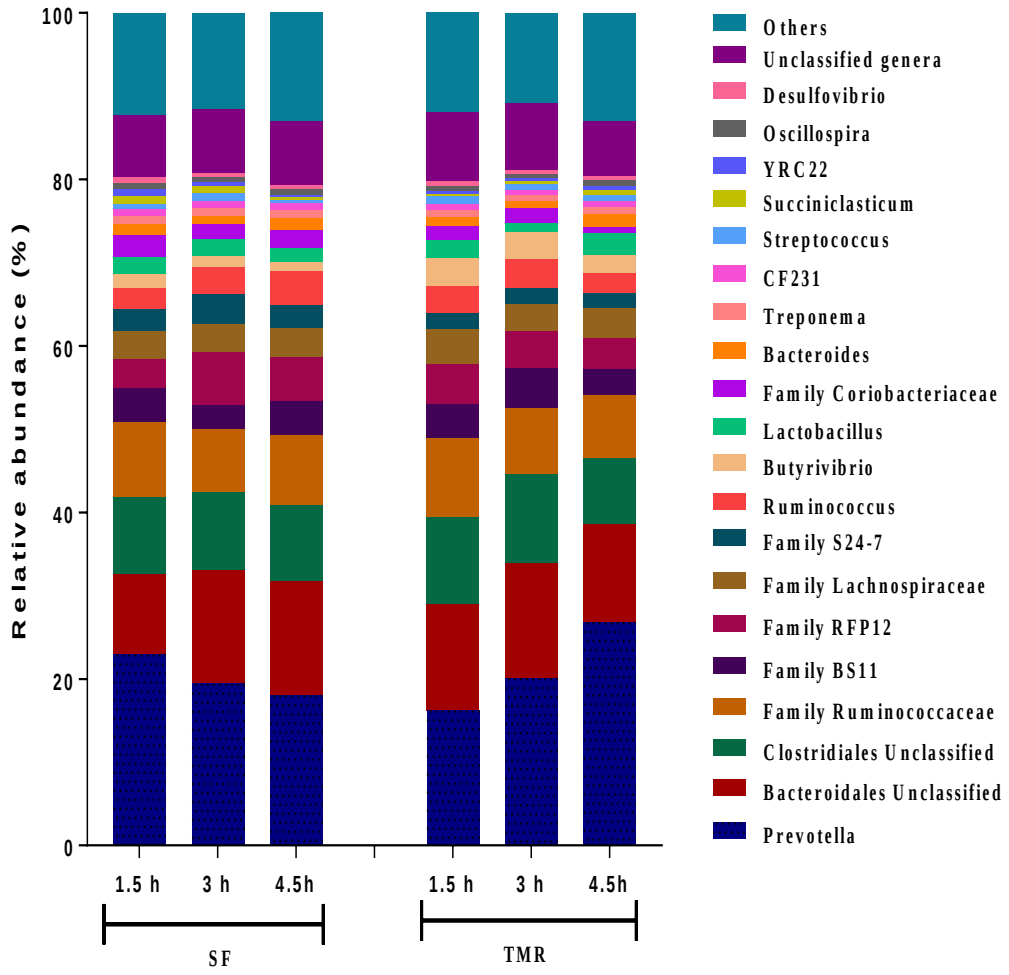


Fig 11a. The taxonomic profiles for the relative genus-level Bacteria abundance in each group at > 0.5% of total sequences

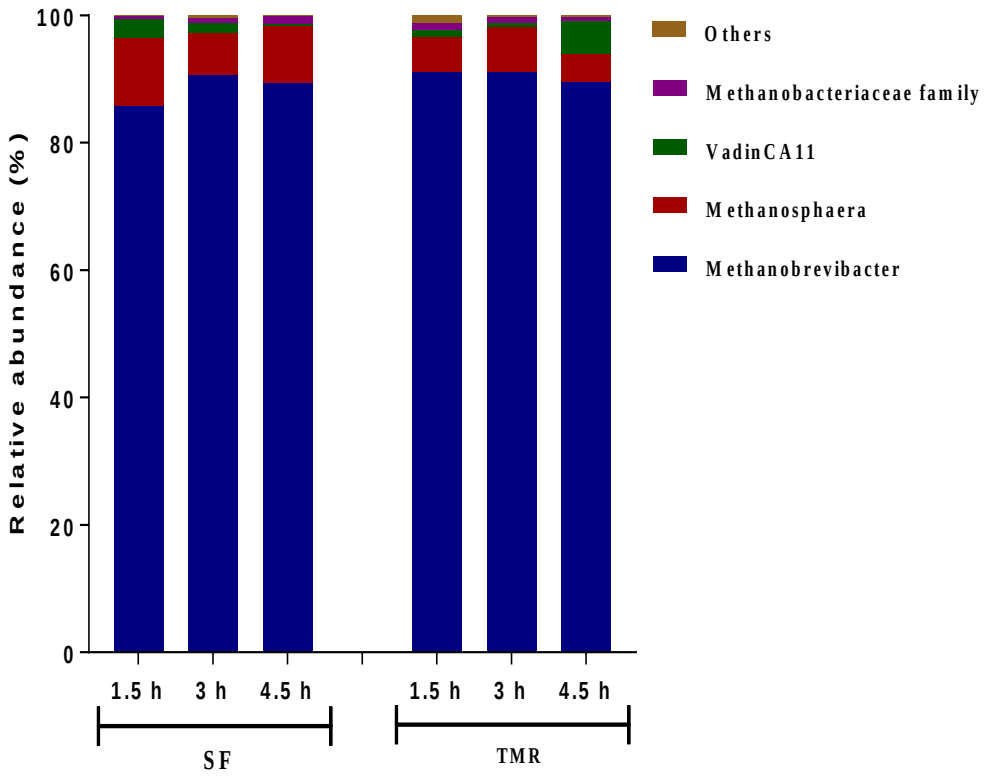


Fig 11b. The taxonomic profiles for the relative genus-level Archaea abundance in each group classified by representation at > 0.5% of total sequences.

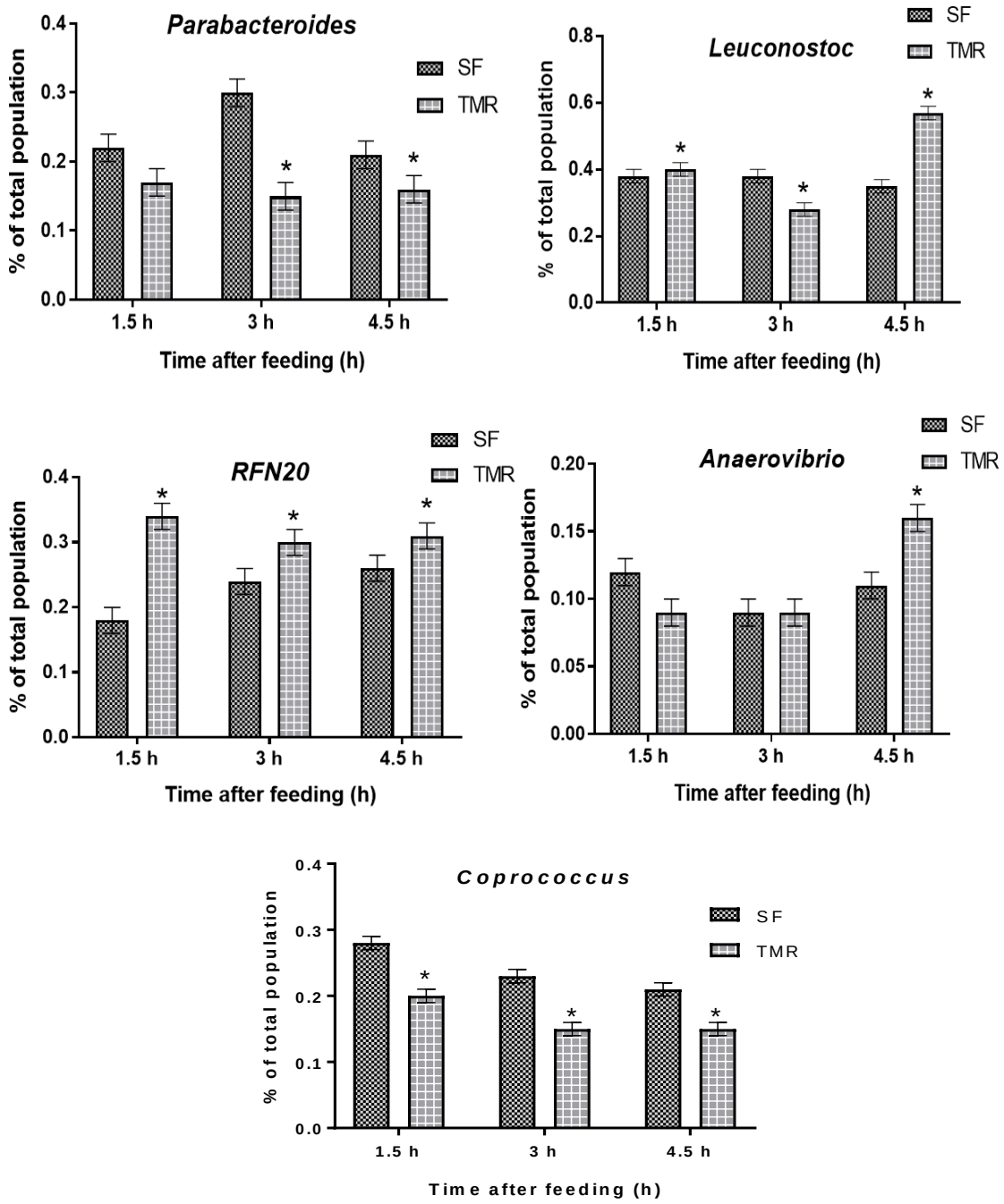


Fig 12. Relative abundance of genera in the two groups representing >0.1% of total sequences that differ significantly (P < 0.1)

Table 11. Relative abundance of taxa in the two groups representing > 0.1% of total sequences

Phylum	Classification	Percentage of total sequences [1,2]						SEM	P value (Feed)	P value (Time)		
		SF			TMR							
		1.5 h	3 h	4.5 h	1.5 h	3 h	4.5 h			1.5 h	3 h	4.5 h
Bacteroidetes		44.97	44.46	43.73	39.36	44.40	49.12	3.86	0.990	0.680	0.990	0.543
	Prevotella	22.99	19.46	18.03	16.26	20.12	26.84	2.53	0.925	0.622	0.928	0.441
	Bacteroides	1.35	1.05	1.44	1.04	0.88	1.58	0.13	0.750	0.658	0.469	0.835
	Parabacteroides	0.22	0.30	0.21	0.17	0.15	0.16	0.02	0.009	0.336	0.026	0.084
	Paludibacter	0.12	0.13	0.20	0.15	0.14	0.19	0.02	0.882	0.573	0.807	0.943
	Family Paraprevotellaceae; Genus CF231	0.88	0.88	0.82	0.71	0.58	0.67	0.06	0.181	0.549	0.214	0.266
	Family Paraprevotellaceae; Genus YRC22	0.90	0.50	0.29	0.26	0.29	0.55	0.07	0.170	0.155	0.112	0.184
	Family Bacteroidaceae; Genus 5-7N15	0.30	0.32	0.34	0.31	0.24	0.36	0.01	0.428	0.939	0.132	0.780
	Family Rikenellaceae	0.14	0.16	0.18	0.15	0.11	0.17	0.01	0.527	0.846	0.276	0.834
	Order Bacteroidales; Family BS11	4.06	2.78	4.11	4.20	4.71	3.11	0.39	0.981	0.912	0.124	0.599
	Order Bacteroidales; Family S24-7	2.64	3.51	2.87	1.90	2.02	1.85	0.56	0.451	0.350	0.457	0.550
	Order Bacteroidales; Family RF16	0.90	0.98	0.73	0.69	0.86	1.05	0.11	0.967	0.621	0.805	0.335
	Bacteroidales Unclassified	9.56	13.58	13.76	12.74	13.72	11.75	0.91	0.854	0.211	0.961	0.635
Firmicutes		37.60	36.52	36.79	42.90	38.05	36.36	1.61	0.579	0.526	0.363	0.935
	Ruminococcus	2.53	3.33	3.94	3.32	3.35	2.39	0.31	0.796	0.463	0.987	0.233
	Butyrivibrio	1.74	1.29	1.15	3.31	3.38	2.21	0.67	0.293	0.409	0.179	0.425

	Lactobacillus	1.99	2.01	1.73	2.19	0.97	2.59	0.16	0.984	0.625	0.119	0.441
	Oscillospira	0.64	0.55	0.67	0.68	0.51	0.63	0.09	0.946	0.857	0.838	0.861
	Succiniclasticum	0.95	0.84	0.34	0.23	0.41	0.54	0.11	0.266	0.132	0.514	0.139
	Streptococcus	0.55	0.91	0.38	1.03	0.69	0.71	0.09	0.325	0.229	0.547	0.103
	Leuconostoc	0.38	0.38	0.35	0.40	0.28	0.57	0.02	0.095	0.571	0.328	0.181
	Weissella	0.27	0.28	0.25	0.28	0.19	0.37	0.02	0.353	0.778	0.289	0.259
	Mogibacterium	0.14	0.11	0.16	0.16	0.17	0.14	0.02	0.777	0.870	0.342	0.804
	Clostridium	0.36	0.41	0.45	0.45	0.34	0.32	0.03	0.184	0.186	0.364	0.155
	Anaerostipes	0.34	0.44	0.65	0.42	0.38	0.25	0.08	0.513	0.753	0.760	0.112
	Blautia	0.22	0.14	0.21	0.23	0.17	0.18	0.01	1.000	0.837	0.117	0.345
	Coprococcus	0.28	0.23	0.21	0.20	0.15	0.15	0.02	0.038	0.261	0.039	0.131
	Dorea	0.13	0.13	0.15	0.14	0.10	0.14	0.01	0.391	0.740	0.434	0.932
	Faecalibacterium	0.17	0.16	0.21	0.13	0.10	0.21	0.02	0.269	0.289	0.327	0.940
	Anaerovibrio	0.12	0.09	0.11	0.09	0.09	0.16	0.01	0.866	0.391	0.963	0.071
	Phascolarctobacterium	0.13	0.12	0.15	0.12	0.11	0.14	0.01	0.486	0.839	0.695	0.944
	Family Clostridiaceae; Genus SMB53	0.07	0.18	0.31	0.20	0.20	0.27	0.05	0.755	0.025	0.916	0.794
	Family Erysipelotrichaceae; Genus p-75-a5	0.25	0.24	0.25	0.26	0.28	0.24	0.03	0.831	0.957	0.576	0.957
	Family Erysipelotrichaceae; Genus RFN20	0.18	0.24	0.26	0.34	0.30	0.31	0.03	0.085	0.002	0.129	0.670
	Family Mogibacteriaceae	0.67	0.63	0.67	0.65	0.63	0.48	0.04	0.395	0.925	0.973	0.116

	Family Christensenellaceae	1.27	0.93	0.86	1.63	1.28	0.87	0.17	0.553	0.660	0.145	0.987
	Family Clostridiaceae	0.28	0.33	0.31	0.36	0.27	0.33	0.02	0.809	0.022	0.281	0.837
	Family Lachnospiraceae	3.35	3.38	3.39	4.16	3.17	3.54	0.34	0.710	0.620	0.612	0.764
	Family Peptostreptococaceae	0.10	0.21	0.21	0.25	0.14	0.21	0.03	0.659	0.069	0.192	0.975
	Family Ruminococcaceae	9.03	7.58	8.40	9.38	7.98	7.56	0.36	0.969	0.867	0.592	0.267
	Family Veillonellaceae	0.54	0.65	0.55	0.44	0.43	0.46	0.06	0.316	0.523	0.272	0.600
	Clostridiales unclassified	9.27	9.41	9.07	10.45	10.72	7.95	0.49	0.702	0.548	0.410	0.521
Verrucomicrobia		4.04	6.85	5.83	5.38	5.13	4.23	0.94	0.790	0.660	0.547	0.481
	Akkermansia	0.21	0.12	0.26	0.16	0.10	0.20	0.02	0.441	0.639	0.391	0.640
	Order LD1-PB3	0.03	0.04	0.03	0.24	0.34	0.13	0.10	0.424	0.430	0.431	0.405
	Family RFP12	3.49	6.45	5.31	4.82	4.54	3.75	0.86	0.756	0.640	0.455	0.498
Actinobacteria		3.06	2.29	2.85	1.97	2.21	1.21	0.47	0.330	0.538	0.937	0.061
	Family Bifidobacteriaceae	0.22	0.33	0.37	0.13	0.22	0.40	0.07	0.878	0.725	0.727	0.954
	Family Coriobacteriaceae	2.58	1.80	2.17	1.68	1.80	0.65	0.46	0.455	0.604	0.989	0.204
Tenericutes		2.65	2.03	2.40	2.21	2.02	1.80	0.25	0.562	0.725	0.988	0.296
	Anaeroplasma	0.10	0.12	0.08	0.14	0.09	0.09	0.01	0.789	0.444	0.569	0.793
	Class Mollicutes; Order RF39	2.47	1.80	2.20	1.99	1.81	1.63	0.24	0.560	0.790	0.991	0.302
Proteobacteria		2.45	1.69	1.94	1.89	1.59	1.96	0.20	0.589	0.395	0.767	0.981
	Desulfovibrio	0.75	0.61	0.56	0.54	0.50	0.56	0.10	0.686	0.634	0.576	0.994

	Succinivibrio	0.10	0.06	0.10	0.05	0.04	0.07	0.01	0.108	0.123	0.581	0.590
	Family Enterobacteriaceae	0.48	0.32	0.37	0.48	0.32	0.49	0.07	0.256	0.991	1.000	0.343
Cyanobacteria		1.08	1.72	1.59	1.90	1.76	1.28	0.19	0.497	0.014	0.827	0.525
	Order YS2	0.96	1.62	1.45	1.74	1.61	1.04	0.12	0.640	0.004	0.966	0.490
Lentisphaerae	Victivallaceae family	0.72	1.07	1.14	1.30	1.37	1.09	0.20	0.406	0.078	0.444	0.904
Spirochaetes		1.04	0.95	0.95	0.88	0.85	1.01	0.14	0.800	0.690	0.485	0.894
	Treponema	1.00	0.92	0.92	0.84	0.80	0.96	0.14	0.747	0.670	0.415	0.922
TM7	Family F16	0.39	0.45	0.58	0.49	0.40	0.37	0.07	0.788	0.631	0.823	0.386
Planctomycetes	Family Pirellulaceae	0.35	0.18	0.36	0.20	0.22	0.14	0.03	0.065	0.316	0.429	0.159
Chloroflexi	Family Anaerolinaceae; Genus SHD-231	0.13	0.20	0.21	0.27	0.29	0.23	0.03	0.344	0.119	0.208	0.880
Fibrobacteres	Fibrobacter	0.13	0.11	0.11	0.13	0.09	0.14	0.01	1.000	0.913	0.632	0.194
	Archaea											
Euryarchaeota	Methanobrevibacter	85.77	90.52	89.29	91.02	90.96	89.46	1.30	0.490	0.253	0.916	0.952
	Methanosphaera	10.63	6.66	9.03	5.59	7.19	4.50	1.05	0.163	0.288	0.872	0.218
	VadinCA11	2.91	1.55	0.30	0.98	0.60	5.11	0.64	0.628	0.232	0.514	0.198
	Methanobacteriaceae family	0.48	0.79	1.13	1.18	0.90	0.63	0.18	0.793	0.440	0.887	0.500

[1] Data is shown as LS Means with standard errors
[2] n = 4 among groups.

Table 12. Relative abundance of taxa in the two groups representing > 0.1% of total sequences that differ significantly (P < 0.1)

Phylum	Classification	Percentage of total sequences [1,2]						SEM	P value (Feed)	P value [3] (Time)		
		SF			TMR							
		1.5 h	3 h	4.5 h	1.5 h	3 h	4.5 h			1.5 h	3 h	4.5 h
Bacteroidetes	Parabacteroides	0.22	0.30	0.21	0.17	0.15	0.16	0.02	**0.009**	0.336	0.026	0.084
Firmicutes	Leuconostoc	0.38	0.38	0.35	0.40	0.28	0.57	0.02	**0.095**	0.571	0.328	0.181
	Coprococcus	0.28	0.23	0.21	0.20	0.15	0.15	0.02	**0.038**	0.261	0.039	0.131
	Anaerovibrio	0.12	0.09	0.11	0.09	0.09	0.16	0.01	0.866	0.391	0.963	**0.071**
	RFN20	0.18	0.24	0.26	0.34	0.30	0.31	0.03	0.085	0.002	0.129	0.670
	Family Clostridiaceae	0.28	0.33	0.31	0.36	0.27	0.33	0.02	0.809	**0.022**	0.281	0.837
	Family Peptostreptococcaceae	0.10	0.21	0.21	0.25	0.14	0.21	0.03	0.659	**0.069**	0.192	0.975
Lentisphaerae	Victivallaceae family	0.72	1.07	1.14	1.30	1.37	1.09	0.20	0.406	**0.078**	0.444	0.904
Planctomycetes	Family Pirellulaceae	0.35	0.18	0.36	0.20	0.22	0.14	0.03	0.065	0.316	0.429	0.159
Cyanobacteria	Order YS2	0.96	1.62	1.45	1.74	1.61	1.04	0.12	0.640	**0.004**	0.966	0.490

[1] Data is shown as LS Means with standard errors
[2] n = 4 among groups.
[3] Bold P-values indicate groups that differ (P<0.1).

Conclusion

The two different feeding system of feeding roughage and concentrate separately or as TMR had no effect on the DMI. The total CH_4 emission (L/d) was high at higher feeding level (2.4% BW) and found to decrease at lower feeding level (2% BW). At lower feeding level separate feeding system exhibited low CH_4 production (L/d) and emission rate (% GEI) when compared to TMR. Neither ruminal pH nor the total nutrient digestibility were altered by the feeding system. The total VFA production was increased by SF system and the percentage of acetate and propionate increased and decreased respectively upon time. The percentage of isofatty acids was high in SF system suggesting a possibility of increase in microbial protein synthesis. Rumen NH_3 concentration was high in SF system. Bacterial genera like *Coprococcus*, involved in propionate production and increasing productivity of animals were noted in abundance in SF system. Likewise, *Leuconostoc*, CO_2 and acetate producing bacteria was found to be in abundance in TMR feeding system. Furthermore, the feeding system was found to have no impact on the total methanogen population.

In conclusion, this experiment suggests that the traditional way of feeding the roughages and concentrates separately instead of high capital requiring TMR feeding system would increase VFA production and reduce the CH_4 production without altering the efficiency of nutrient utilization thereby reducing the GEI loss that could be otherwise used for other productive mechanisms by the animals. However, the differences exhibited in microbial diversity between the feeding system should be studied in detail in future to figure out their role in increasing the productivity of animals that could assist in

the further understanding of rumen microbiology and also facilitate further development of animal husbandry.

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요 약

조사료와 농후사료를 각각 급여하는 분리급여(SF; Separate feeding)와 이들 사료를 혼합(TMR; Total mixed ration)하여 급여하는 방법이 반추가축 장내발효 메탄생산에 어떤 영향을 주는지는 거의 연구된 바가 없다. 따라서 본 연구에서는 6두의 홀스타인 육우(체중 230~570kg)를 공시하여 각각 1일 사료급여량을 체중의 2.4(실험 1) 그리고 2.0%(실험 2) 급여하는 Latin square design의 실험을 연속해서 수행하였다. 공시사료의 조사료와 농후사료 비율은 73 : 27이었으며, 모든 사료원료 구성이 동일하였다. 사료를 체중의 2.4%을 섭취한 실험 1에서는 메탄 생산량의 유의적 차이가 없었지만($P > 0.1$), 체중의 2.0%를 섭취한 실험 2에서는 SF 방법이 TMR 방법 보다 1일 메탄 생산량(114.2 vs. 138.5 L/d; $P < 0.005$) 그리고 메탄에너지 손실 비율(3.39 vs. 4.08%; $P < 0.005$)에서 유의성있게 낮은 값을 보였다. 또한 SF 방법은 TMR 방법과 비교하여 낮은 반추위 pH ($P < 0.05$), 높은 ammonia-N과 total VFA 농도($P <0.05$)를 보였다. 특히 SF 방법에서 acetate:propionate 비율 (2.2 vs 2.6; $P < 0.05$)이 유의성있게 낮았던 것은 SF 방법이 메탄생성보다는 propionate 생성을

위해에 더 많은 수소를 기질로 이용하였다는 것을 설명해주고 있다. 반추위 미생물 16SrRNA 분석 결과는 TMR 방법에서 *Leuconostoc* 과 *RFN20* population, SF 방법에서 *Coprococcus 비율이 높아지는 경향을* 보였다. 본 논문은 SF 방법이 TMR 급여 방법보다 메탄생산량이 적다는 첫번째 보고이지만, 보다 정확한 결론을 위해서는 추가 연구가 수행되어야 할 것으로 생각된다.

주요어: 메탄, 반추위, 분리급여, 혼합급여

학 번: 2015-22097