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June, 2020

Effect of the Fermented Concentrate Feed on *In vitro* Gas Production and Digestibility in Cattle

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List of Acronyms

CF	-	Crude Fibre
CH_4	-	Methane
CO_2	-	Carbon-di-oxide
CP	-	Crude Protein
DM	-	Dry Matter
DMD	-	Dry matter Digestibility
e.g.	-	Example
et al	-	and his associates
FAO	-	Food and Agriculture Organization
GE	-	Gross Energy
GHG	-	Green House Gas
GIT	-	Gastro Intestinal Tract
H_2	-	Hydrogen
IVOMD	-	In Vitro Organic Matter Digestibility
Kcal/kg	-	Kilocalorie per kilogram
LY	-	Live Yeast
mmol/l	-	Millimole per liter
NFE	-	Nitrogen-free Extracts
NH_3	-	Ammonia
NH_3-N	-	Ammonia Nitrogen
OM	-	Organic Matter
Sig.	-	Significance
TDN	-	Total Digestible Nutrients
TG	-	Total Gas
VFA	-	Volatile fatty Acid
Wt	-	Weight

Abstract

The study was conducted to evaluate the effect of the fermented concentrate feeds on gas production, digestibility and fermentation kinetics using *in vitro* fermentation technique in cattle. Concentrate feeds at 1% of total dry matter (DM) was used as substrates for *in vitro* rumen fermentation using molasses-yeast mixture. The study included four experimental diets designated as, C= Diet without molasses-yeast mixture, T_1= Diet containing molasses at 0.1% of concentrate DM, T_2= Diet containing molasses-yeast mixture at 0.2% of concentrate DM and T_3= Diet containing molasses-yeast mixture at 0.4% of concentrate DM. *In vitro* results showed that there were no significant differences ($p>0.05$) in the chemical composition and pH of the fermented and non-fermented concentrate feeds. In case of total gas production, significant differences ($p<0.05$) were observed after 12 h ($p<0.05$), 24 h ($p<0.01$) and 48 h ($p<0.01$) of incubation period. The lowest total gas production was noticed in T_2 (61.8 ml) and the highest in the control (76.8 ml) group after 48 h of incubation. Significant ($p<0.05$) differences were observed in *in vitro* CH_4 production after 12 h of incubation where lowest CH_4 was measured in T_2 (32.8 ml) group. The CO_2 production consistently decreased in fermented groups than the control. The highest CO_2 production was observed in control (22.2 ml) and lowest in T_3 (17.4 ml) group after 48 h of incubation. There were significant ($p<0.05$) differences in the OM digestibility after 6, 12 and 24 h of incubation period. The highest OM digestibility was observed in T_2 (96.4%) than the control (94.5%) group after 24 h of incubation and after 12 h the highest OM digestibility was recorded in T_2 (97.2%) and lowest in T_1 (95.3%) group. Therefore, it is presumed that concentrate feeds fermented at 0.2% of molasses-yeast mixture has the higher digestibility and methane reducing potentiality.

Keywords: Fermented concentrate, *in vitro* fermentation, organic matter digestibility, molasses-yeast mixture.

CHAPTER-I: INTRODUCTION

Feed resources are very important factor for livestock production both in tropical and temperate regions. The security of feed is becoming critical in terms of quantity and quality, particularly protein sources which affect productive performance. Concentrate feed provide nutrients that forage alone cannot provide. This is particularly true in the terms of high-producing animals. Livestock are able to acquire nutrient from plant based feed, cereal, and cereal by products. The increasing demand for meat and milk and their products are driven by continuous growing population led to intensive livestock production. In developing countries like Bangladesh, the reduction of growth in cattle production may be caused by low and inadequate nutrient supply in a high-forage based ration (Suharti et al., 2011). Therefore, it is necessary for cattle production systems to introduce new sources and technologies of feedstuffs. Recently, small stakeholder farmers have been using a variety of concentrate feed ingredients such as rice bran, maize meal as well as concentrate mixture from these concentrate feedstuffs in cattle production (Suharti et al., 2011). Researchers are trying to figure out the alternative protein sources which may help to increase livestock productivity (Wanapat et al., 2007). Many scientists are seeking different strategies to improve and enhance nutritive value of by-products and local feeds such as cassava chip, rice straw, rice bran, soybean meal, etc. Incorporation of microbial additives such as culture of *Saccharomyces cerevisiae* to the diet has become a common practice in ruminant nutrition (Polyorach et al., 2012). The use of fermented concentrate feed in ruminant feeding is seen to be an approach in improving animal performance. Manipulation of rumen eco-system to improve animal performance is the most important goals of animal nutritionist (Patra et al., 2010). These compounds are capable to change *in vitro* rumen fermentation parameter such as decrease the acetate concentration and increase propionate and butyrate concentration, decreasing methane production as well as CH_4: VFA ratio (Busquet et al., 2005).

However, the better outcome of using these available feeds is limited because of insufficient information about nutritive value, digestibility as well as rumen fermentation characteristics and previous studies reported that concentrate feeds differ substantially in their rumen fermentation characteristics (Mills et al., 1999). These

variations may be due to the differences in the physical and chemical characteristics of each concentrate feed ingredients (Orskov, 1986). Concentrate containing high starch is crucial in ruminant nutrition because it is a pragmatic and cost-effective source of energy and driven to influence the functioning of the rumen and nutrient digestion (Krause and Oetzel, 2006; Tahir et al., 2013). The stomach of ruminant animal is a habitat of the essential beneficiary microorganisms. Those microorganisms produce necessary nutrients for the host such as fermentation acids, microbial protein and vitamins. Simultaneously, they produce fermentation by products such as CO_2 and H_2 which ultimately produce CH_4. Among the widely used substrates for yeast production are the molasses, the wastes byproduct of sugar industries from sugarcane and sugar beet. This is because they are cheap raw materials, readily available, and ready for conversion with limited pretreatments as compared with starchy or cellulosic materials, as all sugars are present in a readily fermentable form. The singled cells proteinaceous source of yeasts are the most commonly used microorganisms for concentrate fermentation that decrease cell wall of concentrate feed and increase cellulytic activity in the gut. *Saccharomyces cerevisiae* is one of the well-known fermented product producers.

Rising environmental concentration of CH_4 inspired scientists to explore its sources. It is a very important greenhouse gas that creates considerable environmental problems associated with global warming. Additionally, CH_4 has a global warming potential that is 23-25 times higher than CO_2 (Forster and Artaxo, 2005). The CH_4 emitted from ruminant animal constitutes up to 15% of global CH_4 emissions, as well as 2 to 12% loss of dietary energy by ruminants. There are varieties of factors that influence CH_4 production in ruminants, such as: level of feed intake, feed ingredients, feed processing, feed additives, lipids or ionophores addition, and alterations in the ruminal micro flora. Accordingly, alteration of these factors can reduce CH_4 emissions from cattle. The use of feed variation in ruminant feeding is seen to be an approach in improving animal performance and methane mitigation. The *in vitro* methods provide less expensive and more rapid alternatives (Getachew et al., 2004) and these techniques have been widely used to assess the nutritive value of different feeds. It is not only because these methods are capable of measuring rate and extent of nutrients degradation with less expenditure, but also measure dry matter (DM) and organic

matter (OM) digestibility effectively (Menke et al., 1979; Getachew et al., 2004; Tahir et al., 2013).

1.1 Objectives

The present study is designed to investigate the following objectives:

1.1.1 To evaluate the chemical composition of fermented and non-fermented concentrate feed.

1.1.2 To compare the *in-vitro* parameter of fermented and non-fermented concentrate feed.

1.1.3 To measure the DM and OM digestibility of fermented and non-fermented concentrate feed.

1.2 Research hypothesis

Provision of fermented concentrate in ruminant diet may improve ruminal gastro-intestinal function, reduce gas production and increase digestibility in cattle.

CHAPTER-II: REVIEW OF LITERATURE

Experiment was accompanied to assess the effects of Fermented Concentrate Feed through *in vitro* on gas production and performance parameter. In order to conduct the experiment and converse the result successfully, a comprehensive study on different reviews and literature within the area was done. The aim of this chapter is to represent the previous study, methods and interpretation regarding this study.

2.1 Concentrate feed

Concentrate feed include granular feeds like cereal and legumes, some by-products of technical production, mixed feed-concentrates and animal feeds. According to the composition of their nutritional substances, there are two groups of concentrated feeds distinguished: carbohydrate and protein. Carbohydrate feeds include cereals (such as, corn, barley and oats), which are rich in starch and sugar; mill by-products (like bran, grain cuttings, flour dust); and dried by-products of sugar-beet and starch production. One kg of such feeds contains 0.7–1.3 fodder units and 70–80 g of digestible protein. Moreover, Protein concentrated feeds include leguminous crops (such as peas, beans, soya, and lentils), by-products of macro extraction production (like oilcake and oilseed meal) and by-products of meat-packing combines which includes meat, meat-and-bone and blood meal and of fish-processing products like fish meal. One kg of such feeds contains 0.7–1.2 unit fodders and 180–350 g of digestible protein (encyclopedia).The purpose of concentrated feeds in feeding depends on the species, age, sex and productivity of the animals. For ruminants, whose rations consist basically of coarse, nourished and succulent fodders, concentrates are supplementary and are introduced to increase the level of the total and protein nutrition of the rations.

Concentrate feeds and starch generally provide more digestible nutrients than roughages, which increase the digestibility of feed and generally lift animal productivity. Jiao et al., (2014) conducted a study to determine the effect of concentrate feed level on methane emissions from grazing dairy cows and stated that offering concentrates to grazing dairy cows increased milk production per cow and decreased CH_4 emissions per unit of milk produced. In ruminants, the amount of CH_4 emissions released is determined by the amount and composition of feedstuffs

ingested. Supplementation of diets with concentrates are widely used to increase the production of ruminants (Purwin et al., 2016; Ruiz-Albarran et al., 2016), and is regarded as an effective methane mitigation strategy (Martin and Nisbet, 1992), in particular, in intensive production systems with over 35% grain inclusion in diets (Sauvant and Giger-Reverdin, 2009). Concentrates favor propionate production in the rumen offering an alternative hydrogen sink to methanogenesis and lower ruminal pH, which in turn inhibits methanogens directly and indirectly, as protozoal inhibition also decreases protozoal-associated methanogenesis (Grainger and Beauchemin, 2011). In addition, concentrates supply greater amounts of digestible nutrients than roughages, increasing animal productivity, and consequently, decreasing CH_4 emission intensity (emissions generated for each kilogram of product), a phenomenon called the dilution of maintenance effect (Capper et al., 2009).

Previous research assessing the effects of moderate levels of dietary supplementation with concentrates (1 *vs*. 5 kg as-fed, corresponding to 5% and 23% of concentrate in the diet DM, respectively) found no effects on methane yield (g kg-1 DM intake) or intensity (g kg-1 milk yield;) of grazing dairy cows. However, increasing levels of concentrate supplementation (2, 4, 6, and 8 kg d-1 animal-1 as-fed) resulted in decreased methane yield (Jiao et al., 2014). Diet composition and intake are main factors affecting CH_4 production by ruminants. Ruminant fed forages rich in structural carbohydrates produce more CH_4 than those fed mixed diets containing higher levels of non-structural carbohydrates per unit of fermented material in the rumen (Sauvant and Giger-Reverdin, 2009). This is explained by the different metabolic routes used to ferment the different carbohydrates which result in different VFA profiles that yield more or less metabolic H_2 as the main substrate to produce CH_4. There is a clear relationship between feed organic matter digestibility, concentrate feed or starch intake, and the pattern of ruminal fermentation and CH_4 production.

2.2 The yeast (Saccharomyces cerevisiae)

The yeast is a living organisms- invisibly small ones, microorganisms. As long as they are kept cool and dry, they are not active. But when they are given food, moisture, and warmth, they become active and do many of the things larger organisms do**.** Yeast supplementation of horse diets can influence nutrient digestibility and

microbiota dynamics in the horse hindgut. In some *in vitro* (Elghandour et al., 2016) studies, yeast addition to the diets improved digestion of low-quality forages. It has been shown that yeast supplementation can alter the microbial environment by increasing the total number of hindgut microorganisms (Lattimer et al., 2005). As a result, feed digestion in the hindgut can be enhanced, especially that of the fiber fraction, most likely due to increased numbers of cellulolytic bacteria in the hindgut (Warren and Hale, 2012). In contrast, other studies have reported no effect of yeast addition to equine diets on nutrient digestibility *in vitro* (Lattimer et al., 2005) or in vivo (Glade and Biesik, 1986). It is hypothesized that yeasts can enhance the digestion of poor-quality high-fiber feeds (such as oat straw) in the hindgut of horses. The aim of the present study was to assess how the supplementation of high-fiber diets with yeast could modify the microbial fermentation activity in the hindgut of horses and affect the digestion of a high fiber substrate (oat straw). Feces from horses fed oat straw diets and supplemented with live yeast (*Saccharomyces cerevisiae*) were used to inoculate batch cultures, and total fermentation gas, methane (CH_4), and carbon dioxide (CO_2) produced after incubation *in vitro* were used as indicators of the fermentative activity in the hindgut.

Yeast supplementation significantly ($P<0.05$) increased digestibility of dry matter (DM), organic matter (OM), crude protein (CP), NDF and ADF of tomato pomace where the gross digestibility derived from the supplementation was superior for 4g yeast compared to the control group. In addition, sheep fed yeast had a marked increase in energy digestibility of tomato pomace at 4g level (Newbold and Rode, 2006). Yeast supplementation significantly accelerated the increase in milk yield during early lactation and compared to the pre-experimental period, the cows of the live yeast (LY) group achieved significantly higher milk yield than those of the control group (Rihma et al., 2003). Yeast culture can improve feed efficiency of heat stressed dairy cows in mid lactation. Supplementation of yeast culture (YS) increased dry matter intake (DMI) during the transition period and increased DMI postpartum (Dann et al., 2000). The ruminal digestion would be more easily affected by dietary YS addition when rams consumed a diet rich in forages (Galip, 2006).

The addition of this probiotic increases the number of cellulolytic bacteria in the rumen; the ingestion of dry matter, the ammonium production is stimulated, the

proteolysis is reinforced, the digestion and absorption of nutrients improves and the digestion of the fiber contained in the food increases, which results in an increase of the average milk production of 1.13 kilograms per cow per day (Kg/ cow/d).Higher digestibility values could be explained by a higher population of cellulolytic bacteria, which is one of the most consistent effects of yeast (Wallace and Newbold, 1993). The aim of the current work was to investigate the effect of concentrate to roughage ratio and baker‘s yeast supplementation during summer season on digestibility, rumen fermentation activity, feed intake, feed conversion and economic efficiency of Cows.

2.3 Molasses

Molasses is a dark brown color, viscous liquid produced as a co-product of the production of sugar. At high temperature, after dissolving sugars out and the crystals of sugar settle out as the liquid cools leaving the molasses, much of which was traditionally mixed back with the pulped fibers to produce molasses sugar beet feed (Senthilkumar et al., 2016). It is a sticky dark by-product of processing sugar cane or sugar beets into sugar. Senthilkumar et al., (2016) discovered that molasses can be a source of quick energy and an excellent source of minerals for farm animals. It can also be a key ingredient for cost effective management of feeds and pastures. The calcium content of sugar cane molasses is relatively high (up to one percent), whereas the phosphorus content is low. Cane molasses is also high in other minerals like sodium, potassium, magnesium and sulphur but in beet molasses is higher in potassium and sodium but lower in calcium. Molasses also contains significant quantities of trace minerals such as copper, zinc, iron and manganese. Adding molasses with poor quality hay will increase feed intake and improve palatability. Ruminal microbes break down the sugars in molasses rapidly, which extensively causes a rapid release of energy that makes molasses very useful for balancing other feeds in the dairy diet all year round. Feeding molasses to farm animals will improve digestion of pastures/hay; increase milk production, help maintain body condition and appetite and result in less feed waste (Senthilkumar et al., 2016).

2.3.1 Feeding rates of molasses

Molasses is suitable for addition in the diets of all ruminant livestock that can offer a very cost effective way to increase the palatability of feeds while contributing

sufficient levels of energy and protein. In ruminants specifically for dairy cows, ideal for complete diets added up to 3kg of molasses per head per day. Whereas, in beef cattle up to 10 per cent of molasses can be included in beef diets depending on the nature of other feeds in the mix and subsequent storage facilities for the finished ration (Senthilkumar et al., 2016).

Table 2.1 Nutrient composition of molasses (dry matter basis)

Nutrients	**Amount**
Dry Matter	74%
Crude Protein	6.5%
Oil	Trace
NDF	Nil
Starch	Trace
Sugar	65%
ME (MJ/KG DM)	12.5

2.3.2 Molasses as stock feed

The molasses used with feed ingredients is based mostly on its sugar content (around 50%). In comparison with the carbohydrates in concentrated form, molasses contains a small amount of protein, but it provides also a certain amount of non-protein, non-sugars which have some nutrient value especially for ruminants. In general, molasses should be added to feed when it is essential to compensate for an excess of protein. Molasses has a high mineral content, but it usually lacks adequate calcium and phosphorus. These must be taken into account when preparing mixed feeds and they should be supplied by suitable supplements (e.g. lime) or by a proper combination of feeding materials (Senthilkumar et al., 2016).

2.4 Rumen liquor

Rumen liquor is the liquid phase found in the rumen of ruminant animals where microbial fermentation takes place.

2.4.1 Constituents of rumen liquor

Each millilitre of rumen liquor contain around 10^9 to 10^{11} bacterial population, 10^5 to 10^6 protozoa population and variable numbers of yeast and fungi (Paul et al., 2004). Bacteria are rarely classified by their substrate preference or the end products they produce. Many of them utilize multiple types substrate, although there are some of the major groups which utilize specific type substrate. Each group of bacteria contain multiple genera and species. The groups include cellulolytic bacteria that digest cellulose, hemicellulolytic bacteria that digest hemicellulose, amylolytic bacteria that digest starch and proteolytic bacteria that digest proteins. Rest groups are sugar utilizing bacteria (utilizing monosaccharides and disaccharides), acid utilizing bacteria (utilize lactic, succinic and malic acids), ammonia producers, vitamin synthesizers and methane producers (Odenyo et al., 1999).

The protozoal population is far less than bacterial population, but they are so much larger than bacteria that they may occupy a volume nearly equal to that occupied by bacteria. In general protozoa utilize the same set of bacterial substrate in which different populations of protozoa show distinctive substrate preference as bacteria. Many utilize sugars and some store ingested carbohydrates. Many species of protozoa have been found to consume bacteria, which are thought to play a role in limiting bacteria overgrowth. The fungi are considered significant in the rumen as they have unique ability to break and penetrate the fibrous feed particles and provide more surface area for the action of other microbes. Those fungi produce highly active enzymes for lignocellulose degradation. Thus rumen fungi play an important catalytic role in the digestion of poor quality fibrous feeds in rumen (Paul et al., 2004). Almost all rumen microbes are anaerobes although a few facultative microbes exist, performing a key role in removing oxygen quickly from the rumen. These kinds of microorganisms interact and support one another in a complex ecosystem with products of some species serving as nutrients for other species. Through fermentation they can convert plant materials that could not otherwise be digested to volatile fatty acids (VFAs), methane, carbon dioxide, ammonia and microbial cells. NH3 is used as a nitrogen source for microbial growth and VFAs are absorbed from the rumen and used as a key energy source for the ruminants.

2.4.2 Quality of rumen liquor

The quality of the rumen liquor is assessed by measuring the by-products of the rumen fermentation, such as pH, NH_3-N and total VFAs. The fermentation characteristics of the rumen liquor give information about the microbial population and presence of rumen microbes (Mekasha et al., 2003). The amount of NH_3-N and total VFAs present in the rumen liquor is a reflection of microbial activity and their absorption or passage out of the rumen (Habib and Akbar, 2005). There are minimum concentrations of these fermentation characteristics in which rumen microbes function well. The optimal environmental conditions of the rumen have been noticed to be at the pH of around 6. The pH values for normal microbial activities in the rumen have been proposed by different researchers. McDonald et al., (2010) proposed the optimum pH range of 5.5 to 6.5 for microbial fermentation. Fibre digesting bacteria perform best at pH 6.0 – 6.8 and starch digesting bacteria at pH 5.5 – 6.0 (Russell and Wilson, 1996). The change in the ruminal pH is caused by the type of feed consumed by the animal (Mekasha et al., 2003). If large amounts of soluble carbohydrates are consumed, then the pH may fall. If pH drops to about 5.5, protozoal populations become markedly depressed because of acid intolerance. In the study by Vargas *et al.* (2009) it was found that the type of diet fed to donor animals had a marked effect on the inoculum pH, which was noticeably lower with diet having high concentrate to forages ratio. Ruminal pH variations have direct effects on rumen microbial composition, population and their fermentation activity. The pH ranges below or above the optimal range recommended may directly affect the microbial growth and activity.

Greater pH decline means decreased population and activity of fibrolytic bacteria and protozoa population. Continuous lowering of rumen pH, as can occur with higher feeding of concentrate can destroy many species and have serious consequences to the animal. If ration of the ruminants contain 50 to 60% of concentrate then there is a rist of ruminal protozoa to drop from 10^6 to10^3 and hence reduction in digestibility of the feeds (Calsamiglia et al., 2008). The increase in the amount of total VFA indicates the increased rumen microbial activity (Oosting, 1993). The total VFA concentration in the rumen liquor of the rumen in cattle should be in the range of 70 – 150 mmol/l of rumen liquor for normal function. The NH_3-N concentration in rumen is a limiting

factor for rumen microorganisms that affect the digestion of fibrous feeds. The increase in the concentration of NH_3-N in rumen may show the reduced utilization of ammonia by rumen microbes, which indicates the decrease in intensity of fermentation due to decrease in microbial growth and activity (Mekasha et al., 2003). Given that the constituents of rumen liquor are affected by the diet of animal, then in utilizing rumen liquor from slaughtered cattle for estimating *in vitro* digestibility there is a need to understand if the concentration of the constituents in the rumen liquor to be utilized is in the required level. This will be important since the cattle brought for slaughter in the abattoir are coming from different areas and are feeding on different types and quantities of feeds, which may affect the quality of rumen liquor and hence the results.

2.5 Rumen fermentation

In an experiment done by de Visser et al., (1992) it was revealed that after feeding, rumen fluid contents increased sharply, resulting in dilution of the VFA and buffering the pH to decline. In addition, rate of absorption from the rumen increases with higher VFA concentrations. The ruminant animal has a number of mechanisms which will prevent the average VFA concentration from rising above the maximum of 150 mmol/1, thereby restraining a drop in pH values that inhibit the rate of degradation. It is also been shown that the production of total VFA decreases linearly as intake declined. Rumen fermentation is a result of the activity of microbes namely bacteria, protozoa and fungi. End products of anaerobic microbial fermentation include volatile fatty acids (VFA's) in the rumen and serve as a major energy source for the host animal. The three main VFA's are acetate which is produced in the greatest amount in most diets, propionate which also serves as a hydrogen sink that reduces methane and butyrate. The production of these individual VFA's depend on the substrate consumed by the cow. For example, forage based diets favor acetate and butyrate production however starch based diets favor greater propionate production (Knapp et al., 2014).

2.6 Rumen digestion of fiber

Fiber is defined as the carbohydrate fraction resistant to digestion by enzymes produced by cattle and is the predominant carbohydrate of the plant cell wall. Fiber is

mostly comprised of cellulose, hemicellulose and lignin. Cattle do not produce the enzymes required to break down fiber therefore they rely on microbes to break down the fiber. Cattle contribute to microbial digestion by chewing and ruminating feed particles and this physically breaks the fiber particles and increases surface area available for microbial digestion. Fiber digestion can occur in the rumen and large intestine; however, only a small amount of fiber will be digested in the large intestine. Fibrolytic bacteria ferment the fiber and from this fermentation acetic acid (VFA) is produced and is absorbed through the rumen wall. Acetate or acetic acid is used by the cow for energy and for the synthesis of milk fat. Some of the fiber fermented by the microbes is utilized as energy for the microbial cell (Zicarelli et al., 2011).

The pH in the rumen may also affect fiber digestion. Inadequate fiber concentrations of fiber or fiber that is too fine may result in reduced chewing time therefore reducing salvia production and reduced ruminal pH. Fibrolytic bacteria grow best when the pH of the rumen is 6.2 to 6.8. When rumen pH drops below 6.0 – 6.2, fiber digestion begins to decline because fibrolytic bacteria activity is reduced. If the pH drops below 5.8 – 5.9 fiber digestion may be severely impaired (Lattimer et al., 2007).Meenongyai et al., (2017) reported that ruminal pH was not affected by feeding fermented ration. Compared to non-fiber carbohydrates such as starch, fiber is generally less energy dense and less digestible (Knapp et al., 2014). Furthermore, there are animal factors that affect rumen fiber digestion. For example, at high levels of intake, ruminal fiber digestion may be suppressed because passage rate increases and rumen microbes have less time to digest fiber. There are also plant factors that affect fiber digestion. One limitation is the physical and chemical nature of plants which may serve as a barrier to complete digestion, especially lignin. Lignin is part of the cell wall in forages and it is largely indigestible by rumen microbes and thus cannot be used as an energy source for the animal. As plants mature the concentration of lignin increases and will reduce fiber digestibility. Another limitations cellulose crystallinity. This high order of structure may impair digestibility. Maturity of plants will affect fiber digestion. Immature plants are more digestible than mature plants. Finally, location will influence fiber digestibility. Forages grown in warmer places have more lignin and therefore are less digestible than forages grown in temperate places.

2.7 Methane gas

Methane is one of the major greenhouse gases. Dairy cows contribute CH_4 to the atmosphere due to microbial fermentation of feed in the rumen and hindgut. The production of CH_4 by ruminants also causes energy losses for the animal, corresponding to 2 to 12% of gross energy (GE) intake. The total amount of CH_4 released is dependent on several factors, such as DMI, type of feed, feed quality, and OM digestibility.

2.7.1 Methane production in the rumen

Methane along with carbon dioxide and nitrous oxide are considered greenhouse gases (GHG) that contribute to global warming. There has been a rising concern and emphasis put on ways to mitigate these GHG, especially methane in the livestock industry since ruminants produce more methane than any other livestock. The Innovation Center for US Dairy is striving to reduce GHG from fluid milk by 25% by 2020. Greenhouse gases are either directly (e.g. enteric fermentation and manure management) or indirectly (e.g. feed production activities) produced from livestock (Hopkins and Del Prado, 2007). In 1995, it was estimated that over the subsequent 50 years methane would be responsible for 15 – 17% of global warming while 2% was expected to be from cattle. More recently Knapp et al., (2014) suggested that methane causes 3.3% of the total GHG emissions and cattle contributed 6.3 % of these GHG emissions. Overall agriculture is responsible for 29% of global methane sources, with 17% of methane coming from enteric fermentation, 2% of methane coming from manure production and 7% of global GHG sources (Knapp et al., 2014).

Importantly the world population is growing and because of that livestock numbers are projected to increase also. According to Grainger & Beauchemin, (2011) if methane emissions increase parallel to the projected increase in livestock numbers, then global methane emissions from livestock are expected to increase 60% by 2030. It is clear that ruminants contribute to increasing methane emissions and in turn global GHG emissions. Consequently, there is a need to discover methods to mitigate methane emissions without impacting animal and whole-farm productivity (Grainger and Beauchemin, 2011). Methane production is a natural component of the digestive

processes in ruminants. Microbes occupy the animal‘s digestive system that ferments feed consumed by the animal. This digestive microbial fermentation process is often referred to as enteric fermentation and produces methane as a byproduct. This methane is then ultimately exhaled which can be called eructation or loss via flatulence by animals. The volume of methane an animal will emit and is dependent on characteristics of the individual animal such as size of animal‘s digestive system and the amount or type of feed they consume.

Ruminant animals emit large volumes of methane and this is because of the extent of rumen fermentation. The rumen which is an anaerobic environment allows microbial fermentation to break down the feed ruminants have consumed into specific products that can be absorbed and metabolized. This microbial fermentation in the rumen allows ruminants to digest plant material that non-ruminants cannot and consequently ruminant animals have the highest methane emitted per unit of body mass among all animal types.

Cattle begin to eructate methane at about 4 weeks of age and this coincides with the consumption of solids, a developing reticulorumen and establishment of rumen microbes. Fermentation and methane production rates are rapidly increasing during reticulorumen development. Cattle produce 60 to 160 L of methane, per year, though size of animal and DMI will have an effect. Lactating dairy cattle specifically will produce 109 to 126 L of methane per year (Hattori and Matsui, 2008).

Beauchemin et al., (2008) reported that dairy cattle consuming grain and forage diets produce approximately 500 to 600 L/d of methane. Though methane and carbon dioxide are natural by-products of ruminants, they do require a fair amount of energy from cattle. Generally, 6 to 8%, but up to 12% of the gross energy in feed is converted to methane in the rumen (Beauchemin et al., 2008).Therefore, reducing methane production in the rumen is generally also believed to improve energetic and production efficiency of the cattle.

Microbes have a large effect on daily function of cattle and methane production in the rumen is no different. Methane and carbon dioxide are natural by-products of microbial fermentation of carbohydrates and to a smaller degree amino acids in the

rumen plus the hindgut of farm animals. Enteric methane is produced by ruminants during the process of microbial digestion of feed. Ruminant animals and microbes have a unique relationship that allows for conversion of complex plant carbohydrates to energy that is beneficial to both ruminant and microbes. In the reticulo-rumen, carbohydrates are converted to 5- and 6- carbon sugars by microbial enzymes. Some fermentation occurs in the hind gut but the extent of this activity is much lower than the rumen (Beauchemin et al., 2008).

Carbohydrates in the rumen are then fermented to volatile fatty acids (VFA‘s) (primarily acetate, propionate and butyrate) by microbes including bacteria, protozoa and fungi that obtain energy and produce reducing equivalents (e.g., metabolic hydrogen, NADH or $FADH_2$) in the process. A small amount of these reducing equivalents will be used in lipid synthesis and fatty acid bio-hydrogenation Synthesis of amino acids can use or produce reducing equivalents also (Knapp et al., 2014) Or the reducing equivalents can go to methane production, often referred to as methanogenesis:

$CO_2 + H_2 \rightarrow CH_4$

2.7.2 Enteric methane production and its function in rumen ecosystem

Fermentation of diet components by rumen microbiota results in the production of short chain fatty acids (SCFAs)—an energy source for ruminants—and gases (CO_2 and CH_4) excreted via eructation (Martin et al., 2010). Rumen fermentation involves an oxidation process, generating reduced co-factors (NADH, NADPH, and FADH), which are then re-oxidized (NAD+, NADP and FAD+) by dehydrogenation reactions, releasing hydrogenin the rumen. As an electron acceptor process, methanogenesis removes hydrogen gas (H_2)from the rumen. Methane production is therefore essential for obtaining a high-performing rumen ecosystem because H_2 accumulation, which could inhibit dehydrogenase activity in re-oxidation co-factors, is avoided. An efficient H_2 capture in the rumen contributes to increase the rate of fermentation bythe lack of its inhibitory effect on the microbial degradation of vegetative material (Wolin, 1979; McAllister and Newbold, 2008).

Enteric methane is derived from the activity of the methanogen *Archaea*, a microbial group distinct from eukaryotes (protozoa and fungi), bacteria with its own co-factors (coenzymes M, F420, and F430), and fat (isoprene-glycerol esters). Despite the central function of H_2 in the metabolism, methanogenesis is important to rumen function and animal nutrition although methanogens comprise only a small part of the rumen‘s microbial biomass (Janssen and Kirs, 2008). *Archaea* methanogens are responsible for methane production in ruminants. Therefore, considerable research efforts have been made to gather more information about them (Attwood et al., 2008).

Identification of their metabolic activities and diversity is required for developing strategies to mitigate enteric methane emissions. Sequencing of their genomes will provide important information to develop such strategies (Buddle et al., 2011). Other microorganisms provide an appropriate environment to facilitate methanogen survival or produce substrates that would be available for methanogens.

Metabolic pathway for H_2 production and interspecies relationships between methanogens and other microorganisms of the ruminal ecosystem should be considered in the strategies to control methane emission by ruminants. The H_2 produced bymicrobial fermentation is an energy source to *Archaea* methanogens for methane production. Formate can be used to produce methane by methanogens; however,it is a less important methane precursor than H_2 and is responsible for approximately 18% of the methane produced. Ruminal fermentation products are not equivalent in terms of H_2 production; their amount depends on short chain fatty acid (SCFA) concentration and the relative ratio between acetate, propionate and butyrate. Quantitative mathematic models consider fermentation stoichiometric calculations to balance formation of H_2, SCFAs and other products for predicting methane production (Bannink et al., 2006).

2.8 Strategies to reduce enteric methane emissions from ruminants

Different strategies available to reduce CH_4 emission from enteric fermentation were reviewed by Hopkins & Del Prado, (2007). They categorize them as: dietary changes, direct rumen manipulation and systematic changes. The latter include considerations of breed, livestock numbers and intensiveness of production. More intensive

production may result in lower CH_4 emission, but may be less desirable in terms of other environmental impacts. An overall reduction in CH_4 production (liters/day) per individual animal is the ideal goal. However, given the nature of livestock production systems, the immediate goal should be to reduce CH_4 per unit of product (milk or beef). Decreasing livestock numbers as an approach to reducing CH_4 implies reducing numbers, but holding productivity per animal constant so that CH_4 emissions fall. This strategy has economic consequences as the profit from livestock farms will decline in direct proportion to the reduction in numbers of animals. As milk or beef production per animal increases, CH_4 output per animal also increases, but both the proportion of gross energy used in the production of CH_4 and the amount of CH_4 emitted to produce a given quantity of milk or beef falls (Blaxter and Clapperton, 1965).

2.8.1 Forage quality

An important feed characteristic that can impact enteric CH_4 production is forage quality, specifically its digestibility. As noted by Blaxter & Clapperton, (1965), increased intake of poor-quality, less digestible feeds has little effect on CH_4 production when expressed on a dry matter intake basis. For feeds with higher digestibility, however, increased intake results in a depression in the amount of CH_4 produced per unit of feed consumed. Moreover, it decreases CH_4 produced per unit of product (emission intensity) by diluting maintenance energy. Forages are the feed ingredients with the largest variability in composition and have the largest impact on diet digestibility. Factors, such as plant species, variety, maturity at harvest and preservation can all affect forage quality and digestibility. In general terms, as the plant matures, the content in structural carbohydrates increases and that of more fermentable carbohydrates declines. Harvesting forages at the right time, depending on the type of forage, is important to maximize the amount and digestibility of nutrients supply by forages. Also, the different processes used to conserve forages (hay, silage) may negatively influence the nutritional value if not done properly. In the last decade a strong effort is now in place to develop forage varieties rich in desirable nutrients (lipids, water soluble carbohydrates) that have shown promising mitigation effects.

In general, CH_4 reductions are correlated with greater nutrient quality and digestibility, which are 2 attributes for which forage type and maturity might be indicators. Increasing quality or digestibility of forages will increase production efficiency and this will likely result in decreased CH_4. Keady et al., (2012) provided a comprehensive review of the effects of silage quality on animal performance in various production systems in Ireland. These authors concluded that a 10 g/kg increase in digestible organic matter concentration of grass silage DM could increase 1) daily milk yield of lactating dairy cows by 0.37 kg, 2) daily carcass gain of beef cattle by 28 g/head, 3) daily carcass gain of finishing lambs by 10 g/head, 4) lamb birth weight by 0.06 kg, and 5) ewe BW post-lambing by 1.45 kg. They also pointed to the critical effect of maturity on grass silage digestibility; each 1week delay in grass harvest reduced digestibility by 3 to 3.5 percentage points.

2.8.2 Dietary ingredients

Concentrate feeds and starch generally provide more digestible nutrients than roughages, which increase the digestibility of feed and generally lift animal productivity. Starch is a possibility in some situations but cannot be generalized (i.e. low input systems with slight supplementation with starch). The suitability of this approach for GHG mitigation depends on the access to and availability of feed and potential competition with direct human consumption. By-product feeds with high oil contents, such as distiller grains and meals from the biodiesel industry, can be cost-effective lipid sources. There is a large body of evidence that lipids suppress CH_4 production. The effects of lipids on rumen archaea are not isolated from their overall suppressive effect on bacteria and protozoa. Meta-analyses by Moate et al., (2011) documented a consistent decrease in CH_4 production with fat supplementation. Moate *et al.* (2011) reported the following relationship between dietary fat and CH_4 production per unit of DMI: CH_4 (g/kg DM) = 24.51 (±1.48) – 0.0788 (±0.0157) × fat (g/kg DM). Grainger & Beauchemin, (2011) analyzed 27 studies and concluded that, within a practical feeding rate of less than 8% fat in the diet, a 10 g/kg increase in dietary fat would decrease CH_4 yield by 1 g/kg DMI in cattle. Although supplementing animal diets with edible lipids for the sole purpose of reducing CH_4 emissions is debatable, high-oil by-products from the biofuel industries [dry or wet distillers grains alone or with soluble and mechanically extracted oilseed meals can

naturally serve as a CH_4 mitigating feed, if included in the diet to decrease feed cost (Hales et al., 2012).

2.8.3 Precision feeding

Two main aspects of ruminant nutrition can be related directly to NH_3 emissions from cattle manure: (1) inefficient utilization of feed N in the rumen; (2) inaccurate prediction of the animal degradable and undegradable protein requirements, leading to overfeeding of dietary N. A large portion of the dietary proteins and non-protein compounds entering the rumen are degraded by the ruminal microorganisms to peptides, amino acids, and eventually to NH_3. Available research data indicate that diets fed to animals have profound effects on NH_3 emissions from manure. Overfeeding of rumen degradable protein or metabolizable protein will result in excessive urinary N excretion. Feeding a diet imbalanced in a supply can also result in poor feed N use efficiency because one or more amino acids can limit protein synthesis and thus the productive use of the other amino acids, resulting in increased catabolism of all amino acids. Finally, insufficient diet fermentability can limit N capture in microbial protein in the rumen, and insufficient energy supply to the animal can limit rates of protein synthesis, both of which result in poor feed N efficiency, excessive urinary N output and, consequently, increased NH_3 emissions from manure. Urinary N losses by dairy cows decrease linearly with decreasing dietary CP levels. These reductions can sometimes be achieved with minimal or no effects on yield or composition of milk and milk protein (Hales et al., 2012).

2.8.4 Grass management

Grasslands are an important source of low-cost and high-quality feed for ruminants in Europe. It is estimated that roughly half of the total dry matter intake by livestock at the global level comes from grass and other roughages, albeit with strong regional variations. Grassland soils also store large quantities of carbon and in many regions have the potential to sequester more carbon, while providing a range of other ecosystem services related to habitat and water quality. Improving management practices and breeding/adopting new species and cultivars can improve the quantity and quality of feed to animals and also, in some regions and systems, enhance soil

carbon storage. However, the potential for carbon sequestration and techniques for achieving it are country/region specific, and differ across soil types, management practices and climate. Developing grass varieties with specific traits aimed at improving feed efficiency or directly reducing emissions may be of significant importance for predominantly pasture-based ruminant production systems. The focus on development and subsequent uptake of the so-called high sugar grasses in the UK are one example. These have been shown to improve N utilization by ruminants (Moorby et al., 2006) which would result in less nitrogen excretion and therefore less subsequent N_2O and ammonia emissions. They have also been shown in one UK trial to reduce enteric CH_4 emissions from grazing lambs by 20%, with the reduction hypothesized to be due to a combination of altered carbohydrate metabolism in the rumen towards propionate production (H-sink) and away from acetate formation (H-source) plus improved microbial growth through improved capture of N in the rumen, diverting surplus hydrogen away from CH_4 production and into microbial cells. However, a review by Parsons et al., (2011) was less conclusive on the effects of high sugar grasses and further research is needed to demonstrate both mechanism and effectiveness. Other targets for development include increasing the lipid content of grazed grasses, as lipids are known to suppress

2.8.5 Feed additives, plant compounds

Fundamental understanding of the microbiome and the relation between host animals, methanogens and other micro-organisms is essential to be able to modify the rumen in a way that is consistent with farming practices, economics, and food safety requirements. Some chemical compounds can have an inhibitory effect on methane generating rumen micro-organisms. Laboratory experiments have shown methane reductions *in vitro* of up to 100%. Some compounds have also been demonstrated to be effective in animal trials, with some resulting in almost complete removal of methane emissions; however, these are not commercially viable due to animal health and food safety concerns or prohibitive costs. Research is focussed on examining natural or synthetic compounds that meet the requirements of long-term efficacy (including possible adaptation of the rumen microbial community), no negative effects on productivity, and food and animal safety. It has been suggested that rumen function will be disrupted if methane production is significantly decreased by directly

inhibiting methanogenic archaea without the provision of alternative hydrogen sinks (McAllister and Newbold, 2008), which implies that methane production is unavoidable in ruminant production systems.

However, recent work (Abecia et al., 2012) suggest that methane production ruminants can be significantly decreased by inhibiting the metabolism of methanogenic archaea with little effect on rumen function and diet digestibility. Indeed, studies on the rumen transcriptome suggest that the methane-inhibited rumen adapts to high hydrogen levels by shifting fermentation to alternative H sinks and direct emissions of H_2 from the rumen. Given that methane emissions can be significantly reduced without affecting production and health attention should focus on the practical means by which this might be achieved. The greatest progress has been in the areas of diet and dietary additives to mitigate against ruminal methane emissions, with decreases in excess of 60reported in cattle fed specific dietary additives. Recent data suggest that, in many cases, additives enhance capacity to mitigate against ruminal CH_4 production.

2.9 Alternative feeding strategies to reduce methane in Ruminants

Manipulating the nutrient composition of the diet of ruminants can directly reduce enteric CH_4 output. For example, a high proportion of concentrates (grain based feeds) in the diet tends to reduce the protozoa population in the rumen, reduce rumen pH, alter the acetate: propionate ratio and decrease the amount of CH_4 produced per unit of feed intake (Blaxter and Clapperton, 1965). The proportion of concentrates in the diet needed to bring about this effect may well be over 50%. The direct manipulations of the diet in pasture - based systems by feeding concentrate supplements has economic consequences, which limit their use in many cattle production systems. Developing forages that directly reduce CH_4 is likely to be a better option for reducing CH_4 than feed supplementation based on concentrates.

2.9.1 Selection of plants with secondary compounds

In many studies (*in vitro* and in vivo) it has been demonstrated that with temperate legumes (*Hedysarium coronarium*, *Lespedeza cuneata*, *Lotus corniculatus* and

L.uliginosus) and tropical legumes (*Calliandra calothyrsus, Flemingia macrophylla*) thatcontain secondary compounds such as condensed tannins (CT) it is possible to reduce methanogenesis. Tannins and phenolic monomers have been found to be toxic for some of the rumen microbes, especially ciliate protozoa, fiber degrading bacteria andmethanogenic archaea, and as a result methanogenesis in the rumen can also be reduced. Reports in the literature provide evidence that by feeding legumes with CT there is a reduction of CH_4 production in different ruminant animals. In a review by Ramírez-Restrepo & Barry, (2005) on alternative forages containing secondary compounds for improving sustainable production of grazing ruminants, they indicated that the condensed tannin containing legumes *Lotus corniculatus* and sulla (*Hedysarum coronarium*) promoted faster growth rates in young sheep and deer in the presence of internal parasites, and showed reduced methane production relative to forages without tannins (*Chicorium intybus*). They also reported that grazing on *L. corniculatus* with CT was associated with increases in reproductive rate in sheep, increases in milk production in both ewes and dairy cows and reduced CH_4 production.

2.9.2 Probiotics

Microbial feed additives that have been developed to improve animal productivity by directly influencing rumen fermentation. Wallace & Newbold, (1993) reviewed data from trials involving dairy cows and growing cattle fed high concentrate diets and calculated that probiotics improved productivity by 7 - 8%. Interest in probiotics as a potential technology to reduce CH_4 came from findings that *in vitro* they can directly reduce CH_4 production. However, *in vitro* results on CH_4 reduction have not been consistent (Martin et al., 2010) and there are no reports in the literature on in vivo CH_4 production after supplementation of probiotics. Given that probiotics are feed additives that need to be fed daily, they would appear to be only suitable for systems where feed supplements are given on a routine basis or for lactating dairy cows. This combined with the limited evidence that probiotics directly influence CH_4 emissions indicate that they have limited utility to reduce CH_4 in ruminants.

2.10 Methane research

Research into manipulating methane (CH_4) production as a result of enteric fermentation in ruminants currently receives global interest. Approximately 90% of total enteric CH_4 production in ruminants originates from rumen fermentation of feedstuffs, which implies that nutrition can have a large impact on total CH_4 emissions. For this reason, the topic of nutritional strategies to reduce CH_4 emissions from ruminants has been the subject of several qualitative and quantitative reviews. Metabolizable energy (ME) and Net energy (NE) systems are widely used in feed evaluation for cattle. The ME is the heat of combustion (gross energy; GE) of feed, minus the energy in faeces, urine and gases. To accurately determine ME, losses of energy in CH_4 have to be measured. Methane represents, on average, a loss of 6.5% of GE, but with a wide range (2-12% of GE). Initially, research into manipulating CH4 production was related to the loss of GE represented by CH_4. However, more recently the research focus shifted from enteric CH_4 as inefficiency in animal production, towards the contribution of CH_4 to global greenhouse gas emissions.

Anthropogenic methane production is a significant contributor to the greenhouse effect, and approximately 12% of this is generated by ruminants (Crutzen et al., 1986). Opportunities for amelioration of methane production by ruminants may include: a. selection for relatively less methane producer animals, and/or b. dietary manipulation or management of animal's internal environment which predispose them to the lower levels of methane production.

Enteric CH4 production is associated primarily with production of acetic acid and butyric acid and, in general, the fermentation of predominantly forage diets results in a higher molar proportion of acetic acid than occurs with concentrate-based diets (Orskov, 1986). Conversely, concentrate-based diets normally contain greater proportions of more readily fermentable components that favor propionate production during rumen fermentation, with a consequent reduction in CH4 production per unit of fermentable OM in the rumen. In addition, the more rapid fermentation associated with concentrate-based diets tends to result in a lower rumen pH, and this will also inhibit the growth of methanogens and protozoa (Hegarty, 1999).

Blaxter & Clapperton, (1965) noticed that animals relatively produce more methane per unit energy intake on forage rather than concentrate diet but an important question that has remained unresolved is whether animals that are assessed as high or low CH_4 emitters on one type of diet retain the characteristic or rank on other feed types.

Cattle produce methane from enteric fermentation (85 to 90%) and fecal excretion. About 95% of ruminal methane is excreted via eructation and from the intestines, 89% of ruminal methane produced is exhaled and around 1% excreted *via* the anus. Methane from enteric fermentation represents about 25% of methane anthropogenic emissions (Wuebbles and Hayhoe, 2002). CH_4, N_2O, and CO_2 are being the 3 main GHG emitted from the agricultural sector. In 2011, the EU agriculture sector produced 461,012 kt of CO_2 equivalents, representing approximately 10% of the total EU GHG emissions. With regard to CH_4, the global livestock sector is responsible for 37% of all human-induced CH_4 emissions, with 89% of these livestock-derived emissions arising from enteric fermentation.

Data and prediction equations describing CH_4 emissions from confined dairy cows have been extensively published (Yan et al., 2010), information on CH_4 emissions from grazing cattle reflect, in part, the challenges faced when measuring CH_4 emissions from grazing cattle. In many temperate regions dairy cows spend between 5 and 9 month of the year grazing, and as such, emissions during this period represent a significant part of their annual emissions. Cows offered confinement diets indicate that although total CH_4 emissions increase with increasing concentrate feed levels (Aguerre et al., 2011), emissions per liter of milk produced generally decrease. However, much less evidence exists concerning the effect of concentrate feed level on CH_4 emissions from grazing cows.

Lovett et al., (2005) measured CH_4 emissions from grazing cows offered either 1.0 or 6.0 kg/d of a fiber-based concentrate and found that whereas CH_4 production per kilogram of milk was unaffected by concentrate supplementation, CH_4 production per kilogram of FCM decreased with increasing concentrate feed level. In a more recent study involving 3 concentrate feed levels (2.0, 4.5, and 7.0 kg/cow per day).

2.11 The in vitro techniques

The *in vitro* techniques have been developed to overcome the shortcoming of the *in vivo* technique. The advantages of the *in vitro* techniques are that they are less laborious and are more suitable for a large scale evaluation of ruminant feeds. The most important techniques are the *in sacco* (nylon bag) technique using the fistulated ruminants, *in vitro* gas production technique and the two stage *in vitro* technique, which involve the incubation of feed samples in rumen liquor. These techniques have been used to predict the *in vivo* digestibility of the feeds. The *in sacco* technique (nylon bag technique) involves incubation of feed samples into nylon bags which are placed in the rumen of fistulated animals. In this technique the bags are extracted and weighed at fixed times for measuring the disappearance of feed from the bags, providing information about rate and extent of feed digestion (Kitessa et al., 1999). The technique has been largely employed to evaluate rumen degradability of feeds and found to predict well the *in vivo* digestibility of the feed. However, the technique is criticized for the need of rumen fistulated ruminants.

The *in vitro* gas production technique measures the appearance of fermentation products (gases, volatile fatty acids, NH_3) when feed samples are incubated in rumen liquor. When a feed is incubated with buffered rumen liquor, it is degraded, and the degraded matter is partitioned to yield gases (mainly CO_2 and CH_4) and microbial biomass. It is assumed that gas production is related to the rate and extent of feed digestion.

The two stage *in vitro* technique for estimation of digestibility of feedstuffs for ruminants was introduced by (Tilley and Terry, 1963). This technique attempts to approximate digestion in an artificial environment, where rumen conditions are simulated in a test tube. The first stage involves 48 hours incubation of the feed samples at 39°C in a test tube with buffered rumen fluid under anaerobic condition. In the second stage the residues are incubated for 48 hours at 39°C with pepsin in an acid solution under aerobic condition. The insoluble residues are filtered off, dried and ignited to obtain ash. The contents of organic matter of the feed and residues are obtained by subtracting ash from the dry matter of the feed and residue respectively.

The two stage *in vitro* technique of Tilley & Terry, (1963) provides a quick, in-expensive and precise prediction of *in vivo* or conventionally determined digestibility in ruminants. It produces values that are numerically similar to *in vivo* values for many types of forages. However, there are some technical limitations of using two stage *in vitro* techniques. There are variations in the *in vitro* digestibility values of forages obtained by the technique in different laboratories as shown in Table 2.2. These variations are mainly caused by the quality of rumen liquor which is due to the diet fed to the donor animal for rumen liquor (Mould et al., 2005).

Table 2.2 *In vitro* DMD and OMD of some tropical forage using two stage techniques (The first and second figures indicate values of the 1st and 2nd stages respectively)

Forage name	DMD	OMD
Cenchrus sp.	60-69	53-64
Cenchrus sp.	41.1	39.5
Brachiaria sp.	60.2	56.3
Brachiaria sp.	65.3	66.5
Cynodon dactylon	51.5	39.9
Cynodon dactylon	79.0	69.2
Pennisetum purperum	38.7	38.8
Pennisetum purperum	52.2	38.4

Other limitation of the technique is the need for fresh rumen fluid, which involves the need for fistulated ruminants, such as cattle, sheep and goats available as donor animals. Surgical operation modifies animals for experimentation, which appears to be unkind, harshly and cruel to the animal leading to some countries ban the use of rumen liquor from fistulated ruminants. These concerns raise the need for alternative approach. Using rumen liquor from slaughtered animals is one of such options. Various studies have shown that rumen liquor from slaughtered cattle has a high possibility of being a replacement to fistulated ruminants as source of inoculum for *in vitro* digestibility studies. Chaudhry, (2014) reported the possibility of using slaughtered cattle as a source of inoculum to evaluate supplements for *in vitro* forage

degradation. In addition, a study conducted by Mutimura et al., (2013) found that rumen fluid from slaughtered cattle could be used for feed evaluation using *in vitro* gas production technique.

Though rumen liquor from slaughtered cattle has shown possibility of being the replacement to that from fistulated cattle in *in vitro* techniques, there is still a great challenge on the quality of the rumen liquor from slaughtered cattle, which may affect the results of *in vitro* techniques. The common source of variation of *in vitro* digestibility is the quality of liquor used as inoculum. Since the dietary history of the animal which is brought to the abattoir for slaughter is not known, information on the quality of rumen liquor from slaughtered cattle coming from different areas with different dietary history should be assessed to know its effect on the digestibility values. The effect of diet of cattle before slaughter on the rumen fermentation characteristics can be assessed by measuring the pH, concentrations of the rumen ammonia nitrogen (NH_3-N) and total volatile fatty acids (total VFAs) of rumen liquor from slaughtered cattle reared under different feed sources.

2.11.1 In vitro gas method

The gas measuring technique has been widely used for evaluation of nutritive value of feeds. More recently, the increased interest in the efficient utilization of roughage diets has led to an increase in the use of this technique due to the advantage in studying fermentation kinetics. Gas measurement provides a useful data on digestion kinetics of both soluble and insoluble fractions of feedstuffs. Several gas measuring techniques and *in vitro* gas methods are in use by several groups. Advantages and disadvantages of these methods are discussed by Getachew et al., (2004). The *in vitro* gas method based on syringes (Menke et al., 1979) appears to be the most suitable for use in developing countries. Other *in vitro* methods such as Tilley and Terry and nylon bag methods are based on gravimetric measurements which follow disappearance of the substrate (the components which may or may not necessarily contribute to fermentation), whereas gas measurement focuses on the appearances of fermentation products (soluble but not fermentable products do not contribute to gas production). In the gas method, kinetics of fermentation can be studied on a single sample and therefore a relatively small amount of sample is required or a larger

number of samples can be evaluated at a time. The *in vitro* gas method is more efficient than the *in sacco* method in evaluating the effects of tannins or other anti-nutritive factors. In the *in sacco* method these factors are diluted in the rumen after getting released from the nylon bag and therefore do not affect rumen fermentation appreciably. In addition, the *in vitro* gas method can better monitor nutrient-antinutrient and antinutrient-antinutrient interactions.

A simple *in vitro* approach is described below which is convenient and fast, and allows a large number of samples to be handled at a time. It is based on the quantification of substrate degraded or microbial protein produced using internal or external markers and of gas or short chain fatty acid production in an *in vitro* rumen fermentation system based on syringes (Menke et al., 1979). This method does not require sophisticated equipment or the use of a large number of animals (but one or preferably two fistulated animals are required) and helps selection of feeds or feed constituents based not only on the dry matter digestibility but also on the efficiency of microbial protein synthesis.

In the method of Menke et al., (1979), fermentations are conducted in 100 ml capacity calibrated glass syringes containing feedstuff and a buffered rumen fluid. The gas produced on incubation of 200 mg feed dry matter after 24 h of incubation together with the levels of other chemical constituents are used to predict digestibility of organic matter determined *in vivo* and metabolizable energy. For roughages, the relationships are:

ME (MJ / Kg DM) = 2.20 + 0.136 Gp + 0.057 CP, R2= 0.94
OMD (percent) = 14.88 + 0.889 Gp + 0.45 CP + 0.0651 XA, R2=0.92

Where ME is the metabolizable energy; DM, OMD organic matter digestibility; CP, crude protein in percent; XA, ash in percent; and Gp, the net gas production in ml from 200 mg dry sample after 24 h of incubation and after correction for the day-today variation in the activity of rumen liquor using the Hohenheim standard.

Aiple et al., (1996) compared three laboratory methods (enzymatic, crude nutrient and gas measuring technique) as predictors of net energy (as estimated by equations based

on *invivo* digestibility) content of feeds and found that for predicting net energy content ofindividual feeds, the gas method was superior to the other two methods.

2.12 Gas measurement

A number of different systems have been used to measure gas production. Menke et al., (1979) described a method in which fermentations were conducted in 100 ml gas-tight, ground-glass syringe barrels and gas evolution was measured after 48 h of incubation. The technique was primarily used for end-point digestion studies, but by measuring the rate of assent of the plunger in the syringe barrel, information on the kinetics of digestion of the feedstuff was also obtained. More recently, Theodorou et al., (1994) described a simple gas production method using an electronic measuring procedure employing a pressure transducer to measure gas from incubations in 160 ml gas-tight culture bottles. Gas accumulated in the head-space of the bottles as the fermentation proceeded and was measured at regular intervals by a pressure transducer connected to a digital readout voltmeter, gas-tight syringe and needle. This method, although technically straight forward, was labor intensive since frequent readings were needed, especially over the initial stages of fermentation.

Pell & Schofield, (1993) described a gas production system using a series of closed 50 ml serum bottles, each with its own stirrer. Each bottle had its own individual pressure sensor that remained in place throughout the entire incubation. These pressure sensors were linked to an IBM-compatible computer.

In the system of Cone et al. (1994), each bottle was linked with its own pressure transducer and electric micro-valve. The pressure transducer measured the pressure build up in each bottle until a pre-set upper value was reached (ca. 0.65 kPa). The valve then opened, allowing the pressure to fall back to a set limit (ca. 0.4 kPa). Every valve opening represented a known amount of gas, so the number of valve openings was proportional to gas production. Each valve opened for just a fraction of a second (50 ms) (Cone et al., 1994)

2.13 Methods of estimating digestibility

The ability of feeds to sustain animal performance depends mainly on their digestion efficiency, which is measured by digestibility values. Feed digestibility is affected by its chemical composition and physical characteristics because these properties affect capability of digestive enzymes to colonize and digest the feed particles (Kitessa et al., 1999). Various methods have been used to determine the digestibility of ruminant feeds and this are grouped in *in vitro* techniques. Digestibility is an important measurement tool for nutritive value of feed, can be determined by several methods such as *in vivo*, *in situ* and *in vitro* technique. *In vivo* method is laborious and requires a relatively large number of animals (Zicarelli et al., 2011). While *in situ* has disadvantage of being expensive in terms of labor and analytical costs, and measures feed disappearance but not the actual amount of fermented substrate while *in vitro* methods provide less expensive and more rapid alternatives. There are several *in vitro* techniques available to measure the nutritive value of ruminant feeds at relatively low cost. Use of *in vitro* gas production technique is beneficial for feed evaluation especially in developing countries such as Vietnam because this method is capable of measuring rate and extends of nutrients degradation with less expenditure. When a feed is incubated *in vitro* with buffered rumen fluid, the amount of gas produced reflects the production of VFA, which are a major source of energy for ruminants. On the other hand, measurement of *in vitro* DM digestibility has been widely used to assess the nutritive value of feeds, due to its high correlation with *in vivo* digestibility. More recently, using *in vitro* gas production technique to evaluate nutritive values of ruminant feeds has been increased (Getachew et al., 1998; Soltan et al., 2012; Tahir et al., 2013).

It was observed from this above discussion that concentrate feeds have effects on ruminal methane production. But in none of these above researches, concentrate feeds were not fermented with yeast. So in this research molasses-yeast mixer were used to ferment concentrate feed to evaluate its effect on total gas production and digestibility measurement.

CHAPTER-III: MATERIALS AND METHODS

3.1 Study area

The study was conducted in postgraduate laboratory under the Department of Animal Science and Nutrition, Chittagong Veterinary and Animal Sciences University (CVASU) Khulshi, Chattogram, Bangladesh and different analysis were conducted in Department of Physiology, Biochemistry and Pharmacology and PRTC laboratories of CVASU.

3.2 Study period and climatic condition

The overall research was conducted from July, 2018 to June, 2019. The weather of Chattogram is characterized by tropical monsoon where the pre-monsoon hot summer season from March to May, monsoon season from mid-May to September, the post-monsoon autumn season from October to November, the dry winter season from December to February (Climate Report, 2016).

3.3 Feed collection

The concentrate feed material of the cattle was collected from the feed store room of Chittagong Veterinary and Animal Science University (CVASU) Bangladesh. The proximate composition of the commercial cattle feed used in the experiment had certain quantity. The labeling of the feed suggested that it was constituted of 75-77% total digestible nutrients (TDN), 14-15% crude protein (CP), 1.1% calcium (minimum), 0.8% phosphorous (minimum), and 90% dry matter (DM). Feed powder of less than 1mm (<1mm) was prepared using mortar.

3.4 Optimization of yeast concentration

The optimum quantity of sugar molasses solution was taken in fermentation flask and the pH and temperature were maintained at 4.0 and 35^{o} C and kept in a constant temperature shaker. The quantities of baker's yeast like 2.0gm were added. An anaerobic condition was maintained for 72 h and during this period, the strain

converts sugar into bio-ethanol with the evolution of CO_2 and the fermented solution was analyzed at every 48 h and 72 h intervals. After 72 h of incubation period, were count the yeast cells (*Saccharomyces cerevisie*) was 4.4×10^8 cells/ml in Neubauer chamber at direct 1: 10 fold dilution method. Calculation of yeast cells were counted using this equation: Number of yeast (cells/ml) = no. of counting chamber × sum of yeast cells × depth of counting chamber × dilution factor.

3.5 Rumen fluid collection

Ruminal contents were obtained from 48-month old rumen of deshi cow from slaughter house. The rumen fluid was collected early in the morning, whereas the required buffers were made the day before for time constraint. Immediate collection of rumen fluid is vital after slaughtering of the cow. The collected ruminal fluid was squeezed and the extracted fluids was strained through cheese cloth that had been folded four times and was placed in a glass bottle with cap. The bottles were subsequently capped and immediately transported to the laboratory while maintaining the temperature at 39°C and put it in a water bath at 39°C in the laboratory. On an important note, it is essential to preserve the rumen fluid temperature for the *in vitro* test.

3.6 Buffer preparation

The buffer medium was prepared according to the method described by Asanuma et al., (1999) with the following composition in mg/L: dipotassium phosphate (K_2HPO_4), 450; monopotassium phosphate (KH_2PO_4), 450; magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 190; calcium chloride dehydrate ($CaCl_2.2H_2O$), 120; Sodium chloride (NaCl), 900; cysteine hydrochloride ($C_3H_7NO_2S.HCl$), 600; ammonium sulfate ($(NH_4)_2SO_4$), 900; Trypticase peptone (BBL; Becton Dickinson, Cockeysville MD), 1000; and, Yeast extract (Difco Laboratories, Detroit, MI), 1000. The chemicals were poured in distilled water of one liter. Firstly, all the chemicals were poured and a very small amount of distilled water was put for the solution to mix evenly. Yeast extract and trypticase peptone were dissolved by hands since they clump immediately when these come in contact with air. Thereby, immediate mixture of these chemicals was needed. In this process, a certain pH is required for the

efficient function of the *in vitro* test the required and desired pH is 6.9. The pH was balanced by adding one to two drops of Sodium Hydroxide (NaOH) (Base) and Hydrochloric Acid (HCL) (Acid). Afterwards, the buffer was dispensed with 100 % Nitrogen (N_2) gas for creating anaerobic condition. Lastly, the buffer was autoclaved at 121°c for 15 minutes. Finally, the buffer was collected after almost one hour when the buffer was cooled after autoclaving and preserved till the next day for mixing with freshly slaughtered rumen fluid.

3.7 Preparation of buffered rumen fluid

The rumen fluid was mixed with the buffer the next day after collection of freshly slaughtered cow and rumen fluid. The upper residue of the rumen fluid was removed while the middle portion was collected and used in the experiment. The pooled and particle-free rumen fluid was transferred to a buffer medium bearing pH 6.9 (Hino et al., 1992) in a 1:3 rumen fluid: buffer ratio. 4000 ml of total liquid was required, but excessive 500 ml was prepared in order to prohibit shortage of liquid in case liquid is lost while pouring in serum bottles.

3.8 Preparation of serum bottles

Fifty ml of buffered rumen fluid was anaerobically transferred under a constant flow of N_2 gas atmosphere in order to make it oxygen free as per suggested by Asanuma et al., (1999) to 100 ml serum bottles containing the 0.5g concentrate feed substrate added with molasses and molasses containing yeast at different concentrations. Finally, the rumen fluid buffer was prepared to be poured in 80 different serum bottles for the ultimate *in-vitro* experiment. Sealing with rubber septum stopper and aluminum cap (Asanuma et al., 1999) of the bottles containing the mixed substrate and buffered rumen fluid will follow which will then be incubated subsequently at 39°C for 6, 12, 24, and 48 h in a shaking incubator with 120 rpm (Hattori and Matsui, 2008).

The final bottle setup was made according to the following treatments were: non addition, 0.1% Molasses, 0.2% and 0.4% Molasses-yeast culture and, hereafter referred to as control, treatment 1 (T_1), treatment 2 (T_2), treatment 3 (T_3) and keeping five replication of each treatment. Thereby, the incubation times were 6 hour, 12 hour,

24 hour and 48 hour. As for bottles, four types of bottles were made, where 20 bottles for each control and treatments. There were 5 bottles fixed for every 6 hour, 12 hour, 24 hour, 48 hour at both control and treatments group. Finally, all the bottles of both control and treatments group were put into shaking incubator at 39°C temperature for *in vitro* gas production with 120 rpm (Hattori and Matsui, 2008).

3.9 Layout of the experiment

Table 3.1 Layout of the experiment showing treatments and replications[†]

Hours	Control	T_1	T_2	T_3
	1	1	1	1
	2	2	2	2
6 h	3	3	3	3
	4	4	4	4
	5	5	5	5
	6	6	6	6
	7	7	7	7
12 h	8	8	8	8
	9	9	9	9
	10	10	10	10
	11	11	11	11
	12	12	12	12
24 h	13	13	13	13
	14	14	14	14
	15	15	15	15
	16	16	16	16
	17	17	17	17
48 h	18	18	18	18
	19	19	19	19
	20	20	20	20

[†]C = Diet without molasses-yeast mixture, T_1 =Diet containing molasses at 0.1% of concentrate DM, T_2 =Diet containing molasses-yeast mixture at 0.2% of concentrate DM and T_3 = Diet containing molasses-yeast mixture at 0.4% of concentrate DM.

3.10 Analyses of in vitro fermentation parameters

3.10.1 Total gas measurement

Fermentation parameters were monitored at the end of each incubation time set. Pressure sensor calibrated gas syringe was used to measure TG production from each of the serum bottles during different stages of incubation. Briefly, a needle channel connected to the syringe was extended into the sealed fermentation bottle to measure the positive pressure created by the gas build up in the headspace of the syringe at room temperature and allowing the gas to flow inside a syringe barrel. The plunger was pulled gradually until the pressure the volume of gas trapped inside the barrel was recorded as the TG produced in ml.

3.10.2 CO_2 and CH_4 measurement

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

3.10.3 pH measurement

In addition, pH was determined using a pH meter after opening each serum bottle. Fermentation was halted by swirling the bottles on cold water after different incubation periods.

3.10.4 Determination of in vitro dry matter and organic matter digestibility

Earlier to the *in vitro* rumen fermentation, the Dry matter (DM) and organic matter (OM) of concentrate feed was determined by drying at 105°C for 16 h and ashing at 550°C for 12 h, respectively. The resulting percent DM and percent OM was used to compute the initial DM (DMi) and initial OM (OMi) of the substrate in grams.

Fermenta samples from each serum bottle after the specified incubation period were drained in dried, pre-weighed nylon bags and knotted using nylon thread, then splashed with flowing water for 15 minutes or until the turbidity of water resulting from washing disappeared. The final DM (DMf) and OM (OMf) of the feed were determined using the same conditions applied when determining the initial values (DMi and OMi). The DM and OM digestibility (%) were calculated as ([DMi − DMf]/DMi) × 100 and ([OMi − OMf]/OMi) × 100, respectively.

3.11 Statistical analysis

After collection, data were compiled in MS excel professional 2016. Data were sorted and compiled for further analysis. Data were tested for the outliers and multicolliniarity by inter quartile range test and variance inflation factors. Differences among the fermented and non-fermented feeds were compared by one way ANOVA using Stata 14.1 SE (Stata Corp LP, College Station, Texas, USA). Statistical significance was accepted at $p<0.05$ for all the test statistics.

CHAPTER-IV: RESULTS

4.1 Chemical composition of feed

There were no significant differences ($p>0.05$) in the chemical composition of the fermented and non-fermented concentrate feeds (Table 4.1). However, the fermented feeds (T_2 and T_3) had a numerically higher CP and CF contents than the non-fermented feed.

Table 4.1 Chemical composition of the experimental fermented and non-fermented feeds

Parameter (%)	Dietary treatments†				SEM	Sig.
	C	T_1	T_2	T_3		
DM	97.5	98.1	98.5	98.3	0.22	NS
Ash	6.0	5.9	6.0	6.0	0.02	NS
OM	94.0	94.2	94.0	94.0	0.05	NS
CP	20.2	20.5	20.8	20.7	0.13	NS
CF	7.0	7.3	7.6	7.4	0.13	NS

†C = Diet without molasses-yeast mixture, T_1 =Diet containing molasses at 0.1% of concentrate DM, T_2 =Diet containing molasses-yeast mixture at 0.2% of concentrate DM and T_3 = Diet containing molasses-yeast mixture at 0.4% of concentrate DM; SEM = Standard error of the means; NS = Non-significant ($p>0.05$).

4.2 In vitro fermentation parameters

4.2.1 pH

Similar to chemical composition, there were no significant ($p>0.05$) differences in the pH of the fermented and non-fermented concentrate feeds. However, decreasing trends of pH was observed in case of all the fermented groups following incubation periods (Table 4.2).

Table 4.2 pH from *in vitro* rumen fermentation of the experimental fermented and non-fermented feeds

Incubation Period	Dietary treatment†				SEM	Sig.
	C	T_1	T_2	T_3		
6 h	5.7	5.8	5.8	5.8	0.01	NS
12 h	5.3	5.4	5.4	5.4	0.02	NS
24 h	5.3	5.2	5.2	5.2	0.01	NS
48 h	5.2	5.2	5.2	5.2	0.01	NS

†C = Diet without molasses-yeast mixture, T_1 =Diet containing molasses at 0.1% of concentrate DM, T_2 =Diet containing molasses-yeast mixture at 0.2% of concentrate DM and T_3 = Diet containing molasses-yeast mixture at 0.4% of concentrate DM; SEM = Standard error of the means; NS = Non-significant ($p>0.05$).

4.2.2 Total gas

In case of total gas production, significant difference was observed after 12 h ($p<0.05$), 24 h ($p<0.01$) and 48 h ($p<0.01$) of incubation period. However, there was no difference ($p>0.05$) observed in fermented groups after 6 h but total gas tended to be higher in fermented groups than the control. The lowest total gas production was noticed in T_2 (61.8 ml) and the highest total gas was measured in control (76.8 ml) group after 48 h of incubation (Table 4.3).

Table 4.3 Total gas (ml) production from *in vitro* rumen fermentation of the experimental fermented and non-fermented feeds

Incubation period	Dietary treatment†				SEM	Sig.
	C	T_1	T_2	T_3		
6 h	19.8	20.2	18.0	19.6	2.0	NS
12 h	43.8	47.2	40.8	44.6	3.9	*
24 h	70.4	64.0	60.4	59.2	4.3	**
48 h	76.8	72.8	61.8	66.0	4.7	**

†C = Diet without molasses-yeast mixture, T_1 =Diet containing molasses at 0.1% of concentrate DM, T_2 =Diet containing molasses-yeast mixture at 0.2% of concentrate DM and

T_3 = Diet containing molasses-yeast mixture at 0.4% of concentrate DM; SEM = Standard error of the means; NS = Non-significant ($p>0.05$); * = Significant ($p<0.05$); ** = Significant ($p<0.01$).

4.2.3 CH_4 production

Significant ($p<0.05$) differences were observed in the *in vitro* CH_4 production after 12 h of incubation where lowest CH_4 was measured in T_2 (32.8 ml) group. Though there was no difference difference ($p>0.05$) after 48 h of incubation period, the lowest CH_4 production was measured in T_2 (43.6 ml) and highest in control (54.6 ml) group (Figure 4.1).

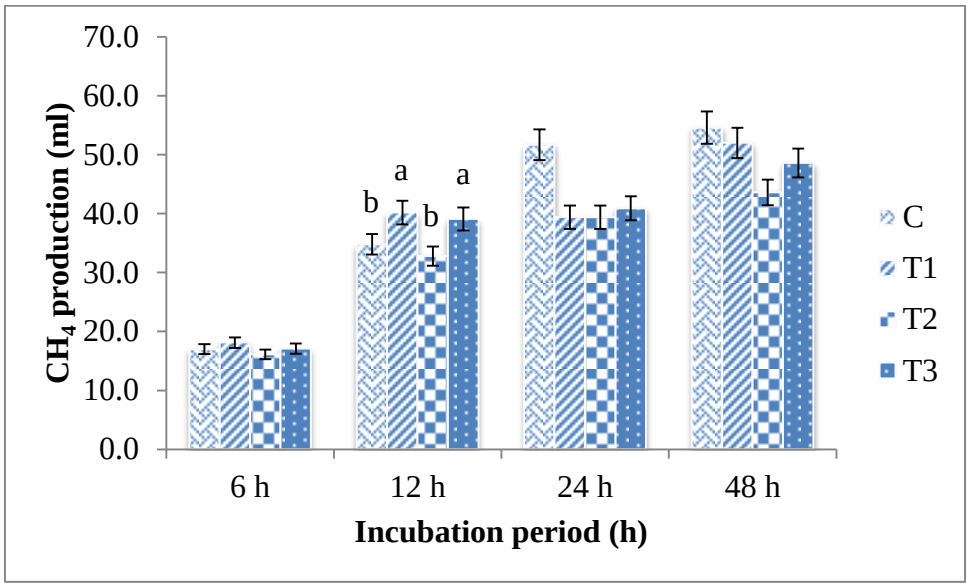

Figure 4.1 CH_4 production (ml/ 0.5 g DM) from different fermented and non-fermented feeds

4.2.4 CO_2 production

There were no differences ($p>0.05$) in CO_2 production at 6 h, 12 h and 24 h except at 48 h of incubation. The CO_2 production consistently decreased in treatment groups than the control. However, the highest CO_2 production was observed in control (22.2 ml) and lowest in T_3 (17.4 ml) group after 48 h of incubation (Figure 4.2).

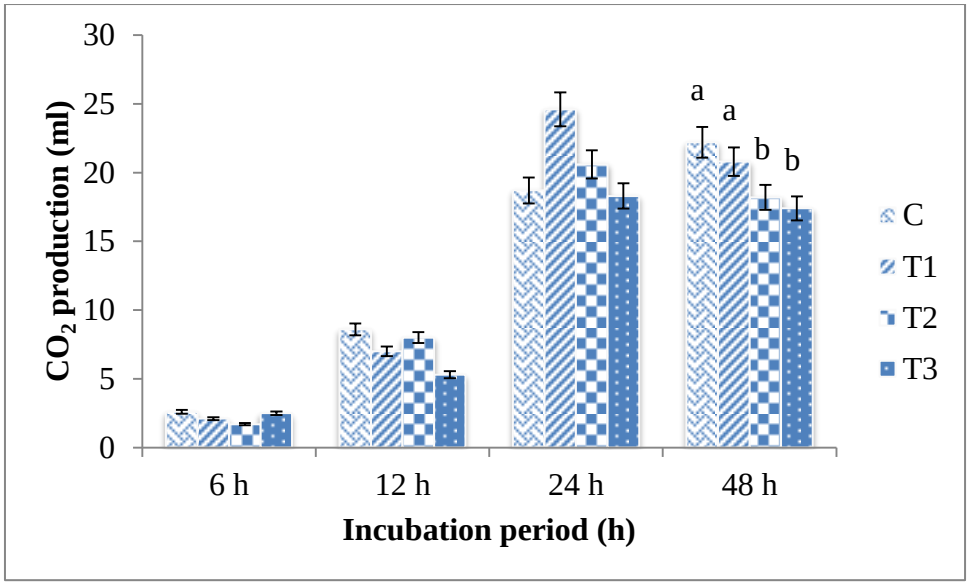

Figure 4.2 CO_2 production (ml/ 0.5 g DM) from different fermented and non-fermented feeds

4.2.5 In vitro organic matter digestibility

There were significant ($p<0.05$) differences in the OM digestibility after 6, 12 and 24 h of incubation period. The highest OM digestibility was observed in T_2 (96.4 %) than the control (94.5 %) group after 24 h of incubation and after 12 h the highest OM digestibility was recorded in T_2 (97.2%) and lowest in T_1 (95.3%) group (Figure 4.3).

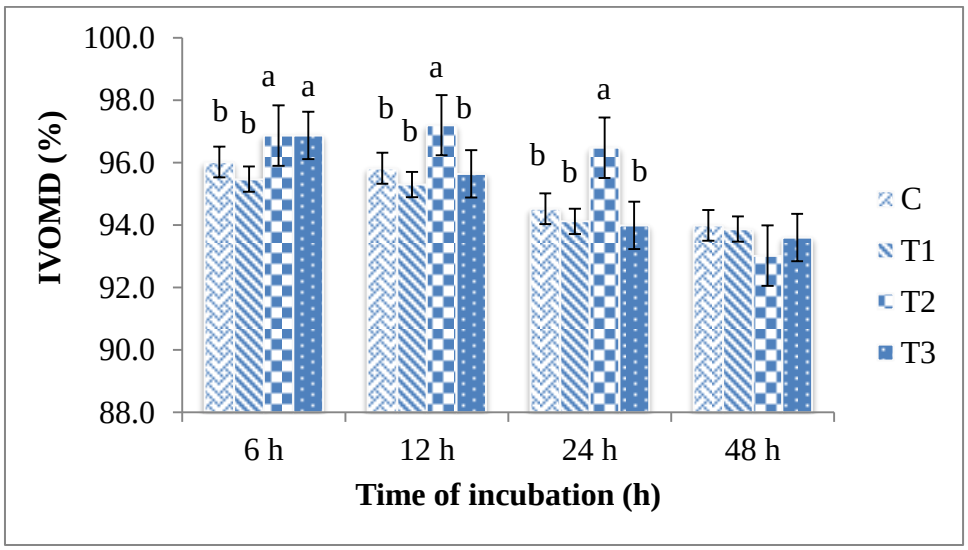

Figure 4.3 *In vitro* organic matter digestibility (IVOMD) of different fermented and non-fermented feeds

4.2.6 In vitro dry matter digestibility

There were no significant differences ($p>0.05$) in DM digestibility but the amount tended to be highest in T_2 (67.3 %) and lowest in T_1 (64.4 %) group after 48 h of incubation (Figure 4.4).

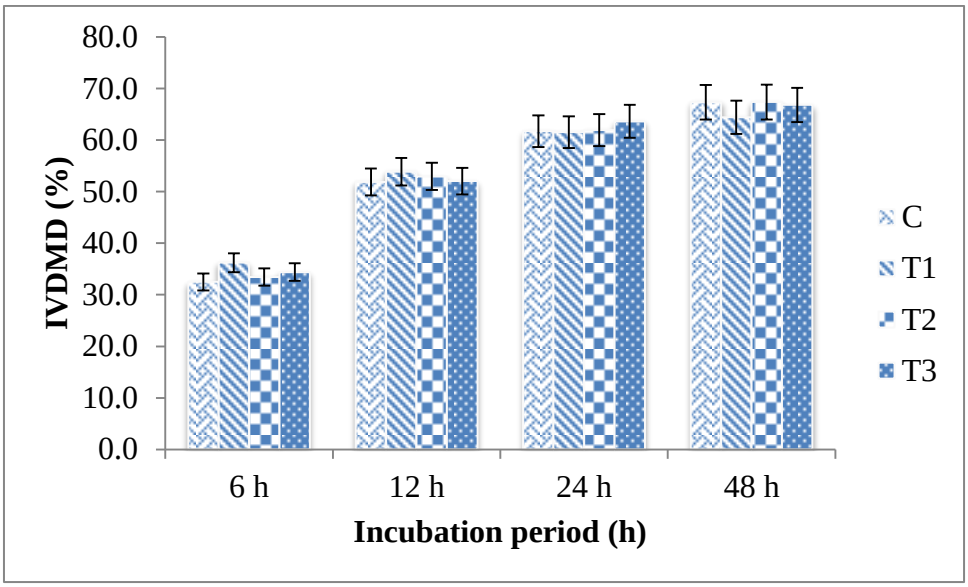

Figure 4.4 *In vitro* Dry matter digestibility (%) of different fermented and non-fermented feeds

CHAPTER-V: DISCUSSION

5.1 pH

In the rumen fermentation process, pH is considered a leading factor affecting rumen microbiome, fermentation and CH_4 production. Ruminant animals solely depend on cellulolytic ruminal microorganisms to digest cellulose. In this study, there was no significant difference in pH among treatments hence, the tended to higher pH observed in fermented concentrate proportion. Specifically, this may led to increased consumption that affecting digestibility and increasing rumination. Vasupen et al., (2005a) reported that ruminal pH was not affected by feeding fermented ration. Meenongyai et al., (2017) also reported that utilizing silage or total fermented ration did not negatively impact on ruminal p^H. Russell & Wilson, (1996) illustrated that the major consequence of ruminal pH<6 is that fibre digestion declines dramatically. This can occur for two reasons, the enzymes necessary for fibre breakdown do not function effectively at pH <6.0, and the growth rate of fibrolytic activity declines markedly at low pH. The lack of difference observed in the present study could be due to the very high buffering capacity of the *in vitro* fermentation processes because four parts of buffer solution were added to one part diluted fecal fluid (Lattimer et al., 2007).

5.2 Total gas

Fermented concentrate feeds digestibility is faster than non-fermented concentrate feeds digestibility due to lower cell wall in fermented feeds (pre-digested), which explains the higher total gas production observed in high proportion of non-fermented concentrate than fermented concentrate feed. The results of the experiment confirmed that gas production increased with the advancing incubation period. But fermented ration feed produced significantly less gas production than non-fermented feeds in each incubation period. Mao et al., (2008) also noted that the total gas production would increase with advancing rumen fermentation period. This consistency illustrates the similarity between present and previous research results. The significance of total gas production indicates a directly proportional relationship between total gas production and the amount of fermented concentrate feed provided. Fermented concentrate feed has lower cell wall components than non-fermented

concentrate feed. Therefore, increasing fermented concentrate diets has been proposed for CH_4 mitigation. However, commercially produced concentrates vary in nutrient compositions, and therefore, differ in CH_4 production (Kim et al., 2013). Less gas production occurred with fermented feed also supported by different reports such as (Cao et al., 2012; Chao et al., 2016; Arangsri et al., 2017).

5.3 CH_4 production

Fermented concentrate feeds play a significant role on rumen fermentation as well as CH_4 emission due to lower total gas production. The greenhouse effect of methane is 20-50 times than that of carbon dioxide (Beauchemin and McGinn, 2005). *In vitro* rumen fermentation technique can be used to determine CH_4 production of different feeds ingredients, substrates, supplements, probiotics, etc. Moreover, the results differ among regions because of the feed variation provided to the ruminants as their source of rumen fluid inoculum for *in vitro* fermentation studies. For example, Lamba et al., (2014); and Mamuad et al., (2014) used the same substrate (rice straw), but the CH_4 production differed. These findings indicate that the rumen fluid inoculum source for *in vitro* fermentation differs depending on the diet provided to the animals. Feed intake, digestibility, species, physiological state, concentrates, and roughage ratio all influence CH_4 production in the rumen (Tiemann et al., 2008).

Fermented concentrate feeds can be used to increase productivity and methane reduction of ruminant animal. In the current experiment, CH_4 production was significantly lowest at 0.2% fermented concentrate feed group. Lower CH_4 production noted with 0.2% fermented concentrate feed group agrees with the findings of (Doležal et al., 2018). They asserted that *Saccharomyces cerevisiae* yeast is used as a feed supplement of ruminant that can improve and modify the rumen environment, due to the decreased amount of oxygen, favoring anaerobiosis and growth stimulation of cellulolytic bacteria by increasing the microbial protein synthesis. Kim et al., (2018) supported that changes in diet influenced the rumen microbiome, CH_4 concentration, and methanogen diversity in cattle. In addition, Smith et al., (2010) reported non- significantly lower CH_4 at 0.2% sulphur supplemented from sodium sulfate (Na_2SO_4) where 92% concentrate where used as substrate. (Blaxter and Clapperton, 1965) noticed that animals relatively produced more methane per unit

energy intake on forage rather than concentrate diet but an important question that has remained unresolved is whether animals that are assessed as high or low CH_4 emitters on one type of diet retain the characteristic or rank on other feed types.

Enteric CH_4 production is associated primarily with production of acetic acid and butyric acid and, in general, the fermentation of predominantly forage diets resulting in a higher molar proportion of acetic acid than occurs with concentrate-based diets (Mitsumori et al., 2012). Conversely, concentrate-based diets normally contain greater proportions of more readily fermentable components that favor propionate production during rumen fermentation, with a consequent reduction in CH_4 production per unit of fermentable OM in the rumen (Jiao et al., 2014). In addition, the more rapid fermentation associated with concentrate-based diets tends to result in a lower rumen pH, and this will also inhibit the growth of methanogens and protozoa (Hegarty, 1999).

Yeast has the ability to shift H_2 utilization from methanogenesis to reductive acetogenesis through the homoacetogenic bacteria that can produce acetate from CO_2 and H_2 (Mwenya et al., 2004). *In vitro* studies have shown beneficial effects of feeding live yeast strain on growth and H_2 utilization and acetate production by acetogenic bacteria isolated from a rumen of lambs, even in the presence of methanogens (Chaucheyras-Durand et al., 1997). (Martin et al., 2010) reported a 20% reduction in CH_4 production after a 48 hours incubation of alfalfa supplemented with a live yeast product. In another study, yeast addition decreased CH_4 by about 58% (Newbold and Rode, 2006). Polyorach et al. (2014) noted that CH_4 production was decreased when animals fed *Saccharomyces cerevisiae* fermented cassava chip protein instead of soybean meal due to the ability of *Saccharomyces cerevisiae* to affect H_2 metabolism in the rumen with altering the fermentation process in a manner that reduces the formation of CH_4.

5.4 CO_2 production

In this study there were no major significant effects observed on CO_2 production but CO_2 production consistently decreased in treatment group that supported the results of

Elghandour et al., (2016) where they observed no effect on CO_2 production as a result of yeast addition at different doses in treatment group.

5.5 Dry matter and Organic matter digestibility

Consideration of the results of other workers (Asplund et al., 1958; Reid et al., 1959; Clark and Mott, 1960; Bowden and Church, 1962). It was evident that a simple *in vitro* technique is capable of providing accurate estimates of *in vitro* digestibility. *In vitro* DM digestibility rose highest at fermented concentrate group, which agrees with results from Soltan et al., (2012). The higher concentrate level in diets contributed to a higher level of soluble substrates which could be the reason for improving DM and OM digestibility. In present study, DM digestibility tended to the highest level at 0.2% fermented concentrate diet treatment, which indicated that, increase in fermented concentrate in diet could improve nutrient digestibility. The OM digestibility resulted for the C and T diet were not different and consistent over time, whereas OM digestibility was reduced by both the diet (from 6 hours onwards. Better digestibility found at every 6h, 12h, 24h and 24h *in-vitro* incubation trial with fermented feed. Cao et al., (2012) reported increased digestibility of fermented ration compared with fresh ration. Effects of fermented ration on diet digestibility have good improvement (Vasupen et al., 2005). The positive effects on digestibility have been confirmed by Poppy et al., (2012) which is also proved with this study. In this study *Saccharomyces cerevisiae* used for the fermentation. The lack of effect of *Saccharomyces cerevisiae* supplementation on DMD coincides with Lattimer et al., (2005) who obtained unaffected *in vitro* DMD with *Saccharomyces cerevisiae* supplementation of a high-concentrate or high-fiber diets. The higher digestibility values could be explained by a higher population of cellulolytic bacteria, which is one of the most consistent effects of yeast (Martin et al., 1989; Wallace and Newbold, 1993).

CHAPTER-VI: CONCLUSION

This research investigated the effects of the fermented concentrate feed on *in vitro* gas production and digestibility in cattle. The final result showed that the increased amount of fermented concentrate feed decreased the total gas and methane (CH_4) production and increasing *in vitro* organic matter digestibility (IVOMD). However, chemical composition and pH value remained non-significant. From both fermented group gave more significant results in comparison with control. It is presumed that the concentrates fermented at 0.2% with molasses-yeast mixture has the higher digestibility and methane reducing potentiality.

CHAPTER-VII: LIMITATIONS AND RECOMMENDATIONS

Limitations

All commercially available feeds were not included in this present study. Preparation of combined molasses and yeast were included under this study rather than individual yeast preparation for fermentation. The study included limited number of samples due to time and fund restrictions. TG, pH, CH_4 and CO_2 production is observed in this study, whereas productions of other VFAs were not possible.

Recommendations

Many other factors like pH, temperature etc. might have effects on ruminal digestion which remained undetected. Advanced studies with better technological supports are required to detect those factors. Further studies are recommended to find the total VFAs production. Further studies with extended time and sufficient fund are required to extend the number of samples and quality of fermented feed.

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