Approaches to increase digestibility of Bangladesh ruminant feed resources in order to mitigate enteric methane production

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LIST OF ABBREVIATIONS

ADF	Acid detergent fibre
ADL	Acid detergent lignin
CA	Crude ash
CF	Crude fibre
CL	Crude fat
CI	Confidence interval
CP	Crude protein
$\mathbf{CH_4}$	Methane
DM	Dry matter
DMI	Dry matter intake
dOM	Digestibility of organic matter
$\mathbf{GP_{24}}$	Gas production at 24 h
GE	Gross energy
***p*GP**	Potential gas production
***c*GP**	Gas production rate constant
GP	Gas production
ME	Metabolisable energy
N	Nitrogen
NDF	Neutral detergent fibre
NEL	Net energy lactation
NPN	Non-protein-nitrogen
$\mathbf{R^2}$	Coefficient of determination
RMSE	Root mean square error
SEM	Standard error of the mean
VFA	Volatile fatty acid

1. INTRODUCTION

According to projections, the world's population will reach 9.7 billion by 2050 and 11.2 billion by 2100, with low- and middle-income nations experiencing the most rapid expansion (FAO 2018, United Nations 2019). Due to the strong demand from emerging global low-and-middle classes, diets will become more affluent and diversified, and the production of animal-sourced foods will grow; milk and meat demand are projected to increase by 73 and 58%, respectively, from their production in 2010 (FAO 2011c). This "livestock revolution" is attributed to the rising living standards of human beings and urbanisation (Thornton 2010, FAO 2018). In this sense, livestock will play a significant role in supplying the growing demand for products originating from animals in order to ensure the continued safety of the world's food supply in the future (FAO 2017, Iannotti et al. 2021).

Two difficulties currently confront the livestock industry in developing nations. First, low animal productivity with poor quality roughages remains a significant problem; second, according to Herrero et al. (2013), these countries account for 50–65% of the world's emissions of greenhouse gases (GHG) from the livestock industry, which is a factor in climate change and global warming. Among these, anthropogenic GHG is the major contributor to GHG emissions. In 2020, the livestock sector of Bangladesh was estimated to produce 30.1 Gg of CH_4 (CO_2e), whereas it emitted 26.7 Gg of CH_4 in 2005; over 15 years, CH_4 production gradually increased due to enteric fermentation (Das et al. 2020). Consequently, potential climate change negatively affects crop and forage quality (IFAD 2010, Chapman et al. 2012, Polley et al. 2013), animal production (Nardone et al. 2010, Henry et al. 2012) and reproduction (Nardone et al. 2010), and indirectly affects the transformation of the planet's ecosystems, which threatens the well-being of present and coming generations (Marino et al. 2015). Environmentalists, researchers, consumers, and legislators are all exerting more pressure on local and international governments to reduce GHGs emissions. Thus, the development of sustainable production systems is a key concern for the livestock sector today, where animal-derived feed can be produced with increased efficiency to accommodate growing demands from an increasing human population, while simultaneously decreasing environmental impacts.

Due to current social, economic, and environmental challenges, conventional feeds, fodders, and feeding patterns are becoming increasingly unsustainable for livestock production. For the stabilisation of CH_4 concentration in the atmosphere, CH_4 production needs to be decreased by 11–30% by 2030 and by 24–47% by 2050 (IPCC 2018). Therefore, reducing CH_4 loss during rumen fermentation of fibrous feeds will not only increase the efficiency of energy utilisation, but also reduce environmental pollution. Researchers believe that to achieve economically viable and environmentally friendly feed production and reduce GHG emissions, there is a notable effort to influence agricultural by-products, tree foliage, and leguminous fodders to supply adequate nutrients and modify feed composition. Additionally, a number of approaches including animal and feed management, diet formulation, and rumen manipulation have been developed to lessen enteric CH_4 production from ruminants to lower global GHG emissions as a way to enhance nutrient utilization (Yuli Yanti and Masato Yayota 2017, Haque 2018). Hence, this research aims to perform an overview of existing information and provide new data on reducing CH_4 production by considering Bangladeshi feed resources side by side to enhance the digestibility of low-quality roughages.

2. LITERATURE REVIEW

2.1. Overview of feedstuffs

2.1.1. Rice straw (*Oryza sativa*)

After harvesting the rice grains, what remains of the vegetative part of the rice plant is the rice straw. In rice-producing countries such as Southeast Asia, rice straw is the predominant agricultural by-product used as livestock feed (Kadam et al. 2000).

According to Göhl (1982) and Drake et al. (2002), the poor quality of rice straw is determined by a number of parameters, such as variety, N-fertilization, and lignin concentration of the cell membrane, which is induced by the stage of maturation, harvesting time, and storage, as well as the local climate. It is deficient in crude protein (2–7%) and has a large amount of silica (Van Soest 2006), which acts as a barrier to digestion by silicifying the leaves cuticular layer more than the stem (Drake et al. 2002). The lack of P, Cu, Zn, Ca, and NaCl in rice straw does not satisfy the nutritional needs of animals (Gowda and Prasad 2005). Additionally, it has low metabolisable energy (ME) and palatability (Odai et al. 2002) compared to maize silage, which reduces nitrogen (N) utilisation. It also encompasses a high neutral detergent fibre (NDF) concentration that may result in reduced dry matter (DM) intake and directly affect animal growth and milk yield (Kanjanapruthipong and Thaboot 2006). Another obstacle is retaining the high level of oxalates (1–2% of DM), which is the major concern for livestock production because it passes out calcium (Ca) from the body through faeces and urine (Jackson 1979, Van Soest 2006). Therefore, rice straw reduces the rumen's overall digestibility, passage rate, N fermentation, and by-pass protein.

To offset the deficiency or nutritious value of rice straw, supplementation or treating approaches are utilised. For producing optimal milk and meat output, rice straw must be supplemented with energy and protein; 7–10% CP is required for good roughage quality to meet animal needs (Hariyadi and Santoso 2010). Legumes are often used as supplementary protein sources, and Akbar et al. (2000) showed that legume fodder (*Lathyrus sativus*) seed broadcast with standing Aman rice intensified fodder yield (11.0 tons/ha), soil organic matter, and N, as well as increased milk yield (20% and 14%) in on-farm and research station studies, respectively. Kusmartono (2007) clarified that Gliricidia (*Gliricidia sepium*) or cassava leaves together with jackfruit wastes (JFW) as energy sources have positive impacts on the dietary value of a rice straw-based diet, as well as on animal performance with cassava leaf hay. The addition of Gliricidia (*Gliricidia sepium*) and

Leucaena (*Leucaena leucocephala*) to ammoniated rice straw (ARS) resulted in increased DM intake and digestibility, subsequently improving the N utilisation and liveweight gain at the rate of 19.3, 34.6, and 33.9 g/d for the ARS, *Leucaena* and *Gliricidia*, respectively (Orden et al. 2000). Green plants, such as Napier grass, are an excellent source of protein when supplemented with rice straw to minimise feed expenditures and enhance milk fat without compromising milk output or other components (Ngo Van Man and Wiktorsson 2001, Wittayakun et al. 2005, Kusmartono 2007). Significantly enhanced feed intake and digestibility was found when maize silage was fed in addition to rice straw (Liu Xiao Hui et al. 2006).

Various processes, including mechanical, chemical, heat, and pressure, are utilised to enhance the dietary quality of rice straw, consequently enhancing feed intake, rumen fermentation, and digestibility. For example, chopping and of milling rice straw may improve the rumen passage rate and feed intake (Doyle et al. 1986). Urea treatment is the most common and easiest method for smallholder farmers, which may improve by 18% the digestibility of the rice straw (Van Soest 2006). Additionally, the buildup of treated rice straw and grass or legumes improved roughage quality, which led to an increase in dairy cow milk production (Khan et al. 1990, Bhaskar et al. 1992, Ngo Van Man and Wiktorsson 2001, Oddoye et al. 2002, Wittayakun et al. 2005).

2.1.2. Maize stover (*Zea mays*)

After harvesting the mature maize cobs, what remains of the vegetative part of the maize plant is the maize stover, which includes the stalks, leaves, and husks. It is typically left in the farmed area and ground to improve soil health (Chenost et al. 1991) and is also utilised as fuel or livestock feed (Suttie 2000, Bwire and Wiktorsson 2002) due to its higher ME and CP content compared to rice straw. However, it contains a high level of fibre, which affects the digestibility and palatability of the feed and may necessitate supplementation or treatment to improve its nutritive value.

It is assumed that 0.8 tons of dry stover are produced from one ton of dry maize grain (Lizotte et al. 2015) and that the global maize grain production in 2017 was 1,100 million tons (FAO 2019), which is approximately 900 million tons of dry maize stover. Similarly, Bangladesh produced 16.03 MM tons of maize stover, mitigating 26% of the total DM requirement (BBS 2016) of livestock rearing.

Maize stover is a fibrous feed, although it has less lignin than other straws, which improves its digestion and animal performance. It is commonly considered an adequate source of energy for livestock (Russell 1986, Jordan 1990, Kyle 2011, 2015, Jean et al. 2017a, 2017b). Nevertheless, supplementation with legume crops is important to improve the protein and mineral supply (Jordar 1990, Bwire et al. 2002, CNC 2002). The wild rye grass, maize silage, or maize grain may be replaced by the combination of dried distiller grains with CaO-treated maize stover to a level of 15% DM without affecting DM intake, milk production, and 4% fat-corrected milk (FCM) yield (Donkin et al. 2013, Shi et al. 2015, Casperson et al. 2018). Supplementing maize stover with cottonseed cake effectively improved intake and milk yield (Methu et al. 2001). Ensiling maize stover with cassava peel improved the silage's gas production and nutritive value (Onaleye et al. 2018).

2.1.3. Napier grass (NG)

Napier grass (*Pennisetum purpureum*) is a high-yielding, valuable, and popular grass in the tropics and subtropics. It is versatile and can grow under dry and wet conditions and in small- or large-scale agriculture (FAO 2015). It is a perennial fodder, and temperatures of 25–40 °C, with over 1,500 mm of rainfall annually, is suitable for its vigorous growth.

Vegetative propagation occurs through stem cuttings that contain at least two nodes, one of which is buried in rows. After planting, NG grows faster and can reach 4 m in 90 d (Skerman and Riveros 1990). However, it requires a high fertiliser and water supply level and produces 20–80 tons of DM/ha (Francis 2004, Sarker et al. 2018). Cutting can be made at 45- to 90-d intervals, depending on the location (FAO 2015).

NG is considered an ideal grass for smallholder dairy farms in the tropics. However, their energy and protein content are extremely sensitive to environmental conditions, maturity, and regrowth days, which reduces protein content and increases fibre content (Krishnamoorthy et al. 1995). For example, after 30 d of regrowth, organic matter (OM) digestibility was measured in sheep at 65% and 60% for 70 d (Butterworth 1965). Therefore, 30 to 35 d of regrowth is the standard for maintaining high nutritional value of NG (17 to 21% CP DM) (Machado et al. 2008). Therefore, supplementing high-protein-containing leguminous fodder with NG will result in good dairy performance. For instance, adding Ipil-ipil to NG supported a milk yield of 7–8 L/day/cow (Muia et al. 2000b). Furthermore, supplementation of Ipil-ipil with maize bran increased the DM intake and efficiency of nitrogen utilisation and allowed a higher milk yield (Muinga et al. 1995) compared to NG alone. On the other hand, NG supplied at 1.25% of the body weight resulted in live weight gain of 0.85 kg/day in crossbreed steers (Neumann et al. 2005).

2.1.4. German grass (GG)

German grass (*Echinochloa polystachya*) is a longstanding or semi-water logging forage that provides highly palatable fodder in tropical and sub-tropical natural wetlands (Cook et al. 2005, Hannan-Jones and Weber 2008). It evolved in the tropics or subtropics of America, from the southern United States to Argentina. Its propagation method is identical to that of Napier grass. In Bangladesh, Australia, and South America, the annual biomass output of German grass ranged from 17 to 20 tons DM/ha depending on soil health, N-fertilization, plant age as well as climatic conditions (Cook et al. 2005, Huque et al. 2019).

The dOM and CP of German grass range from 59 to 63% and 9 to 15%, respectively. The addition of Ipil-ipil to German grass, according to researchers, reduced total lipids and cholesterol levels, resulting in the production of low-fat meat that is nutritionally beneficial for the customer (Rodas-Gonzalez et al. 2007, Uzcategui-Bracho et al. 2008). Huque et al. (2019) indicated that a sole feed of German grass resulted in a higher average gain (107 vs 64.0 g/day) than a sole feed of maize silage. Adding soybean meal (SBM) to German grass resulted in higher digestibility than wheat bran with German grass and rice straw (Habib et al. 2011).

2.1.5. Ipil-ipil

Ipil-ipil (*Leucaena leucocephala*) is a fast-growing, evergreen, long-living perennial, high-quality, and palatable legume fodder tree in the tropics (Chander Datt et al. 2008, Ecoport 2009, FAO 2009, De Angelis et al. 2021). In the 16th century, Ipil-ipil was introduced in the Philippines and Southeast Asia and spread throughout the Asia-Pacific region. An average annual temperature and rainfall of 25–30 °C and 1,500 mm, respectively, are suitable for optimum growth and production.

After the sapling stage of Ipil-ipil becomes less bushy compared to the second year, average annual biomass production is ranged from 3 to 30 tons DM/ha, depending on the soil health, environmental temperature, cutting interval (6-8 weeks), and moisture conditions (Cook et al. 2005).

It can fix a large amount of N (150–300 kg/ha), thus making it promising for use in grass or maize production (Ecoport 2009). It is incredibly palatable for cattle, sheep, and goats and grows well in association with different tropical and subtropical grasses (Cook et al. 2005). It contains mimosine (up to 9% DM in young shoots), a toxic amino acid that has detrimental effects on non-ruminants; however, mimosine is detoxified by rumen microbes (3,4 and 2,3 dihydroxy-pyridine).

Ipil-ipil is a good source of CP (25%–35%), β-carotene, amino acids (isoleucine, leucine, phenylalanine, and histidine), branched-chain fatty acid, calcium, phosphorus, sulphate, peptides and other minerals (Hume 1970, Vietmeyer et al. 1977, Ter Meulen et al. 1979, Elliott and Armstrong 1982, Brewbaker et al. 1985, Cotta and Hespell 1986, Scapinello et al. 2000, Figueredo et al. 2019, Lou et al. 2019). This makes fodder comparable to alfalfa, soybean meal, and fish meal (D'Mello and Thomas 1977, Ndlove and Buchanan-Smith 1985). However, it is generally deficient in sodium (Brewbaker et al. 1985, Garcia et al. 1996, Figueredo et al. 2019). Supplementation with Ipil-ipil may increase OM intake and digestibility (Bamualim et al. 1984). This indicates that rumen microbial activities are promoted. It contains bioactive compounds such as tannins and saponins in the leaves and stem, which reduce DM digestibility but enhance by-pass proteins (FAO 2009, Soltan et al. 2012, De Angelis et al. 2021) and reduce CH_4 production (Soltan et al. 2012, Rusmana Wijaya Setia Ningrat et al. 2019, Irawan et al. 2020). Additionally, increased OM digestibility results in faster rumen fermentation and produces a significant proportion of propionate in total volatile fatty acids (VFAs) (Pacheco et al. 2014, Van Lingen et al. 2016). This is associated with decreased enteric CH_4 emissions, given the negative relationship between propionate formation and enteric CH_4 production.

Supplementing Ipil-ipil with poor quality roughages increased the live weight of ruminants compared to grass pastures during the dry or winter season (Jones 1979). The average daily gain ranged from 0.36 kg (315-d period) to 1.10 kg (90-d period), and even reaching 1.44 kg when the diet contained a high concentration of Ipil-ipil, without the detrimental effects of mimosine (Shelton and Brewbaker 1998). In dairy animals, milk production has increased by 10–14%, and milk fat and protein have also increased (Paschal and Agnes 1997). Ipil-ipil foliage is also a promising feedstuff for sheep and goats, resulting in better replacement of concentrate, DM intake, weight gain, and reproductive performance (Espinoza et al. 2005, Kanani et al. 2006).

2.1.6. Gliricidia

Gliricidia (*Gliricidia sepium*) is also a perennial, high protein tropical forage tree. It originated from Central America's seasonally dry coast and is now widespread throughout the tropics. Temperatures between 20 °C and 29 °C and annual precipitation between 900 and 1,500 mm are optimal conditions for growing *Gliricidia* (Ecocrop 2009). It takes 14 months for establishment from the seedling to the first cutting; after that, looping can be done every 6–8 weeks. The average biomass yield ranges from 9.0 to 16 tons DM/ha in a fodder plot. The plate is thus considered a soil improver for fixing N.

Supplementation of *Gliricidia* to *Panicum maximum*, rice straw, sorghum, Rhode grass, or natural grass tends to rise CP content in the diet, which enhances dOM, DM and nutrient absorption, resulting in a good influence on daily live weight gain and milk production (Abdulrazak et al. 2006, Rusdy et al. 2020). Furthermore, *Gliricidia* leaf meal can be substituted for soybean meal and copra meal in a cassava-based supplement, thereby improving the daily gain of Ongole bulls and, subsequently, farmers' revenue (Winarti et al. 2022). For intensive sheep production, soybean meal can also be substituted for *Gliricidia* without impacting animal growth (Archimede et al. 2009). Incorporating 30% Enterolobium cyclocarpum into the diet of G. sepium decreases CH_4 production without altering DM intake or rumen microbial fermentation (Molina-Botero et al. 2019).

2.2. Physiological properties in the rumen

2.2.1. Function of the rumen

The reticulorumen is the key compartment of the forestomach, where fermentation processes are carried out (Tharwat et al. 2012). Producing enzymes in the rumen through microorganisms helps to ferment the feed eaten by the animal (Aschenbach et al. 2011). For better fermentation of the feed and microbial growth in the rumen, 39 °C temperature with a ruminal pH of approximately 6.7 is required (Arelovich et al. 2014, Ruiz-Albarrán et al. 2016, Zhu et al. 2017). Saliva production is a constant process that provides bicarbonate and phosphate into the rumen, which maintains the rumen pH range from 5.5–7.0 (Krause and Oetzel 2006), relying on the type of feed, level of feed intake, exchange of bicarbonate and phosphate and absorption of VFA (Russell and Strobel 1989, Aschenbach et al. 2011).

The reticulorumen is the important section of ruminants where dietary digestion takes place and feed particles are broken down by the most diverse and numerous microbial populations (Belanche et al. 2012, Benchaar et al. 2012). Rumen microbes receive essential nutrients for their growth from animal feed, and through the fermentation of feed, provide VFAs which is the final fermentation product used as an energy source for ruminants (Xie et al. 2018). These microorganisms also produce proteins that contribute to the protein supply at the animal intestine.

2.2.2. Fibre degradation in the rumen

Roughage with a relatively high cell wall content is the mainstay of ruminant diets; concentrates have a small quantity of cell walls. Cell walls are structural carbohydrates classified biologically as insoluble NDF, including cellulose, hemicellulose, lignin, and a tiny amount of N-containing compounds (Ribeiro et al. 2016, Hatfield et al. 2017). As a substrate component, pectin contributes to NDF. The main rumen function is the fermentation of dietary fibre through the combined activities of bacteria, archaea, protozoa, and fungi. Bacteria and fungi are responsible for around 80% of the degradation (Cai et al. 2010), while protozoa carry out the remaining 20% (Sirohi et al. 2013). These fermentation characteristics are affected by a number of variables, including maturation stage, growing season, soil quality, and fertilisation rate (Valk 2002). It also includes the chemical constitution of roughages, including lignification of NDF. A range of fibrolytic bacteria, the most common of which are *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*, are responsible for the breakdown of the plant cell wall (Forsberg and

Cheng 1992). Ruminal fungi produce the most potent fibrolytic enzymes, such as cellulases and xylanases, which are capable of degrading the most resistant plant cell wall polymeric materials (Trinci et al. 1994). In addition, it can also penetrate the cuticle and lignified cell wall tissues of plant surfaces (Akin 1989).

The degradation of fibre to hexoses and pentoses precedes the fermentation to pyruvate and volatile fatty acids (Fig. 1). Transketolase and transadolase processes of the pentose cycle convert pentoses to hexose and triose phosphate, so that the bulk of dietary carbohydrate metabolism occurs via hexose, which is virtually completely metabolised to pyruvate via the Embden-Meyerhof glycolytic route. Acetyl-CoA is the intermediary step for synthesising acetate and butyrate from pyruvate, whereas the succinate pathway is responsible for propionate formation.

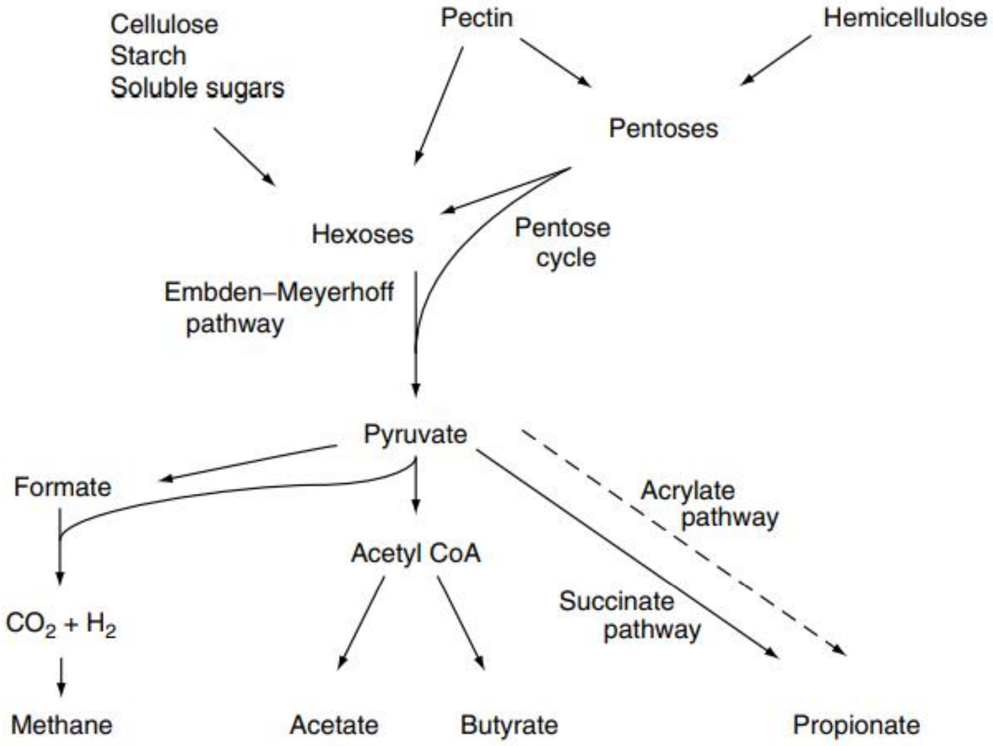

Fig. 1: Diagram of rumen carbohydrate metabolism (France and Dijkstra 2005)

In tropical areas, ruminants depend on feeding of crop residues and agro-industrial by-products, which are known to have a poor nutritive value, rich in carbohydrates such as cellulose and hemicellulose, low energy and N content, and low intake and digestibility (Kumar et al. 2015, Beigh et al. 2017); therefore, animal production performance is low (Van Soest 2006) and for many years, researchers have struggled to enhance nutritional quality using biological, physical, chemical, and enzymatic treatments of agricultural by-products as feed for ruminants. Increasing the nutritional quality of agricultural by-products should increase animal production and productivity and should thus be considered to reduce environmental burden.

2.2.3. Acidosis and rumen pH

Rumen fermentation products supply energy to ruminants by converting consumed feed (Ahmad et al. 2020). The feed comprises plant cells along with non-structural carbohydrates such as starch and fructans, as well as cell walls consisting of cellulose, hemicellulose, lignin, and pectin. Rumen microbes break down these substrates to produce VFAs, microbial proteins, vitamins, and other nutrients vital for the animal's upkeep and productivity (Li et al. 2019). Substrate fermentation generates compounds that affect the microbial population in the rumen considerably (Jaramillo-López et al. 2017). Diversified rumen microbiomes may be linked to animal productivity and health, including milk output and composition, feed efficiency, CH_4 emission, and ruminal acidification (Jami et al. 2014, McCann et al. 2016, Li and Guan 2017).

Studies have demonstrated that feeds containing highly biodegradable components, such as grains and soluble carbohydrate-based feed, provide a substantial amount of energy for milk production in dairy cows (Plaizier et al. 2008). However, many biodegradable substrates are detrimental to dairy animals because during decomposition, the rumen produces organic acids as the main end products. According to a number of studies, the accumulation of organic acids such as lactic acid in the rumen may induce acidic conditions, which may result in ruminal acidosis (Millen et al. 2016, Humer and Zebeli 2017). Ruminal acidosis is typically associated with elevated hydrogen ion or proton (H^+) concentrations that reduce ruminal pH (Dehkordi and Dehkordi 2011).

Ruminal acidosis is a metabolic disease produced by the accumulation of organic acid in the rumen, which can have disastrous effects on animal health (Alarcon et al. 2019). The formation of organic acids decreases the pH level to <5.5, which could potentially stimulate the growth of lactic acid-producing bacteria, producing a huge quantity of lactic acid as the primary metabolite

(Darwin et al. 2018, Darwin and Blignaut 2019, Darwin and Cord-Ruwisch 2019). Acute ruminal acidosis is an extreme form of a metabolic disease caused by the excessive synthesis of lactic acid in the rumen, which lowers the blood pH and bicarbonate levels (Hernández et al. 2014).

Recent findings suggest that the risk of subacute ruminal acidosis (SARA) rises when rumen pH falls below 5.6 for more than three hours per day or below 5.8 for more than five to six hours per day (Plaizier et al. 2008, Zebeli et al. 2008). Long-term exposure to SARA is related with an increased incidence of metabolic illnesses such as fatty liver, displaced abomasum, laminitis, liver abscesses, and downer cow syndrome, which has a negative impact on health and productivity in dairy cattle (Plaizier et al. 2008 and 2012, Zebeli and Metzler-Zebeli 2012). Therefore, dairy cattle feed must have physically effective fibre to enhance chewing activity and salivary buffer, which prevents SARA and supports the proper functioning of the rumen environment (Allen 1997, Zebeli et al. 2012).

2.2.4. Volatile fatty acids production and uptake (VFAs)

Ruminants rely mostly on VFAs for their energy needs, and it is estimated that these acids provide up to 75% of the total ME (Bergman 1990). In most cases, the fermentation of OM in the rumen results in the production of VFAs. In the 1940s, VFAs were found, and research has revealed that the concentration and relative proportion of VFA production are connected to the composition of the feed. This discovery was significant from a nutritional standpoint (Bergman 1990). Rumen microorganisms are responsible for the production of three different types of VFAs: acetate (CH_3COOH), propionate (C_2H_5COOH), and butyrate (C_3H_7COOH). Fibre fermentation primarily releases acetate, while grain fermentation is responsible for producing a larger amount of propionate. Butyrate is generated in lower amounts than acetate and propionate. The ratio of VFA produced is significant because it can be used to generate ATP in the animal's metabolism and used as a precursor for glucose production and fatty acid production. Propionic acid, in particular, is an important factor in the process of hepatic gluconeogenesis in cattle that are not fasting (Huntington 1990). According to Thomas and Martin (1988), the individual supply of VFA to dairy cattle has a significant impact on both the yield and composition of milk. For example, there was a favourable impact on both milk production and fat content when acetic acid supplies were increased. Butyric acid supplementation enhanced the fat content of milk. On the other hand, propionic acid had the reverse effect, decreasing the amount of milk fat while raising the amount of milk protein. This occurs as a result of the glucogenic or ketogenic character of the infused VFA as well as the repartitioning effects of VFA caused by changes in hormone levels.

In the rumen, both the lumen's state (such as pH and the outflow rate of rumen contents) and the mucosa's condition (such as tissue mass, surface area, and blood flow) influence the speed at which VFAs are absorbed. The rumen wall is the most important location for transporting volatile fatty acids, particularly in the epithelial cells (Dijkstra et al. 1993). The connection between VFA transport and rumen wall tissue's metabolic activity has been primarily investigated *in vitro* (Bugaut 1987, Bergman 1990, Remond et al. 1995). The energy involved with ion transport to maintain intercellular homoeostasis (Gabel et al. 2002) and the proliferative response of the epithelial cell layer will all rise in response to moreVFA reaching the rumen wall (Fig. 2).

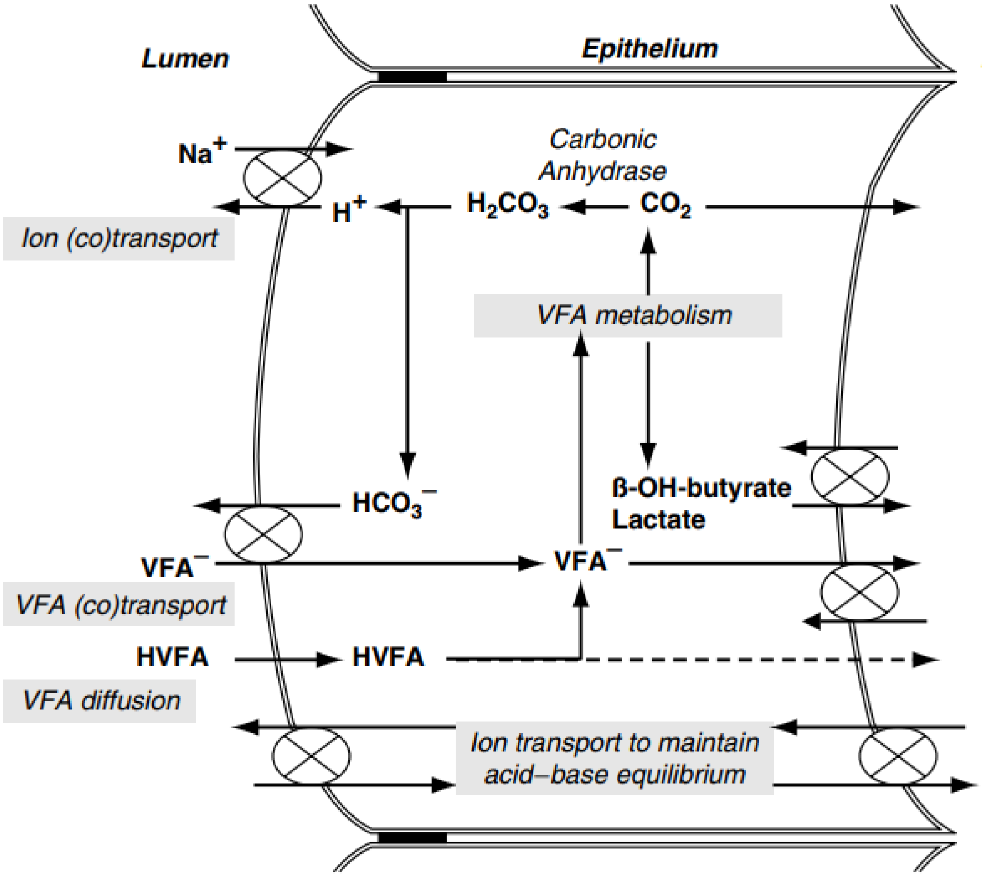

Fig. 2: Graphic illustration of VFA transport, ion transport, and VFA metabolism in rumen epithelial cells (from Gabel et al. 2002).

2.2.5. Methane (CH_4) production in the rumen

Methanogenesis is the process by which CH_4 is produced. This process takes place in the absence of oxygen (O_2) and involves the reduction of CO_2 by H_2 with the assistance of methanogenic archaea. As a result of the degradation of carbohydrates, this is a continuous process in which bacteria, protozoa, and fungi each contribute varying degrees of CO_2 and H_2. Through the interaction of different species H_2 transfer systems, it modulates the metabolic processes of acetogenic and fermentative bacteria (Iyer and Ferry 2005, Tapio et al. 2017). The microbial fermentation process in the rumen is characterized by processes shown in Table 2.1.

Table 2.1: VFA production and rumen reduction mechanism from (Kohn et al. 2000).

Substates		Products	ΔG (KJ)[1]	Reactions
Glucose	→	Pyruvate + 4 H		
VFA production				
$C_6H_{12}O_6 + 4\ H^+$	→	$2C_3H_6O_3 + 2H_2O$		Propionate production
$C_6H_{12}O_6 + 2H_2O$	→	$C_2H_4O_2 + 2CO_2 + 8\ H^+$		Acetate production
$C_6H_{12}O_6$	→	$C_4H_8O_4 + 2CO_2 + 4\ H^+$		Butyrate production
Reductive process				
$2CO_2 + 4\ H_2$	→	$CH_4 + 2H_2O$	-67.4	Methane production

[1] ΔG = free energy change indicates how energetically favourable the reaction is, i.e. the greater ΔG, the greater energy consumption is; a negative ΔG implies energy release.

Propionate synthesis engulfs four H^+ atoms; four H^+ atoms are consumed per molecule of glucose if the end product is propionate. When acetate is produced, an additional two H^+ atoms are produced; therefore, if acetate is the final product, then four H^+ atoms are produced for every molecule of glucose that is consumed. During the fermentation process that results in butyrate, four H^+ atoms are generated for every molecule of glucose. Methanogenic archaea then convert the excess H_2 and CO_2 to CH_4. Potential CH_4 production depends on the type of VFA produced, which means that propionate decreases CH_4 and acetate and butyrate increase CH_4 (Knapp et al. 2014, Buccioni et al. 2015, Tapio et al. 2017).

2.3. Influencing factors for CH_4 emissions

2.3.1. Feed composition and intake rate

Animal feedstuff include two types of feed such as roughage and concentrate. Roughage consisting of material containing cell walls composed of slowly fermentable carbohydrates, such as cellulose and hemicellulose, is associated with higher *in vitro* CH_4 production (Santoso and Hariadi 2009). The proportion of dietary roughage significantly increased physically effective neutral detergent fibre (peNDF), stimulating acetate formation in the rumen and releasing more H^+ ions. The major methanogenic bacteria in the rumen utilise H^+ to reduce CO_2 to CH_4. Therefore, higher acetate formation with microbial fermentation of feed is positively correlated with CH_4 production in the rumen (Janssen 2010). When ruminants consume diets that contain a high level of starch or soluble carbohydrates such as glucose, it tends to indicate increased propionate production, which is negatively correlated with CH_4 production, and diminished acetate to propionate ratio (Demeyer and Van Nevel 1995, Sutton et al. 1988, Kristensen et al. 2003). It is also reported to reduce rumen pH more than that generated by feeding roughages, which may hinder methanogenic bacteria and rumen ciliates (Molina-Botero et al. 2020).

Leguminous fodder contains a high percentage of CP which is considered a supplement feed for poor-quality roughages (Hegarty et al. 1964, Jones 1979, Reed et al. 1990, Siaw et al. 1993, Richards et al. 1994, Irawan et al. 2020). Fodder with a higher CP content has a promising capacity to reduce CH_4 production compared with grass forage (Jayanegara et al. 2009, Melesse et al. 2017, Yonas Berhanu et al. 2019). Additionally, it possesses secondary metabolites such as condensed tannins (CT) and saponins, both of which are capable of inhibiting CH_4 emissions in their own way (Tavendale et al. 2005, Patra 2010, Rira et al. 2019).

2.3.2. Decomposition of fibre and Methanogens

Methanogens do not produce fibrolytic enzymes. However, they enhance the energy efficiency with other ruminal microbes by limiting the accumulation of reduced nucleotides (e.g. NADH) through interspecies hydrogen transfer. This leads to an increase in the amount of fibre digestion (Williams et al. 1994). The presence of a low concentration of H_2 in the methanogen promotes the transfer of electrons to coenzyme F_{420}, and a small amount of ethanol and succinate are produced. As a result, the ATP yield of cellulolytic bacteria and methanogens increases when acetate production increases (Wolin and Miller 1988) (Section 2.3.1). In addition to cellulolytic bacteria, other bacteria are also responsible for interspecies hydrogen transfer. Non-cellulolytic bacteria, including *Selenomonas ruminantium*, protozoa (Finlay et al. 1994), and fungi have been shown to interact with methanogens (Marvin-Sikkema et al. 1990). Methanogens are closely associated with the hyphae of fungi, pellicles of protozoa, and are endosymbiotic with some ciliates. Both rumen fungi and protozoa produce hydrogen in organelles that are bound by membranes and are called hydrogenosomes (Yarlett et al. 1986). It is likely that methanogens in these organelles simplify hydrogen transfer (Wolin and Miller 1988). Cellulolytic bacteria, various genera of rumen fungus, and a variety of rumen methanogens are responsible for a reduction in the production of ethanol, succinate, and lactate, all of which contribute to a 5–28% increase in the efficiency with which cell wall components are digested (Joblin et al. 1990).

2.4. Associative effects between roughage and concentrate

2.4.1. Tropical grasses and legume

To establish feed uniqueness, supplementing low-quality roughage with leguminous forage is an effective strategy (Chapter 2.3.1). In many tropical countries, ruminant productivity is limited because their feed contains low N and high fibre. The supplementary feed offers the necessary nutrients to rumen microorganisms, promoting their proliferation and fibrolytic activity. These nutrients, particularly N, are necessary for producing ammonia, which is also a prerequisite for synthesising protein, vitamins, and minerals by microbes (Anantasook et al. 2016).

In addition to enhancing the rumen environment by raising ammonia-N and the rate of passage of inedible fractions, legumes have direct impacts on fibre degradation and the consumption of low-quality hay and crop residues (Paterson et al. 1982, Ndlovu and Buchanan Smith 1985, Brandt and Klopfenstein 1986, Hunt et al. 1988, Atwell et al. 1991, Haddad 2000). These interactions have the ability to alter the digestive processes that take place in the gastrointestinal tract of ruminants, particularly in the rumen. As a result, the digestibility and intake of a combination of forages may be higher (positive associative effects) or lower (negative associative effects) than the balanced median values obtained when the forages are fed separately.

Mixed tropical grasses and legumes exhibit a positive association with NDF digestibility *in vitro* due to a reduction in the lag time required to initiate fibre digestion (Brown et al. 1991). Furthermore, the legume fodder's high concentration of fermentable cellulose and hemicellulose promotes the growth of fibrolytic microbes, which in turn stimulates the digestibility of other, less degradable types of fibre (Silva and Orskov 1988). According to a number of studies, the ideal proportion of Ipil-ipil combined with crop residues to create favourable interaction effects is approximately 25% (Hunt et al. 1988, Atwell et al. 1991, Haddad 2000).

Research has shown that grass/legume combinations improve animal performance by increasing dry matter (DM) consumption of animals compared to feeding grass alone. For example, milk yield is linked to an increase in DMI, in particular for the combination of ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) in grazed swards (Harris et al. 1998, Phillips and James 1998, Ribeiro Filho et al. 2003), as well as silage feeding (Castle et al. 1983). Nevertheless, a number of investigations focusing on the association between grasses and legumes have shown that the implications of interaction effects on DMI vary depending on the amount of legumes and the plant species in the mixture. For instance, with increasing quantities of legumes, the DMI of lambs increased linearly in some combinations (orchard grass and red clover) and quadratically in others (ryegrass/legume) (Reid et al. 1987). Lagasse et al. (1990) found that the beneficial relationship between lucerne and digestible OM intake in steers differed depending on the grass that was linked with it (percentage of lucerne and rate of recurrence of lucerne feeding to animals). To top it all off, 6–7% more DMI was achieved by incorporating 25 or 50% legumes into the diet (Reid et al. 1987). On the other hand, if an excess amount of legumes is included in the diet, the benefits may diminish due of the overwhelming amount of nitrogen. This can cause substantial liver urea synthesis and an increase in the amount of nitrogen that is excreted. Lower levels of NDF in legumes compared to grasses enhance the DMI and have a positive association due to rapid digestion of the soluble fraction of legumes, high particle breakdown, and a greater passage rate through the rumen (Moseley and Jones 1984, Waghorn et al. 1989). When examined *in vitro*, a combination of whole corn silage and lucerne was found to have associative effects that were beneficial to both OM digestibility and NDF digestibility (Moss et al. 1992).

2.4.2. Low-quality forages and complementing plants

Supplementing low-quality forage with leaf from fodder trees and shrubs, which has a high protein content, is an effective approach (Barry et al. 1997, Rosales et al.1998). In addition, certain plants can induce favourable associative effects on DMD and/or NDFD and voluntary intake (Norton1994, Liu et al. 2002, Doran et al. 2007). Added mulberry (*Morus alba*) and sesbania leaves used in tropical areas or various tree foliage have been recorded as effective complementary plants for ruminant feeding (Doran et al. 2007, Melaku et al. 2003, Tessema and Baars 2004, Bobadilla et al. 2002). Synchronizing the digestion rates of the distinct ingredients of a mixture of tree leaf was used to administer the favourable associative effects of the mixture. This is also connected to the chemical constituents' potential to ferment, particularly proteins, carbohydrates, and cellulose (Rosales 1996). Some of the tree foliage has anti-nutritional effects by secondary plant metabolites which optimises the animal response (Barry et al. 1997).

2.4.3. Secondary metabolites and digestion

Some plants, primarily legumes, contain secondary metabolites and bioactive substances that alter intake and the rumen environment in a positive or negative manner (Barry and McNabb 1999, Rochfort et al. 2008). Therefore, depending on the composition and concentration of the diet, it might bring nutritional and environmental benefits or drawbacks. In 2006, the European Union outlawed the use of antibiotics as growth promoters in animal feed, and efforts were undertaken to identify plants and compounds that have a positive effect on fermentation efficiency, animal health, and environmental emissions (Makkar et al. 2007). The key objective of this research was to decrease the rate of ruminal proteolysis as well as the formation of CH_4 in the rumen (Martin et al. 2009).

Tannins and saponins are natural anti-nutritional factors for ruminants (Kumar and Singh 1984). Dietary condensed tannins (CT) and saponins are prevalent, especially in legumes like Ipil-ipil and sainfoin, which can react with proteins and block their breakdown during fermentation (McSweeney et al. 2001b). When the dietary protein concentration is low and that of fibre is high, CT has a negative impact on digestibility, consumption, and animal productivity because it reduces microbial proliferation (Min et al. 2005). However, when the protein concentration in the diet exceeds animal requirements, animal performance can be improved (Barry and McNabb 1999). Some CT can reduce the proteolytic bacteria in the rumen, which leads to better utilisation of the feed N as a result of increased non-ammonia–N and protein supply to the small intestine (Min et al. 2000). Waghorn et al. (2002) discovered that two CT-containing plants, lotus (*Lotus pedunculat*us) and sulla (*Hedysarum coronarium*), inhibited methanogenesis in growing lambs by 50% and 30%, respectively, without having any deleterious consequences.

As a result, the associative effects of low-quality roughages and high-quality forages may contribute to optimising feed-use efficiency, leading to increased output, decreased animal emissions, and more environmentally sustainable animal production.

2.5. Adaptation and CH_4 mitigation options

The mitigation of enteric CH_4 is a practical approach to reducing the contribution of agricultural livestock to climate change (Fouts et al. 2022). It represents an update on enteric CH_4 mitigation intrusions by incorporating dietary manipulation (Hristov et al. 2022, Tseten et al. 2022, Fouts et al. 2022), which influences the composition of feedstuffs, and an inexpensive approach to forward the rumen fermentation process to reduce CH_4 emissions (Haque et al. 2018). It includes:

- Improving roughage quality
- Decreasing the relative proportion of roughage to concentrate
- Plant secondary metabolites

2.5.1. Improvement of roughage quality

Roughage comprises a significant portion of the feed for ruminants. Improving the quality and digestibility of roughage is a possible intervention for CH_4 reduction (Hristov et al. 2013). Increasing roughage digestibility decreases CH_4 intensity by enhancing the digestible energy available to animals (Jung and Allen 1995). However, poor roughage increases maturity and lignin on the cell wall, decreasing cell wall degradability and polysaccharide hydrolysis. Therefore, low-quality roughages can increase CH_4 yield and intensity owing to a higher C: N ratio and low digestibility (Milich 1999).

High-quality forage can reduce CH_4 emissions from the rumen (Boadi and Wittenberg 2002). Quality forage, young plants, contain a higher amount of readily fermentable carbohydrates and less NDF, reducing enteric CH_4 emission intensity by changing the fermentation pathway (Hristov et al. 2013b, Hills et al. 2015). It also increases digestibility and passage rate. For example, dairy cattle's digestibility was raised by 25% when grass silage or herbage was added to low-quality roughage. In comparison, CH_4 yield and intensity were also reduced by 10% and 19%, respectively, due to increased passage from the rumen and animal productivity (Van Gastelen et al. 2019).

Geographical location is another factor affecting roughage digestibility and CH_4 production. For example, ruminant diets in temperate regions often consist of C3 grasses and cool-climate legumes, whereas C4 grasses and warm-climate legumes are utilised in tropical regions. C4 grasses produced 10–17% more CH_4 than C3 grasses, which was linked to the reduced lignin and NDF content and higher rumen passage rate of C3 grasses (Archimède et al. 2011). Substituting grass silages with legume silages may reduce CH_4 emissions due to their lower fibre concentrations (Waghorn et al. 2002, Archimède et al. 2011) and presence of bioactive compounds (Eugène et al. 2021, Baihaqi et al. 2022).

Commonly, the grass is harvested at a later maturity stage, resulting in reduced sugar, N, and dOM concentrations. After grass silage is made, lactate is produced in the silo and often added to the silage via an ensiling process (Tamminga et al. 2007). Subsequently, grass silage-fed animals emit higher CH_4 than maize silage (Fouts et al. 2022, Hristov et al. 2022). This is because maize silage or other small grains containing whole crop silage usually provide higher contents of readily digestible carbohydrates, such as starch, which increases DMI and enhances animal performance (Beauchemin et al. 2008), resulting in lower CH_4 production from animals (Benchaar et al. 2015, Ramin et al. 2021). There are three potential approaches to minimise CH_4 generation in the rumen from maize or whole-crop silage. First, a high starch concentration produces propionate instead of acetate. Second, it increases total DMI and passage rate, diminishes ruminal retention time and fermentation, and enhances post ruminal digestion. Thirdly, replacing grass silage with maize silage reduces CH_4 emissions per unit of animal output, hence improving animal production efficiency (O'Mara et al. 1998). When alfalfa hay was replaced with 100% corn silage, CH_4 production decreased, as reported by Hassanat et al. (2013).

2.5.2. Decreasing the relative proportion of roughage to concentrate

Roughage alone is insufficient to enhance animal performance and concentrates are commonly added to feed in different proportions (Agle et al. 2010, Jiao et al. 2014). CH_4 emissions went down due to changes in patterns of rumen fermentation and the proportions of VFAs production that happened when the ratio of roughage to concentrate was decreased. VFAs and CH_4 losses also differed according to the degradation of different carbohydrates in the rumen. Roughage is composed of complex carbohydrates, such as cellulose and hemicellulose, which promote the formation of acetate and butyrate, hence increasing the availability of H_2 for methanogenesis (Ungerfeld 2020). Conversely, concentrates are composed of non-structural carbohydrates, such as starch and sugar, which increase propionate production (Agle et al. 2010). Propionate, as a glucose and lactose precursor, uptakes H_2 and raises metabolic energy compared to CH_4 belching (Newbold et al. 2005). It has been reported that adding 35 or 60% concentrate to feed reduces CH_4 production and enhanced productivity (McGuffey et al. 2001). In contrast, researchers have shown that high levels of concentrates can promote lactic acid and VFAs formation in the rumen, which contributes to health disorders such as SARA (Owens et al. 1998, Humer et al. 2018, Ogata et al. 2019, Elmhadi et al. 2022, Darwin et al.2022) (Section 2.2.3).

The production of CH_4 fell by 26% in beef cattle, 14% in dairy cattle, and 6% in sheep when concentrate (386 g/kg DM) was added to their diets (Van Gastelen et al. 2019). Additionally, the inclusion amount of dry-rolled maize increased from 225 to 838 g/kg DM in beef steers, resulting in a decrease in CH_4 emission and an improvement in the conversion efficiency from digestible energy to metabolisable energy (Fuller et al. 2020).

The grazing ruminant diet has a better ability to raise high fermentable carbohydrate intake; however, it depends on the baseline intake of high-quality forage, which influences enteric CH_4 emissions (Jiao et al. 2014, Muñoz et al. 2015, Van Wyngaard et al. 2018a, 2018b). For instance, when poor-to-moderate quality roughage for dairy cattle was supplemented with 281 g/kg DM and 461 g/kg DM concentrate, CH_4 production increased linearly, whereas CH_4 yield, and intensity declined (Van Wyngaard et al. 2018b). Therefore, dietary concentrate inclusion offers a greater possibility of lowering enteric CH_4 emissions when the basal diet is constituted of low-quality roughages (Zubieta et al. 2021).

2.5.3. Plant secondary metabolites

Due to the increasing antimicrobial resistance of methanogenic bacteria for CH_4 production, secondary plant metabolites, such as tannins and saponins, are of substantial interest for CH_4 mitigation (Niderkorn et al. 2020, Begum et al. 2021). It also rationally modulates the rumen microbiome and modifies its function, reducing feed energy as CH_4 in ruminants, which increases microbial protein synthesis and fibre degradation in tropical feedstuffs (Ugbogu et al. 2019, Kuvera et al. 2020).

2.5.3.1. Condensed tannins

Using tannin-containing plants or agricultural industrial waste is an innovative approach to suppress CH_4 production in ruminants (Naumann et al. 2015, Albores-Moreno et al. 2019, Deuri et al. 2019, Baihaqi et al. 2022). Tannins are classified into two types: hydrolysed tannins (HT) and condensed tannins (CT). It works in two ways: directly inhibiting methanogens and indirectly limiting H_2 availability through the reduced feed degradation in the rumen (Tavendale et al. 2005). Identifying tannin-rich plants, such as clover and other legumes including trefoil, vetch, sulla, chicory, and Ipil-ipil, is advantageous for minimising CH_4 reduction (Tamminga et al. 2007). Furthermore, *in vitro* data also suggest it can be used to reduce methane production, lending credence to its potential as an anti-methanogenic substance (Goel and Makkar 2012). Condensed tannins have the ability to limit the growth of methanogens and protozoa in addition to controlling the activity of ruminal microbes through bactericidal or bacteriostatic processes (Liu et al. 2011, Bodas et al. 2012). The production of CH_4 dropped to 55% when tannin-rich forages like Ipil-ipil, sulla, red clover, chicory, and lotus were utilised (Ramirez-Restrepo and Barry 2005). Although tannins appear to have the potential for CH_4 reduction, at high concentrations, they impair forage digestibility and animal production (Beauchemin et al. 2009). However, further research is required to find the optimum balance between the lowering of CH_4 and the dose of tannin and its potential anti-nutritional side effects. Another major consequence is that it has a positive impact on protein digestion. Some tannins, for instance, can bound with proteins to produce a tannin–protein complex that is not destroyed in the rumen and increases the protein supply to the small intestine, also known as by-pass protein, which is digestible in the small intestine and nourishes the animal (McSweeney et al. 2001).

2.5.3.2. Saponins

Saponins are large, complex, surface-active glycosidic molecules present in numerous cultivated and wild plant species; they inhibit CH_4 synthesis in the rumen (Tamminga et al. 2007, Patra and Saxena 2009). It possesses anti-protozoal effect by generating complex sterols in the cell membranes of protozoa (Goel and Makkar, 2012) and bacteriolytic activity in the rumen (Moss et al. 2000). It also inhibits bacterial and fungal species that limit the availability of H_2 for methanogenesis in the rumen (Patra and Saxena, 2009), hence decreasing CH_4 production (Bodas et al. 2012). According to Patra and Saxena (2009), the addition of saponin to the diet lowered CH_4 emission by 50%.

2.6. OBJECTIVES OF THE THESIS

To accurately formulate a balanced diet and meet the nutrient needs of ruminant animals, detailed information is required on rumen fermentation characteristics, CH_4 output, and the feeding value of frequently available single feeds. In addition, it is also vital to comprehend any possible interactions between individual feeds and leguminous fodder in feed mixes that could impair the precision of diet formulation. For example, single concentrate and compound feed are commonly fed together with forage in the form of total mixed rations (TMR). However, information on rumen fermentation, CH_4 production, and possible interactions (associative effects) of the feeding values of single feeds in compound feeds is scarce.

The aims of the doctoral work were:

- To investigate the characteristics of common feedstuffs in terms of nutritional composition and their attributes and to assess the *in vitro* gas production kinetics, CH_4 production, and CH_4 concentrations of gas, and the correlation between CH_4 production, chemical composition, and gas production (Study 1).
- To explore the most promising combinations of different roughages (rice straw, German grass, Napier silage, and maize silage) with graded levels of leguminous fodder (Ipil-ipil and *Gliricidia*) selected for ruminant animals to enhance dOM and CH_4 production and their sound associative effects (Study 2).
- To identify the best combination of single concentrates and compound feeds with forages in the form of mixed feed (MF) and total mixed ration (TMR) with respect to rumen fermentation, CH_4 reduction potential, and associative effect (Study 3 and Study 4).

3. MATERIALS AND METHODS

Overview of study outline

Four studies were conducted as part of this doctoral project. These studies included screening and characterisation of single feedstuffs, evaluation of the combination effects of roughage and supplemented leguminous fodder, formulation of mixed feed (MF) with single concentrate, and formulation of total mixed ration (TMR). The materials and methods used in each of the four studies are described separately in the following sections.

3.1. Study **1: Screening and characterizing of single feedstuffs**

In tropical countries, ruminant production systems are associated with low feed efficiency and high emission intensities because of inadequate nutrition (Van Kuijk et al. 2015, Beigh et al. 2017) and low digestibility (Van Soest 2006). With the rise of the human population and climate vulnerability, the main challenge for developing countries such as Bangladesh is increasing production without affecting the environment caused by excess livestock. Better characterisation of tropical feedstuffs and related features of potential methane (CH_4) production is required to further formulate the mixed feed needed to increase animal productivity (Soltan et al. 2012, Omar et al. 2018, Berhanu et al. 2019). The digestibility of organic matter (dOM), metabolisable energy (ME), net energy for lactation (NEL), and CH_4 production of available feedstuffs in Bangladesh has not been determined because of the lack of laboratory facilities. The dOM and ruminal fermentation are related to CH_4 production (McDonalled et al. 2011). Therefore, *in vitro* gas production techniques, those reported by Menke and Steingass (1988), can be utilised to analyse CH_4 production from a wide variety of feedstuffs; these procedures are relatively cheap and are widely used for feed evaluation (Tavendale et al. 2005). In contrast, *in vivo* procedures are expensive, laborious, and time-consuming (GfE 2017). In Bangladesh, there is also a need for more data on the potential CH_4 production of common tropical feedstuffs and how it relates to the nutrient components of these feeds. Therefore, this study was conducted to gather data on the prevalent feedstuffs utilised in Bangladesh for ruminant animals. The main purposes of the investigation were as follows: i) to characterise the common feedstuffs in terms of chemical composition and related attributes; ii) to assess the *in vitro* gas production kinetics, CH_4 production, and CH_4 concentration of gas; and iii) to relate CH_4 production and CH_4 concentration of produced gas to their chemical composition, digestibility, and *in vitro* gas production attributes.

3.1.1. Sample collection and preparation

Eighteen commonly available feedstuffs in Bangladesh for ruminant feeding, including crop residues like rice straw (*Oryza sativa*), urea molasses treated straw (UMS), and maize stover (*Zea maize*); silages like maize silage (*Zea maize*) and Napier silage (*Pennisetum purpureun*); common grasses like German grass (*Echinochloa polystachya*), para grass (*Brachiara mutica*) and Napier grass; leguminous fodder like Ipil-ipil (*Leucaena leucocephala*), Gliricidia (*Gliricidia sepium*), alfalfa hay (*Medicago sativa*), and moringa tops (*Moringa oleifera*), and concentrates such as crushed maize (*Zea mays*), crushed wheat (*Triticum aestivum*), wheat bran, kashari bran (*Lathyrus sativus*), rice bran (*oryza sativa*) and mustard oil cake (*Brassica juncae Coss*) were collected from Bangladesh Livestock Research Institute (BLRI), Saver, Dhaka-1341 Bangladesh. The feedstuffs collection and preparation procedure are given below:

Feedstuffs	Collection and preparation procedure
Rice straw	After harvesting the grain, the straw was sun-dried on an open field for 3 to 4 consecutive days. After, it was chopped using an electric chopper (Borna electronic, 65/C Shatmatha, Bogra, Bangladesh) to achieve particles with a length of 5 to 6 cm.
UMS	Urea molasses rice straw (UMS) was prepared as the following mix: 10 kg dry chopped rice straw + 5 kg water + 1.7 kg molasses + 300 g urea.
Maize stover	Maize stover is the residues of maize plant grown for grain that includes stalks, leaves, and husks. After harvesting, it was cut as described for the rice straw.
Maize silage	After harvesting the milky stage of maize plants, it was cut using the electric chopper to achieve particle lengths of 10 to 12 cm and kept in the silo pit for 30 days following the BLRI practices of pit silage preparation.
Napier silage	For Napier silage preparation, 45- to 50-day-old Napier grass was harvested from 5 to 6 cm above the ground level, chopped into a length of 6 to 8 cm and ensiled in a silo for 30 days.
Common grasses	The common grasses (Napier, German, and para grass) were cultivated following the standard agronomical practices as recommended by the BLRI. At 45- to 50-

	day intervals, the common grasses were harvested and cut to 5 to 6 cm and supplied to the animals.
Leguminous fodder	Every 65 to 75 days, the leaves with twinges of Ipil-ipil, *Gliricidia* and moringa tops were harvested and sun-dried on a smooth concrete floor with a polythene sheet for two consecutive days. Material was transferred into the polythene bags, sealed, and stored in a well-managed storeroom.

In Bangladesh, all feedstuffs were oven-dried at 63 °C for 48 h and milled (Retsch GmbH, 5657 HAAN, Germany) through a 2.0-mm sieve to prepare the samples. After the arrival of dry samples in Germany, all feedstuffs were milled again using Wiley Mill (Dietz-Motoren, KG, Elektromotorenfabrik, 7311 Dettinger-Tech, Germany) and screened with a 1.0-mm mesh sieve for the Hohenheim Gas Test (HGT) and 0.5-mm sieve for chemical analysis in the Nutrition Lab, Department of Animal Nutrition, Institute of Animal Science, University of Hohenheim, Stuttgart, Germany. All the grinding materials were stored in a glass bottle for further use.

3.1.2. Nutrient composition analysis

Analysis of chemical fractions was performed in the animal nutrition laboratory following the official methods in Germany [Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) 2007]. Dry matter (DM) was determined by oven-drying for 4 h at 103 °C (method 3.1), followed by combustion at 550 °C for 4 h to measure the crude ash (CA) (method 8.1). To determine the crude protein (CP) concentration, the nitrogen concentration was analysed by the Kjeldahl method comprising acid digestion of samples with sulfuric acid (H_2SO_4), steam distillation, and determination of ammonium formed by titration. The resulting nitrogen (N) concentration was multiplied by 6.25 to get the CP concentration of the feed sample (method 4.1.1), neutral detergent fibre pre-treated with heat-stable amylase (NDF) (method 6.5.1), acid detergent fibre (ADF) (method 6.5.2), and acid detergent lignin (ADL) (method 6.5.3). A bomb calorimeter (C 200; IKa-Werke GmbH and Co. KG Staufen, Germany) was used to assess the gross energy (GE) applying benzoic acid as a standard. To ensure the accuracy of the data, two replications were performed for each feedstuff.

3.1.3. *In vitro* studies and determination of nutritive values

3.1.3.1. Animals and diet

Fresh rumen fluid was collected from two ruminally fistulated Jersey cows that were operated on as donor animals. Cows were fed a TMR prepared with 32% maize silage, 23% grass silage, 20% concentrate mixture (17% maize, 20% soybean meal, 25% barley, 28% wheat, 4% molasses, and 6% vitamin-mineral premix), 10% hay, 2% barley straw, 1% mineral mixture, and 12% water over the period of eight weeks. The animals lived in groups and were allowed free access to feed and water at all times.

3.1.3.2. Gas production (GP) kinetics

In vitro GP kinetics were evaluated using HGT as described by Menke and Steingass (1988). In brief, approximately 200 ± 5 mg of DM of feed samples was weighed and placed into the bottom of the 100 mL syringes, ensuring that the sample did not stick to the wall of the syringe, and holding the silicon tube to prevent the feed sample from being lost from the vessel. The syringes were closed airtight with greased plungers and pre-warmed (39 °C) in an incubator for one day. The medium of the solution without rumen fluid was prepared the day before incubation using various solutions (distilled water, 620 mL; micro-mineral solution, 160 µL; buffer solution, 310 mL; macro-mineral solution, 310 mL; and resazurin solution, 1600 µL for 57 pistons). It was kept in a water bath at 39 °C under continuous stirring with a magnetic stirrer and CO_2 flow. Starting on the study day, the reduction solution (distilled water, 62 mL; sodium hydroxide (NaOH), 2.6 mL; and sodium sulfide ($Na_2S \times 7H_2O$), 373 to 400 mg for 57 pistons) was freshly prepared and added to the solution medium. After adding the reduction solution to the solution medium, the colour changed slightly from blue to pink, and after a certain period, the solution became colourless. Before the morning feeding, fresh rumen fluid was collected from the two Jersey cows, mixed at a 1:1 ratio, and filtered through a double cheesecloth. Rumen fluid (650 mL) was added to the solution. After 15 min, the rumen fluid with the medium of the solution was pumped into the syringe (30 mL) and carefully handled to ensure that no air bubbles on the surface developed and placed into the incubator for 72 h at 39 °C. Five experimental runs with two replicates per sample in each run were performed.

Furthermore, each run contained four syringes without feed (blanks), three syringes of roughage standard feed, and three syringes for the concentrate feed standard with known gas production. The arrangement of the syringes in the oven was randomised. The cumulative GP was recorded after 2, 4, 6, 8, 12, 24, 32, 48, and 72 h of incubation. The GP for standards and blanks was used to correct the GP of the feed samples at each incubation time. The following nonlinear regression was applied to the collected GP data according to Seifried et al. (2016):

$$Y = p\text{GP} \cdot (1 - e^{-c\text{GP} \cdot 0.01t})$$

where GP is the GP after t h of incubation (mL/200 mg DM), pGP is the potential GP (mL/200 mg DM), cGP is the GP rate constant (%/h), and t is the incubation time (h).

The dOM, ME, and NEL were estimated using GP after 24 h of incubation and data on nutrient composition using the following equations by Menke and Steingaß (1988):

12f- $\text{dOM (\%)} = 14.88 + 0.8893\ \text{GP}_{24} + 0.0448\ \text{CP} + 0.0651\ \text{CA}$

43f- $\text{ME (MJ/kg DM)} = 1.24 + 0.1457\ \text{GP}_{24} + 0.0070\ \text{CP} + 0.0224\ \text{CF}$

$\text{NEL (MJ/kg DM)} = -0.22 + 0.1062\ \text{GP}_{24} + 0.0048\ \text{CP} + 0.0132\ \text{CF}$

where GP_{24} is gas production within 24 h of incubation (mL/200 mg DM), CP is crude protein (g/kg DM), CA is crude ash (g/kg DM), and CF is crude fat (g/kg DM).

3.1.3.3. CH_4 production and CH_4 concentration

Another incubation was performed to measure CH_4 production. The medium of the solution for gas production was similar to that described previously, with some modifications; the incubation period was 24 h and the amount of sample was 120 mg DM. Four runs, with two replications per run, were performed for each feed. Each run contained four blanks, three standard hay samples, and three standard concentrate samples.

After 24 h of incubation, the GP was first recorded, and the CH_4 concentration was measured using an infrared-methane analyser (Pronov Analysentechnik GmbH Co. KG, Berlin, Germany) calibrated with a reference gas (13.0 vol % CH_4; Westfalen AG, Münster, Germany). The CH_4 volume (mL) was assessed by multiplying the total gas (mL) by CH_4 production (%) divided by 100 and standardised with a 120 mg DM feed sample per syringe. The CH_4 and gas production were corrected for the CH_4 and gas production of the blanks. The CH_4 concentration (%) of the produced gas was calculated as the CH_4 volume (mL/120 mg, DM) divided by the total GP (mL/120 mg, DM) and multiplied by 100.

3.2. Study 2: Combination effects of roughages and supplemented leguminous fodder

In the tropical and subtropical areas of developing countries, the productivity of livestock production is limited because of the low nutritional value of feed, which is characterised by a high lignified polysaccharide content (Archimède et al. 2014) and low digestibility (<50.0%; Piñeiro et al. 2017) from poor and N-restricted native pasture and crop residues (Olivares et al. 2011, Goel and Makkar 2012, Soltan et al. 2012, Albores-Moreno et al. 2019). Nutrient-deficient low-quality roughages cannot enhance ruminal fermentation by improving the ratio of acetic acid to propionic acid (Ellis et al. 2012) which impairs possible reduction of CH_4 production (Archimède et al. 2014, Valencia-Salazar et al. 2018, Gaviria-Uribe et al. 2020). Reducing enteric CH_4 emissions and improving roughage quality are key objectives for mitigating greenhouse gas (GHG) emissions from the livestock sector (Gerber et al. 2013, Hristov et al. 2013).

In this regard, leguminous tree foliage seems to be a promising option for overcoming the constraints of feed sources in many parts of the tropics. Trees can be grown by small-scale farmers, and products can contain a higher amount of degradable and undegradable protein, vitamins, minerals, and amino acids than grasses (Goel and Makkar 2012, Njidda et al. 2017, Halmemies-Beauchet-Filleau et al. 2018, Albores-Moreno et al. 2019). This source of N can increase rumen microbial fermentation and by-pass protein, contributing to the amino acid supply in the small intestine of host animals (Leng 1997). These legumes are also promising for reducing CH_4 emissions because of their low fibre content, faster passage rate, and, in some cases, the presence of condensed tannins (CT) (Bhatta et al. 2012). Several studies have indicated that legume CT can directly suppress ruminal methanogenesis through its anti-methanogenic and anti-protozoal activities (Bhatta et al. 2012, Goel and Makkar 2012). Additionally, increased OM digestibility indicates intensified fermentation in the rumen and is related to a higher proportion of propionate in total volatile fatty acids, which is associated with a decreased CH_4 proportion given the negative relationship between propionate formation and CH_4 production.

Against this background, the hypothesis of study 2 was: "Comparative evaluation of different combinations of roughages with leguminous fodder will yield candidates to reduce methanogenesis while improving the nitrogen supply compared with grasses". To test this hypothesis, rice straw (RS), German grass (GG), Napier silage (NS), and maize silage (MS) were chosen and combined with graded levels of Ipil-ipil and Gliricidia. The objectives of this study were as follows: i) to determine which legumes and their combinations with roughage are suitable for ruminant animals to enhance dOM and reduce CH_4 production, and ii) to assess the associative effects of feedstuffs on the ruminal fermentation characteristics.

3.2.1. *In vitro* studies and determination of nutritive values

3.2.1.1. Animals and diet

Fresh rumen fluid was collected from two ruminally fistulated Jersey cows that were operated on as donor animals. The study was conducted over a period of six weeks, and the cows were offered a TMR composed of 30% maize silage, 25% grass silage, 24% concentrate mixture, 16% hay, 3% barley straw, 1% mineral mixture, and 1% limestone (by dry matter [DM]). The concentrate mixture consisted of 20% maize, 20% soybean meal, 25% barley, 28% wheat, 1% molasses, and 6% mineral. Feed and water were supplied for *ad libitum intake*.

3.2.1.2. Design of the experiment

Following the hypothesis, RS, GG, NS, and MS were replaced by Ipil-ipil and *Gliricidia* separately at levels of 10%, 20%, 30%, and 40%, respectively (Table 3.1). A feed sample of 200 mg was used to measure gas production; for the CH_4 production, 120 mg of the feed sample was used.

Table 3.1: Combination of different roughages with graded level of Ipil-ipil and *Gliricidia* and their calculated CP value (%)

Legume fodder	Treat	Roughages (%)				§Calculated CP of the mixed feed (%)			
	(%)	**RS**	**GG**	**NS**	**MS**	**RS**	**GG**	**NS**	**MS**
Ipil-ipil	**0**	100	100	100	100	4.40	14.7	9.40	10.6
	10	90	90	90	90	7.10	16.4	11.6	12.7
	20	80	80	80	80	9.80	18.0	13.8	14.8
	30	70	70	70	70	12.5	19.7	16.0	16.8
	40	60	60	60	60	15.2	21.4	18.2	18.9
Gliricidia	**0**	100	100	100	100	4.40	14.7	9.40	10.6
	10	90	90	90	90	6.40	15.6	10.9	11.9
	20	80	80	80	80	8.30	16.6	12.3	13.3
	30	70	70	70	70	10.3	17.5	13.8	14.6
	40	60	60	60	60	12.2	18.4	15.2	16.0

RS, Rice straw; GG, German grass; NS, Napier silage; MS, Maize silage; § CP concentration was calculated based on the CP concentrations of the single feeds analysed in Study 1.

3.2.1.3. Gas production (GP) kinetics

The procedure for *in vitro* gas production was the same as that described in Study 1, with some modifications, and three experimental runs with two replicates per sample in each run were performed. Each run contained four syringes without feed (blanks), three syringes of hay standard feed, and three syringes for concentrate standard feed with known gas production.

3.2.1.4. CH_4 production and CH_4 concentration

Another incubation was conducted to measure CH_4 production. The gas and methane production procedures were the same as those described in Study 1. Four runs with two replicates for each feed sample were performed.

3.2.1.5. Calculation of associative effect

Associative effects were calculated by comparing the measured and estimated values of the composite feed used in this study. The measured values were obtained from the fermentation of the composite feeds (RS, GG, NS, and MS replaced by Ipil-ipil and *Gliricidia* at 10%, 20%, 30%, and 40%, respectively), and the estimated values were the weighted mean of the values for the fermentation of the individual feeds as obtained in Study 1. The percentage differences between the measured and estimated values were calculated as follows: % difference = 100 × [(measured value – estimated value)/ estimated value].

Positive or negative values indicate positive or negative associative effects between the ingredients contained in the mixed diet.

3.3. Study 3: Formulation of mixed feed with a single concentrate

Feeding mixed feed (MF) to livestock is an important and economical way to increase productivity. Feed scarcity and unbalanced feeding practices are the main offenders that negatively impact livestock productivity. Minimising feed and labour costs and maximising production is essential for farmers, and this can be achieved by bending concentrates, mainly considering locally available by-products and roughage to form MF; or a mixed diet synonymously called total mixed ration (TMR). Moreover, MF is required to stabilise the rumen environment for optimal fermentation, with minimal loss and ammonia release fluctuations. Feeding MF ensures higher consumption, thereby reducing waste (Yasir et al. 2017) to avoid selective refusal of unpalatable dietary portions.

Based on this, the hypothesis of Study 3 was that optimal combinations of single roughages with concentrates can be identified to reduce methane production while increasing digestibility and energy value of the feed. Four roughage combinations from Study 2 were selected and mixed with a single concentrate to test this hypothesis.

3.3.1. *In vitro* studies and determination of nutritive values

3.3.1.1. Animals and diet

Fresh rumen fluid was collected from two ruminally fistulated Jersey cows that were operated on as donor animals. The research was conducted over a period of four weeks, and the cows were offered a TMR composed of 35% maize silage, 25% grass silage, 10% concentrate mixture (20% winter barley, 20% maize, 20% beans,15% peas, and 25% rapeseed), 8% hay, 2% barley straw, 3% toasted soybean, 3% soybean cake, 1% mineral mixture, and 13% water. The animals had *ad libitum* access to feed and water.

3.3.1.2. Design of the experiment

Twelve mixed feeds were formulated (three mixed feeds per roughage). To prepare the mixed feed, 60% roughage/Ipil-ipil combinations were mixed with concentrates. The roughages were not used alone, but at optimum ratios with Ipil-ipil (I_P) as determined in Study 2: 60% rice straw (RS) + 40% I_P, 90% German grass (GG) + 10% I_P, 70% Napier silage (NS) + 30% I_P, and 70% maize silage (MS) + 30% I_P. The rest of the mixture (40%) came from a single concentrate: rice bran (RB), wheat bran (WB), and kashari bran (KB). A total of 200 mg of feed sample was used to formulate the MF. The combinations of feeds are shown in Tables 3.2–3.5.

Table 3.2: Composition of rice straw-based mixed feed (RS_{MF}) in Study 3 and calculated nutritional values

Ingredients	**RS_{MF}**		
	RS_{MF}-RB	**RS_{MF}-WB**	**R_{MF}-KB**
60% from the combinations of roughages (mg)			
Rice straw	72	72	72
Ipil-ipil	48	48	48
40% from single concentrate (mg)			
Rice bran	80	0	0
Wheat bran	0	80	0
Kashari bran	0	0	80
mg of CP in 200 mg	24.2	28.6	31.3
CP (% of DM)	**12.1**	**14.3**	**15.6**
GE (MJ/kg DM)	**18.9**	**18.6**	**18.1**
ME (MJ/kg DM)	**5.40**	**8.30**	**7.60**

RS_{MF}, Rice straw-based mixed feed (60% rice straw, 40% Ipil-ipil); **CP**, Crude protein; **GE**, Gross energy; **ME**, Metabolisable energy; **RB**, Rice bran; **WB**, Wheat bran; **KB**, Kashari bran; **RS_{MF}-RB**, 60% RS_{MF} + 40% rice bran; **RS_{MF}-WB**, 60% RS_{MF} + 40% wheat bran; **RS_{MF}-KB**, 60% RS_{MF} + 40% kashari bran.

Table 3.3: Composition of German grass-based mixed feed ($\mathbf{GG_{MF}}$) in Study 3 and calculated nutritional values

Ingredients	**GG_{MF}**		
	GG_{MF}-RB	**GG_{MF}-WB**	**GG_{MF}-KB**
60% from the combinations of roughages (mg)			
German grass	108	108	108
Ipil-ipil	12	12	12
40% from single concentrate (mg)			
Rice bran	80	0	0
Wheat bran	0	80	0
Kashari bran	0	0	80
mg of CP in 200 mg	25.6	30.0	32.7
CP (% of DM)	**12.8**	**15.0**	**16.3**
GE (MJ/kg DM)	**18.5**	**18.1**	**17.6**
ME (MJ/kg DM)	**6.00**	**8.90**	**8.20**

GG_{MF}, German grass-based mixed feed (90% German grass, 10% Ipil-ipil); **CP**, Crude protein; **GE**, Gross energy; **ME**, Metabolisable energy; **RB**, Rice barn; **WB**, Wheat bran; **KB**, Kashari bran; **GG_{MF}-RB**, 60% GG_{MF} + 40% rice bran; **GG_{MF}-WB**, 60% GG_{MF} + 40% wheat bran; **GG_{MF}-KB**, 60% GG_{MF} + 40% kashari bran

Table 3.4: Composition of Napier silage-based mixed feed (NS_{MF}) in Study 3 and calculated nutritional values

Ingredients	**NS_{MF}**		
	NS_{MF}-RB	**NS_{MF}-WB**	**NS_{MF}-KB**
60% from the combinations of roughages (mg)			
Napier silage	84	84	84
Ipil-ipil	36	36	36
40% from single concentrate (mg)			
Rice bran	80	0	0
Wheat bran	0	80	0
Kashari bran	0	0	80
mg of CP in 200 mg	25.2	29.6	32.2
CP (% of DM)	**12.6**	**14.8**	**16.1**
GE (MJ/kg DM)	**18.9**	**18.6**	**18.0**
ME (MJ/kg DM)	**6.10**	**9.00**	**8.30**

NS_{MF}, Napier silage-based mixed feed (70% Napier silage, 30% Ipil-ipil); **CP**, Crude protein; **GE**, Gross energy; **ME**, Metabolisable energy; **RB**, Rice barn; **WB**, Wheat bran; **KB**, Kashari bran; **NS_{MF}-RB**; 60% NS_{MF} + 40% rice bran; **NS_{MF}-WB**; 60% NS_{MF} + 40% wheat bran; **NS_{MF}-KB**; 60% NS_{MF} + 40% kashari bran.

Table 3.5: Composition of maize silage-based mixed feed (MS_{MF}) in Study 3 and calculated nutritional values

Ingredients	**MS_{MF}**		
	MS_{MF}-RB	**MS_{MF}-WB**	**MS_{MF}-KB**
60% from the combinations of roughages (mg)			
Maize silage	84	84	84
Ipil-ipil	36	36	36
40% from single concentrate (mg)			
Rice bran	80	0	0
Wheat bran	0	80	0
Kashari bran	0	0	80
mg of CP in 200 mg	26.2	30.6	33.2
CP (% of DM)	**13.1**	**15.3**	**16.6**
GE (MJ/kg DM)	**19.5**	**19.2**	**18.7**
ME (MJ/kg DM)	**5.90**	**8.80**	**8.10**

MS_{MF}, Maize silage-based mixed feed (70% maize silage, 30% Ipil-ipil); **CP**, Crude protein; **GE**, Gross energy; **ME**, Metabolisable energy; **RB**, Rice barn; **WB**, Wheat bran; **KB**, Kashari bran; **MS_{MF}-RB**, 60% MS_{MF} + 40% rice bran; **MS_{MF}-WB**, 60% MS_{MF} + 40% wheat bran; **MS_{MF}-KB**, 60% MS_{MF} + 40% kashari bran.

3.3.1.3. Gas production (GP) kinetics

The gas production process was the same as that described in Study 1, but two experimental runs with three replications per sample were performed for GP.

3.3.1.4. Production of CH_4, ammonia (NH_3-N), and volatile fatty acids (VFAs)

Additional incubation was performed to measure CH_4 production, NH_3-N, and VFA. The same amount of feed sample was used in this study following the same procedure described in Study 1 for the production of CH_4. After 24 h, the gas and CH_4 production were recorded. To analyse the NH_3-N concentration, 30 mL of the liquid sample was collected from a conical flask that contained the total amount of liquid sample from the two syringes of each feed. This was kept in a specific levelling plastic bottle and stored in an icebox to stop microbial fermentation. Steam distillation with subsequent titration (Vapodest 50; C. Gerhardt GmbH & Co. KG, Königswinter, Germany) was performed. The residue was transferred to digestion flasks for this complete incubation, and 15 mL of 0.25 M phosphate buffer (90 g $Na_2HPO_4 \cdot 12\ H_2O\ L^{-1}$, adjusted to pH 11.0, using sodium hydroxide) was added. Distilled NH_3 was trapped in 3% (w/v) boric acid and titrated with 0.05 N HCl (titre = 0.05 M).

For VFA analysis, after measuring the gas production, a liquid sample of two syringes for each feed was kept in conical flasks with continuous stirring, and 5 mL sample was placed in a plastic bottle and stored at –30 °C. Sample preparation and VFA analysis were performed as previously described (Wischer et al. 2013). In brief, samples were collected from the fridge, thawed, stirred, and centrifuged at 4,000 *g* for 5 min. The supernatant of one milliliter was shifted into the Erlenmeyer flask and was added to the standard solution (0.1 mL internal; 80 mM 2-methyl valeric acid in 50% formic acid). Consequently, the samples were retained in an alcohol bath to maintain a temperature of -20° C and vacuum distilled. The VFA were determined using a gas chromatograph (Hewlett-Packard 6890; Agilent, Waldbronn, Germany) equipped with a flame ionisation detector and an HP-FFAP fused silica capillary column (25 m × 0.32 mm, film thickness 0.5 µm, HP 7683; Agilent). Individual amounts of VFA were adjusted for the VFA contained in the blanks. Total VFA (mmol/L) was the sum of acetate, propionate, iso-butyrate, butyrate, isovalerate, and valerate (mmol/L).

3.4. Study 4: Formulating total mixed ration (TMR)

When animals are fed TMR with different roughages and concentrates, digestive interactions can occur among the substances (Niderkorn and Baumont 2009, Ling Sun et al. 2020, Jin et al. 2021). These interactions are called associative effects and can modify the metabolic processes in the digestive tract, especially in the rumen; thus, the animal response to the combination of roughages can differ from the individual feed. This response can positively impact nutrient utilisation by animals, CH_4 production, and digestibility.

Associative effects of roughage and concentrate supplements have been reported in the literature (Berge and Dulphy 1985, Huhtanen 1991, Doyle et al. 2005). For example, the concentrate feed contains high quantities of readily fermentable carbohydrates that decrease the rumen pH, negatively impacting the cellulolytic activities and subsequently digestibility of plant cell walls (Mould et al. 1983). These consequences are called negative associative effects. However, associative effects can also be positive; for instance, straw with protein supplementation may improve digestibility and voluntary intake, thus enhancing N deficiency and promoting the growth of rumen microbes (Church and Santos 1981, Mawuenyegah et al. 1997). In addition, forage legumes containing secondary metabolites may produce positive digestive interactions (Niderkorn and Baumont 2009) and reduce CH_4 production.

Based on this background information, the hypothesis of Study 4 was that TMR formulation could produce associative beneficial effects. The objective of this *in vitro* study was to assess how different TMR rank in regard to associative effects on ruminal fermentation characteristics and the reduction of enteric CH_4 production.

3.4.1. *In vitro* studies and determination of nutritive values

3.4.1.1. Animals and diet

Fresh rumen fluid was collected from two ruminally fistulated Jersey cows that were operated on as donor animals. The research was conducted over six weeks, and the cows were offered a TMR, as described in Study 3.

3.4.1.2. Design of the experiment

Twenty-four TMR were formulated (6 TMR for each roughage). A total of 200 mg feed sample was used, which consisted of 60% supplemented roughage from the selected four combinations of Study 2 (60% rice straw + 40% I_P, 90% German grass + 10% I_P, 70% Napier silage + 30% I_P, and 70% maize silage + 30% I_P) and 40% different combinations of concentrates (60% rice bran + 10% wheat bran + 30% kashari bran, 30% rice bran + 10% wheat bran + 60% kashari bran, 10% rice bran + 60% wheat bran + 30% kashari bran, 10% rice bran + 30% wheat bran + 60% kashari bran, 60% rice bran + 30% wheat bran + 10 kashari bran, 30% rice bran + 60% wheat bran + 10% kashari bran) for each roughage. The combination of feeds is shown in Table 3.6–3.9.

Table 3.6: Composition of rice straw-based TMR (RS$_{\text{TMR}}$) in Study 4 and calculated nutritional values

Ingredients	**RS$_{\text{TMR}}$**					
	RS$_{\text{TMR}}$-1	**RS$_{\text{TMR}}$-2**	**RS$_{\text{TMR}}$-3**	**RS$_{\text{TMR}}$-4**	**RS$_{\text{TMR}}$-5**	**RS$_{\text{TMR}}$-6**
60% RS$_{\text{MF}}$ (mg)						
Rice straw	72	72	72	72	72	72
Ipil-ipil	48	48	48	48	48	48
40% from concentrates (mg)						
Rice bran	48	24	8	8	48	24
Wheat bran	8	8	48	24	24	48
Kashari bran	24	48	24	48	8	8
mg of CP in 200 mg	26.8	28.9	29.0	29.8	26.3	27.6
CP (% of DM)	**13.4**	**14.5**	**14.5**	**14.9**	**13.1**	**13.8**
GE (MJ/kg DM)	**18.7**	**18.4**	**18.5**	**18.3**	**18.8**	**18.7**
ME (MJ/kg DM)	**6.30**	**7.00**	**7.80**	**7.60**	**6.50**	**7.30**

RS$_{\text{MF}}$, Rice straw-based mixed feed (60% rice straw, 40% Ipil-ipil); **CP**, Crude protein; **GE**, Gross energy, **ME**, Metabolisable energy; **RB**, Rice bran; **WB**, Wheat bran; **KB**, Kashari bran; **RS$_{\text{TMR}}$-1**, 60% RS$_{\text{MF}}$ + 40% concentrates (60% RB + 10% WB + 30% KB); **RS$_{\text{TMR}}$-2**, 60% RS$_{\text{MF}}$ + 40% concentrates (30% RB + 10% WB + 60% KB); **RS$_{\text{TMR}}$-3**, 60% RS$_{\text{MF}}$ + 40% concentrates (10% RB + 60% WB + 30% KB); **RS$_{\text{TMR}}$-4**, 60% RS$_{\text{MF}}$ + 40% concentrates (10% RB + 30% WB + 60% KB); **RS$_{\text{TMR}}$-5**, 60% RS$_{\text{MF}}$ + 40% concentrates (60% RB + 30% WB + 10% KB); **RS$_{\text{TMR}}$-6**, 60% RS$_{\text{MF}}$ + 40% concentrates (30% RB + 60% WB + 10% KB).

Table 3.7: Composition of German grass-based TMR (GG_{TMR}) in Study 4 and calculated nutritional values

Ingredients	GG_{TMR}					
	GG_{TMR}-1	**GG_{TMR}-2**	**GG_{TMR}-3**	**GG_{TMR}-4**	**GG_{TMR}-5**	**GG_{TMR}-6**
60% GG_{MF} (mg)						
German grass	108	108	108	108	108	108
Ipil-ipil	12	12	12	12	12	12
40% from concentrates (mg)						
Rice bran	48	24	8	8	48	24
Wheat bran	8	8	48	24	24	48
Kashari bran	24	48	24	48	8	8
mg of CP in 200 mg	28.2	30.3	30.4	31.2	27.7	29.0
CP (% of DM)	**14.1**	**15.2**	**15.2**	**15.6**	**13.8**	**14.5**
GE (MJ/kg DM)	**18.2**	**17.9**	**18.0**	**17.8**	**18.3**	**18.2**
ME (MJ/kg DM)	**7.00**	**7.60**	**8.40**	**8.20**	**7.10**	**8.00**

GG_{MF}, German grass-based mixed feed (90% German grass, 10% Ipil-ipil); **CP**, Crude protein; **GE**, Gross energy; **ME**, Metabolisable energy; **RB**, Rice bran; **WB**, Wheat bran; **KB**, Kashari bran; **GG_{TMR}-1**, 60% GG_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); **GG_{TMR}-2**, 60% GG_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); **GG_{TMR}-3**, 60% GG_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); **GG_{TMR}-4**, 60% GG_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); **GG_{TMR}-5**, 60% GG_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); **GG_{TMR}-6**, 60% GG_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB).

Table 3.8: Composition of Napier silage-based TMR (NS_{TMR}) in Study 4 and calculated nutritional values

Ingredients	NS_{TMR}					
	NS_{TMR}-1	**NS_{TMR}-2**	**NS_{TMR}-3**	**NS_{TMR}-4**	**NS_{TMR}-5**	**NS_{TMR}-6**
60% NS_{MF} (mg)						
Napier silage	84	84	84	84	84	84
Ipil-ipil	36	36	36	36	36	36
40% from concentrates (mg)						
Rice bran	48	24	8	8	48	24
Wheat bran	8	8	48	24	24	48
Kashari bran	24	48	24	48	8	8
mg of CP in 200 mg	27.8	29.9	30.0	30.7	27.2	28.5
CP (% of DM)	**13.9**	**14.9**	**15.0**	**15.4**	**13.6**	**14.3**
GE (MJ/kg DM)	**18.6**	**18.3**	**18.5**	**18.3**	**18.7**	**18.6**
ME (MJ/kg DM)	**7.10**	**7.70**	**8.50**	**8.30**	**7.20**	**8.10**

NS_{MF}, Napier silage-based mixed feed (70% Napier silage, 30% Ipil-ipil); **CP**, Crude protein; **GE**, Gross energy; **ME**, Metabolisable energy; **RB**, Rice bran; **WB**, Wheat bran; **KB**, Kashari bran; **NS_{TMR}-1**, 60% NS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); **NS_{TMR}-2**, 60% NS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); **NS_{TMR}-3**, 60% NS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); **NS_{TMR}-4**, 60% NS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); **NS_{TMR}-5**, 60% NS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); **NS_{TMR}-6**, 60% NS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB).

Table 3.9: Composition of maize silage-based TMR (MS$_{\mathrm{TMR}}$) in Study 4 and calculated nutritional values

Ingredients	**MS$_{\mathrm{TMR}}$**					
	MS$_{\mathrm{TMR}}$-1	**MS$_{\mathrm{TMR}}$-2**	**MS$_{\mathrm{TMR}}$-3**	**MS$_{\mathrm{TMR}}$-4**	**MS$_{\mathrm{TMR}}$-5**	**MS$_{\mathrm{TMR}}$-6**
60% MS$_{\mathrm{MF}}$ (mg)						
Maize silage	84	84	84	84	84	84
Ipil-ipil	36	36	36	36	36	36
40% from concentrates (mg)						
Rice bran	48	24	8	8	48	24
Wheat bran	8	8	48	24	24	48
Kashari bran	24	48	24	48	8	8
mg of CP in 200 mg	28.8	30.9	31.0	31.8	28.2	29.6
CP (% of DM)	**14.4**	**15.4**	**15.5**	**15.9**	**14.1**	**14.8**
GE (MJ/kg DM)	**19.2**	**19.0**	**19.1**	**18.9**	**19.4**	**19.3**
ME (MJ/kg DM)	**6.80**	**7.50**	**8.30**	**8.10**	**7.00**	**7.80**

MS$_{\mathrm{MF}}$, Maize silage-based mixed feed (70% Maize silage, 30% Ipil-ipil); **CP**, Crude protein, **GE**, Gross energy; **ME**, Metabolisable energy; **RB**, Rice bran; **WB**, Wheat bran; **KB**, Kashari bran; **MS$_{\mathrm{TMR}}$-1**, 60% MS$_{\mathrm{MF}}$ + 40% concentrates (60% RB + 10% WB + 30% KB); **MS$_{\mathrm{TMR}}$-2**, 60% MS$_{\mathrm{MF}}$ + 40% concentrates (30% RB + 10% WB + 60% KB); **MS$_{\mathrm{TMR}}$-3**, 60% MS$_{\mathrm{MF}}$ + 40% concentrates (10% RB + 60% WB + 30% KB); **MS$_{\mathrm{TMR}}$-4**, 60% MS$_{\mathrm{MF}}$ + 40% concentrates (10% RB + 30% WB + 60% KB); **MS$_{\mathrm{TMR}}$-5**, 60% MS$_{\mathrm{MF}}$ + 40% concentrates (60% RB + 30% WB + 10% KB); **MS$_{\mathrm{TMR}}$-6**, 60% MS$_{\mathrm{MF}}$ + 40% concentrates (30% RB + 60% WB + 10% KB).

3.4.1.3. GP kinetics, CH_4 production, and VFA

The GP kinetics procedure was similar to that in Study 1 with some modifications, and three experimental runs with two replicates per sample in each run were performed. Furthermore, each run contained four syringes without feed (blanks), three syringes of roughage standard feed, and three syringes for the concentrate feed standard with known gas production. The CH_4 production procedure was the same as that in Study 1and the VFA production procedure was the same as that in Study 3.

3.4.1.4. Calculation of associative effect

Associative effects were calculated by comparing the measured and estimated values of the compound feed used in this study. The measured values were from the fermentation of the TMR and the estimated values were the weighted mean of the values for the fermentation of the individual feeds obtained in Study 1. The percentage differences between the measured and estimated values were calculated as follows: % difference = 100 × [(measured value – estimated value)/ estimated value].

Positive or negative values indicate positive or negative associative effects between the ingredients of the mixed diet.

3.5 Statistical analysis

Analysis of variance for chemical analysis of different feedstuffs, dOM, ME, NEL, CH_4 production, and CH_4 concentration, CH_4/dOM, and CH_4/DM was carried out subjecting one-factorial analysis of PROC MIXED of SAS (SAS Institute Inc., Cary, NC, USA). Significant differences between individuals means were tested by using Duncan's Multiple Range Test, and differences among means at 5% level of significance were accepted as significant. The Pearson method was used to calculate the correlation coefficients among the variables and the CH_4 production. The stepwise multiple regression method was used to develop prediction equations using chemical constituents and gas production of 24 h incubation with observations 72 (12 feedstuffs with 6 replications) for each variable/estimate against 24 h CH_4 production (mL/120mg DM).

A paired t-test was used to compare the associative effect. Estimated and measured values of composite feeds were regressed using the procedure of REG (version 9.4 of SAS system for Windows SAS Institute, Cary, NC, USA). The REG procedure was also used to calculate if slope and intercepts were significantly different from 1 and 0, respectively, by determining 95% CI to detect a possible associative effect.

4. RESULTS

4.1. Screening and characterization of single feedstuffs

4.1.1. Nutrient composition and gross energy (GE) of single feedstuffs

The nutrient concentrations and GE of the studied feedstuffs are listed in Table 4.1. Among the roughages, CP concentration was the highest in leguminous fodder (166–314 g/kg DM), followed by common grasses (52–147 g/kg DM) and silages (94–106 g/kg DM), while concentrations of ADF, NDF, and ADL were the lowest in leguminous fodder. Ipil-ipil contained the highest concentration of CP (314 g/kg DM) and the lowest concentrations of NDF, ADF, and ADL (380 g/kg DM, 187 g/kg DM, and 74 g/kg DM). The opposite trend was observed for crop residues, which demonstrated the lowest CP concentration (44–70 g/kg DM) and the highest concentrations of fibre fractions. The CA concentration was the highest in German grass (155 g/kg DM), and maize stover contained the lowest amount of CA (64.2 g/kg DM). The GE concentration of leguminous fodder was higher than that of crop residues, common grass, and concentrates. For concentrates, kashari bran contained the highest CP (163 g/kg DM) and the lowest ADL (38.4 g/kg DM), whereas rice bran showed the opposite trend, with the lowest CP (75.1 g/kg DM) and the highest ADL (144 g/kg DM).

Table 4.1: Analysed nutrient composition and gross energy of single feedstuffs

Feed-stuff group	Name of feed sample	DM (%)	Chemical fractions (g/kg DM)							GE (MJ/kg DM)
			CA	CL	CP	CF	NDF	ADF	ADL	
Roughages	**Crop residues**									
	Rice straw	93.7	136	6.0	44.3	190	743	547	41.6	16.9
	UMS	94.1	141	5.9	69.7	136	580	424	28.6	16.7
	Maize stover	94.7	64.2	8.1	54.1	146	682	432	44.6	18.6
	Silage									
	Maize silage	95.7	80.9	8.5	107	159	712	484	54.3	18.9
	Napier silage	96.1	71.0	8.3	93.7	206	771	559	84.3	18.8
	Common grasses									
	Napier grass	95.2	87.9	10.2	52.2	188	721	528	75.5	18.3
	German grass	94.6	155	11.3	147	163	626	485	67.6	17.4
	Para grass	96.1	90.7	8.7	67.4	173	695	486	77.1	16.6
	Leguminous fodder									
	Gliricidia	90.7	117	24.7	239	140	524	500	301	20.2
	Ipil-ipil	92.7	92.1	38.4	314	70.3	380	187	74.2	21.1
	Alfalfa hay	93.1	106	14.0	166	189	586	536	122	18.8
	Moringa tops	95.7	66.0	14.0	125	175	563	497	83.4	19.1
Concentrates	Crushed maize	91.3	23.3	20.9	83.9	5.4	86.4	34.5	10.9	19.7
	Crushed wheat	91.5	15.7	62.2	118	8.1	121	40.2	9.10	18.8
	Wheat bran	90.9	41.6	18.7	131	39.8	276	167	44.3	18.7
	Kashari bran	90.9	111	25.4	163	95.8	409	312	38.4	17.3
	Rice bran	93.5	143	47.3	75.1	188	636	533	144	19.5
	M. oil cake	94.5	389	83.6	104	54.7	462	363	57.2	13.3

UMS, Urea molasses treated rice straw; M. oil cake, Mustard oil cake; DM, Dry matter; CA, Crude ash; CL, Crude fat; CP, Crude protein; CF, Crude fibre; NDF, Neutral detergent fibre; ADF, Acid detergent fibre; ADL, Acid detergent lignin; and GE, Gross energy.

4.1.2. *In vitro* ruminal fermentation characteristics and energy value of single feedstuffs

The *in vitro* gas production at 24 h (GP_{24}) and other kinetic parameters are listed in Table 4.2. Within roughages, crop residues of maize stover, common grasses of German grass, and Napier grass produced significantly ($p<0.05$) higher volumes of gas at 24 h (34.0 mL/200 mg DM, 33.7 mL/200 mg DM, and 32.8 mL/200 mg DM, respectively) than that of other roughages. The lowest GP_{24} among the roughages was observed in *Gliricidia* with 15.2 mL/200 mg DM. The highest potential gas production (*p*GP) was recorded for rice straw (60.0 mL/200 mg DM), which was significantly ($p<0.05$) greater than that of all roughages. The leguminous fodder of *Moringa* tops showed the same trend of gas production rate constant (*c*GP) among all roughages ($p<0.05$), which was 12.1% per hour (h). Among the concentrates, the crushed maize and crushed wheat showed the highest GP_{24} (78.9 mL/200 mg DM and 74.9 ml/200 mg DM) and *p*GP (95.3 mL/200 mg DM and 93.6 ml/200 mg DM), whereas the lowest GP_{24} and *p*GP were obtained for rice bran with 13.5 mL/200 mg DM and 16.3 mL/200 mg DM.

The calculated values of dOM, ME, and NEL for the different feedstuffs are listed in Table 4.3. All roughages had different dOM, ME, and NEL, with German grass (61.6%, 7.43 MJ ME/kg DM, and 4.21 MJ NEL/kg DM, respectively) and Ipil-ipil (58.9%, 8.22 MJ ME/kg DM, and 4.65 MJ NEL/kg DM, respectively) exerting the significantly highest, and rice straw the lowest values (47.9%, 5.32 MJ ME/kg DM, and 2.72 MJ NEL/kg DM, respectively). The dOM, ME and NEL of crushed maize (90.3%, 13.8 MJ ME/kg DM, and 8.83 MJ NEL/kg DM) and crushed wheat (87.8%, 14.4 MJ ME/kg DM, and 9.12 MJ NEL/kg DM) were significantly ($p<0.05$) higher than those of other concentrates. Rice bran (39.5%, 4.78 MJ ME/kg DM, and 2.19 MJ NEL/kg DM) showed the lowest values among all tested feedstuffs.

Table 4.2: *In vitro* gas production kinetics of single feedstuffs

Feedstuff group	Name of feed sample	GP_{24} (mL/200 mg DM)	*p*GP (mL/200 mg DM)	*c*GP (%/h)
Roughages	**Crop residues**			
	Rice straw	25.0^{g}	60.0^{c}	2.02^{k}
	UMS	31.1^{de}	53.0^{de}	3.80^{i}
	Maize stover	34.0^{d}	57.2^{cd}	4.00^{ghi}
	Silage			
	Maize silage	32.5^{de}	53.3^{de}	3.80^{i}
	Napier silage	27.8^{f}	49.8^{ef}	3.03^{j}
	Common grasses			
	German grass	33.7^{d}	51.1^{ef}	3.88^{hi}
	Para grass	32.0^{de}	47.6^{f}	4.68^{f}
	Napier grass	32.8^{d}	49.6^{ef}	4.58^{fg}
	Leguminous fodder			
	Ipil-ipil	27.0^{fg}	34.0^{g}	7.39^{d}
	Gliricidia	15.2^{i}	23.8^{i}	3.98^{ghi}
	Alfalfa hay	21.2^{h}	31.1^{h}	4.55^{fgh}
	Moringa tops	29.5^{ef}	33.4^{gh}	12.1^{b}
Concentrates	Crushed maize	78.9^{a}	95.3^{a}	5.94^{e}
	Crushed wheat	74.9^{a}	93.6^{a}	7.60^{d}
	Wheat bran	60.6^{b}	73.4^{b}	8.50^{c}
	Kashari bran	47.3^{c}	67.7^{b}	4.93^{f}
	Rice bran	13.5^{j}	16.3^{k}	7.65^{d}
	M. oil cake	21.5^{h}	21.5^{j}	21.3^{a}
	SEM	3.61	0.69	0.40
	P-value	<0.05	<0.05	<0.05

M. oil cake, Mustard oil cake; GP_{24}, Gas production at 24 h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; SEM, Standard error of the mean; NS, Not significant; $^{a\text{-}k}$ means with different superscripts in the same row are significantly different at ($p < 0.05$).

Table 4.3: Digestibility of organic matter, metabolisable energy, and net energy lactation of single feedstuffs

Feedstuff group	Name of feed sample	dOM (%)	ME (MJ/kg DM)	NEL (MJ/kg DM)
Roughages	**Crop residues**			
	Rice straw	47.9^{i}	5.32^{l}	2.72^{j}
	UMS	54.9^{f}	6.39^{hi}	3.49^{gh}
	Maize stover	51.6^{h}	6.73^{fgh}	3.74^{fg}
	Silage			
	Maize silage	53.8^{fg}	6.91^{fg}	3.85^{f}
	Napier silage	48.5^{i}	6.13^{i}	3.29^{h}
	Common grasses			
	German grass	61.6^{d}	7.43^{e}	4.21^{e}
	Para grass	52.3^{gh}	6.57^{gh}	3.61^{fg}
	Napier grass	52.1^{gh}	6.60^{gh}	3.64^{fg}
	Leguminous fodder			
	Ipil-ipil	58.9^{e}	8.22^{d}	4.65^{d}
	Gliricidia	46.8^{i}	5.69^{k}	2.87^{ij}
	Alfalfa hay	48.1^{i}	5.80^{j}	3.01^{i}
	Moringa tops	50.9^{h}	6.72^{fgh}	3.69^{fg}
Concentrates	Crushed maize	90.3^{a}	13.8^{a}	8.83^{a}
	Crushed wheat	87.8^{a}	14.4^{a}	9.12^{a}
	Wheat bran	77.3^{b}	11.4^{b}	7.08^{b}
	Kashari bran	71.5^{c}	9.84^{c}	5.92^{c}
	Rice bran	39.5^{j}	4.78^{m}	2.19^{k}
	Mustard oil cake	64.1^{d}	6.98^{f}	3.67^{fg}
	SEM	0.468	0.077	0.055
	P-value	<0.05	<0.05	<0.05

dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; SEM, Standard error of the mean; NS, Not significant; $^{a-m}$ means with different superscripts in the same row are significantly different at ($p < 0.05$).

The cumulative gas production patterns from the *in vitro* fermentation of different roughages and concentrates are shown in figures 3 and 4. The total volume and pattern of gas production varied among all feedstuffs; however, the observed differences were not consistent for the different incubation periods, except for crushed wheat and crushed maize, which consistently produced the highest volume of gas across all incubation times. The lowest gas production was measured for *Gliricidia* and rice bran from 8 to 72 h compared to all other feedstuffs.

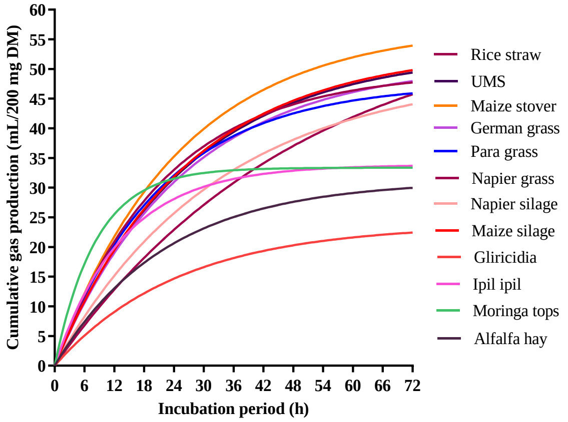

Fig. 3: Cumulative gas production profile of different roughages. The *in vitro* gas production profile has been fitted using the equation $Y = pGP \cdot (1- e^{-cGP \cdot 0.01t})$.

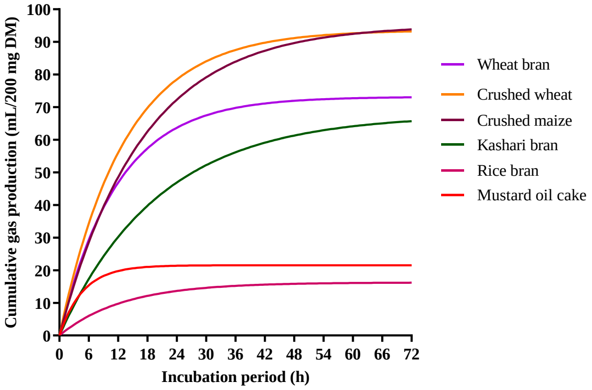

Fig. 4: Cumulative gas production profile of the concentrates. The *in vitro* gas production profile has been fitted using the equation $Y = pGP \cdot (1- e^{-cGP \cdot 0.01t})$.

4.1.3. *In vitro* methane production and related traits

CH_4 production and related traits of the different feedstuffs after *in vitro* incubation with rumen fluid for 24 h are shown in Table 4.4. The values of CH_4 production (0.94 mL/120 mg DM), CH_4 concentration (13.5% of GP), CH_4/dOM (18.7 L CH_4/kg dOM), and CH_4/DM (7.88 L CH_4/kg DM) of rice bran were the lowest, while the loss of energy in the form of CH_4 (13.1% of GE) was the highest ($p<0.05$) compared to all other feedstuffs. *Gliricidia* produced the lowest CH_4 (1.62 mL/120 mg DM), but the CH_4 concentration and CH_4 energy loss were significantly ($p<0.05$) the highest among all roughages. The CH_4 concentrations in total gas of maize silage and Ipil-ipil were significantly ($p<0.05$) lower (14.3% and 14.7%, respectively) than for the other roughages. However, the wheat bran produced 2.11 mL/120 mg DM of CH_4 and lost 1.78% of GE as CH_4, significantly the lowest among all feedstuffs. The opposite trend was recorded in crushed wheat, crushed maize, and kashari bran, which produced the highest concentrations of CH_4 and CH_4/dOM; nevertheless, the GE loss as CH_4 was very low (1.40%, 1.92%, and 2.34%) ($p<0.05$).

Table 4.4: CH_4 production of single feedstuffs

Feed-stuff group	Name of feed sample	CH_4 (mL/120 mg DM)	CH_4 conc. in GP (%)	L CH_4/ kg dOM	L CH_4/ kg DM	CH_4 energy (% GE)
Roughages	**Crop residue**					
	Rice straw	2.79^{efg}	15.9^{cde}	47.3^{cde}	23.3^{fgh}	4.32^{def}
	UMS	3.31^{d}	16.3^{bcd}	49.7^{cde}	27.6^{d}	3.63^{fg}
	Maize stover	3.43^{d}	15.6^{cde}	54.8^{cd}	28.6^{d}	3.50^{g}
	Silages					
	Maize silage	2.95^{def}	14.3^{ef}	44.2^{def}	24.7^{def}	4.08^{efg}
	Napier silage	2.70^{fg}	14.6^{def}	44.6^{def}	22.5^{fgh}	4.46^{de}
	Common grass					
	German grass	3.23^{de}	15.1^{def}	42.9^{def}	26.9^{de}	3.73^{efg}
	Para grass	3.13^{def}	15.6^{cde}	49.3^{cde}	26.1^{def}	3.84^{efg}
	Napier grass	3.29^{d}	15.9^{cde}	51.0^{cde}	27.5^{d}	3.65^{fg}
	Leguminous fodder					
	Ipil-ipil	2.46^{gh}	14.7^{def}	34.8^{fg}	20.5^{gh}	4.86^{d}
	Gliricidia	1.62^{i}	18.2^{ab}	28.8^{gh}	13.5^{j}	7.41^{b}
	Alfalfa hay	2.41^{gh}	17.3^{abc}	41.2^{ef}	20.2^{hi}	4.97^{cd}
	Moriga tops	2.82^{efg}	15.9^{cde}	43.6^{def}	23.5^{efg}	4.29^{def}
Concentrates	Crushed maize	6.36^{b}	14.8^{def}	58.2^{bc}	53.1^{b}	1.92^{hi}
	Crushed wheat	8.54^{a}	18.3^{ab}	81.1^{a}	71.2^{a}	1.40^{i}
	Kashari bran	6.72^{b}	18.7^{a}	58.9^{bc}	56.0^{b}	2.34^{h}
	M. oil cake	5.12^{c}	17.5^{abc}	26.5^{h}	42.7^{c}	5.73^{c}
	Rice bran	0.94^{j}	13.5^{f}	18.7^{i}	7.88^{k}	13.1^{a}
	Wheat bran	2.11^{h}	17.3^{abc}	73.2^{ab}	17.6^{i}	1.78^{hi}
	SEM	0.121	0.489	1.96	1.00	0.13
	P-value	<0.05	<0.05	<0.05	<0.05	<0.05

CH_4, Methane; CH_4 conc., Methane concentration; GP, Gas production at 24 h, dOM, Digestibility of organic matter; DM, Dry matter; GE, Gross energy; SEM, Standard error of the mean; NS, Not significant; $^{a-k}$ means with different superscripts in the same row are significantly different at ($p < 0.05$).

4.1.4. Associations between CH_4 production, chemical constituents, and fermentation characteristics

Across all feedstuffs studied, CH_4 production was negatively correlated with CP (–0.86**), ADF (–0.191), and ADL (–0.36) concentrations of the feedstuffs (Table 4.5). A significant positive correlation was observed between CH_4 production and the NDF concentration (0.67*) and GP (0.94*) at 24 h. The concentrations of other nutrients were not significantly correlated with CH_4 production.

Table 4.5: Pearson correlation between *in vitro* CH_4 production and chemical constituents

Feed fractions	**CH_4**	**Nitrogen and gas production**	**CH_4**
Dry matter (DM)	0.56	Crude protein (CP)	–0.86**
Crude ash (CA)	0.04	Gas production (GP) at 24 h	0.94*
Neutral detergent fiber (NDF)	0.67*		
Acid detergent fiber (ADF)	–0.19		
Acid detergent lignin (ADL)	–0.36		

CH_4, Methane; NS, Not significant. *$p < 0.05$; **$p < 0.01$

Linear regression equations were derived to predict CH_4 production from the chemical constituents and *in vitro* ruminal GP, as shown in Table 4.6. The cell wall fractions alone, including NDF, ADF, and ADL, were poor indicators of CH_4 production (R^2=0.57) in the linear regression ($p<0.06$) for all feedstuffs. The consideration of CP and NDF concentrations as predictors for CH_4 production could improve the equation accuracy ($p<0.01$); however, there was still moderate precision for the prediction of CH_4 production (R^2=0.76). Linear regression also indicated that the CP content alone was a good predictor of CH_4 production (R^2=0.72) and had a significant relationship ($p<0.01$). GP alone showed a positive relationship with CH_4, with an R^2 value of 0.89.

Table 4.6. Linear regression equations to predict CH_4 production by single feedstuffs of Study 1 from chemical constituents and GP_{24}

Equations	R^2	P- value
CH_4= 0.20 NDF + 0.03ADF - 0.66ADL + 14.0	0.57	<0.06
CH_4= - 0.10 NDF - 0.56CP + 37.0	0.76	<0.01
CH_4= - 0.44 CP + 29.1	0.72	<0.01
CH_4= 0.25 NDF + 7.65	0.45	<0.02
CH_4= 0.70 GP_{24} + 3.70	0.89	<0.01

CH_4, Methane (L/kg DM); NDF, Neutral detergent fibre (g/kg DM); ADF, Acid detergent fibre (g/kg DM); CP, Crude protein (g/kg DM); GP_{24}, Gas production at 24 h (mL/200 mg DM).

4.2. Study 2: Combination effects of roughages and supplemented leguminous fodder

4.2.1. Supplementation of Ipil-ipil and *Gliricidia* to rice straw

Supplementation of rice straw with Ipil-ipil and *Gliricidia* affected *in vitro* gas production and related traits, as presented in Table 4.7. The GP_{24} and fermentation rate increased ($p<0.05$) as the level of Ipil-ipil added to the rice straw was increased. Increasing the Ipil-ipil level resulted in a gradual increase in dOM, ME and, NEL ($p<0.05$) of the mix, reaching maximum values (59.1%, 7.60 MJ ME/kg DM, and 4.31MJ NEL/kg DM, respectively) when Ipil-ipil was added at 40% DM. The dOM, ME, and NEL increased more than 15%, 30%, and 40%, respectively, with 0–40% Ipil-ipil in the diet (Fig. 5), which was significant ($p<0.05$). The increasing level of *Gliricidia* in the mix with rice straw gradually decreased the GP_{24} ($p<0.05$). The dOM, ME, and NEL were depressed by the supplementation of rice straw with *Gliricidia* ($p<0.05$).

Interaction effects of Ipil-ipil and *Gliricidia* addition with incubation time on *in vitro* cumulative GP were recorded for the different combinations of rice straw (Fig. 6). From 0 h to 72 h of incubation, the cumulative GP was higher when Ipil-ipil was added to the rice straw, rather than *Gliricidia*. At the end of 72 h of incubation, 40% Ipil-ipil with rice straw produced the highest volume of gas (more than 45 mL/200 mg DM) compared to other combinations, indicating better fermentation of the rice straw.

No significant differences were observed in the CH_4 reduction potential when Ipil-ipil and *Gliricidia* were added to rice straw (Table 4.8). However, the CH_4 concentration and CH_4/dOM decreased gradually with the increase in Ipil-ipil to the rice straw by a trend ($p>0.05$); the lowest CH_4 concentration was recorded (14.0% in GP) when the diet contained 40% Ipil-ipil. Maximal reductions in CH_4 concentration and CH_4/dOM were observed (6.89% and 11.7% relative to the control) when 40% Ipil-ipil was added to the rice straw ($p<0.05$) (Fig. 7). *Gliricidia* did not cause any significant differences in CH_4 production ($p>0.05$).

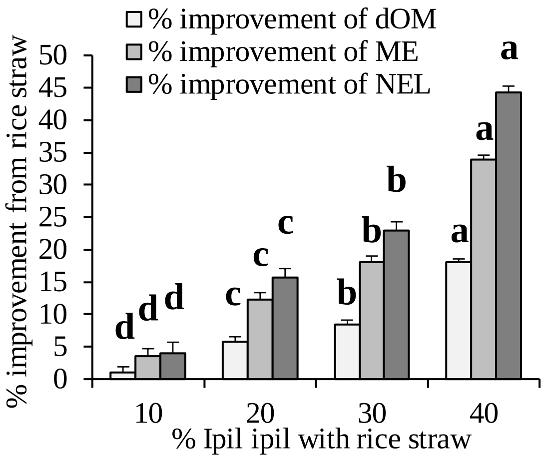

Fig. 5: % improvement of dOM, ME and NEL with the supplementation of Ipil-ipil to the rice straw

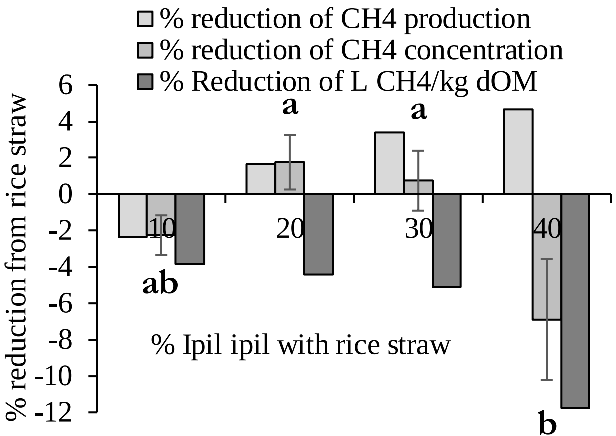

Fig. 7: % reduction of CH_4 production, CH_4 concentration, and CH_4/dOM with the supplementation of ipil-ipil to the rice straw

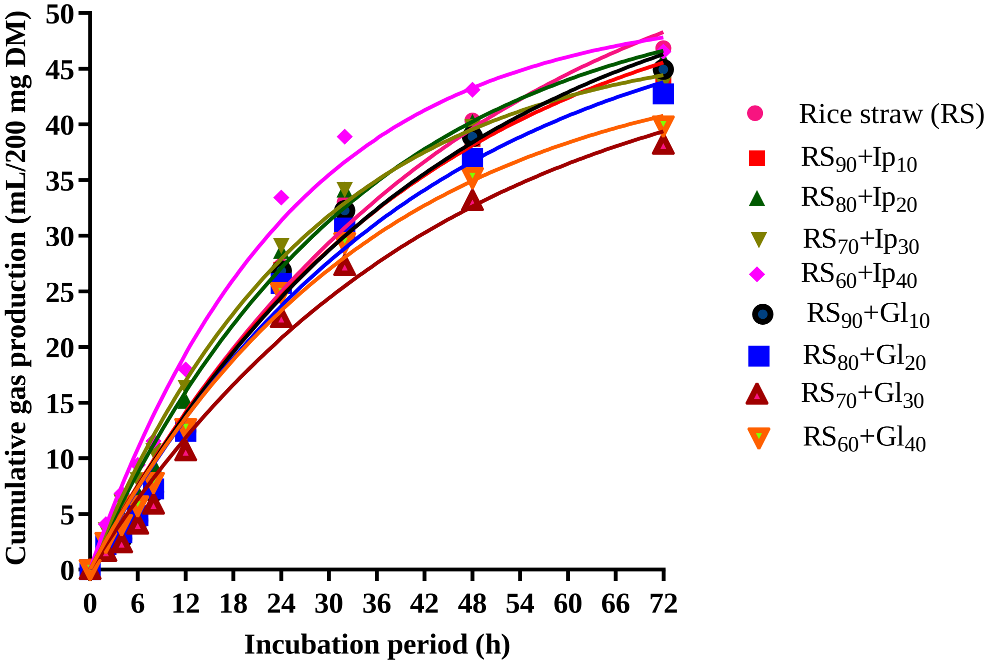

Fig. 6: Cumulative gas production profile of different combinations of rice straw. The *in vitro* gas production profile has been fitted using the equation $Y = p\mathrm{GP}\cdot(1 - e^{-c\mathrm{GP}\cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in Table 4.7.

Table 4.7: ***In vitro*** **ruminal fermentation characteristics of different combinations of Ipil-ipil and** ***Gliricidia*** **with rice straw**

Parameters	Ipil-ipil (%)					SEM	Sig.	*Gliricidia* (%)					SEM	Sig.
	0	10	20	30	40			0	10	20	30	40		
GP_{24} (mL/200 mg DM)	27.3^{a}	27.0^{a}	28.6^{a}	29.0^{a}	33.4^{b}	0.73	$P<0.05$	27.3^{c}	26.9^{bc}	25.7^{bc}	22.6^{a}	24.9^{ab}	0.73	$P<0.05$
*p*GP (mL/200 mg DM)	61.6^{c}	55.2^{b}	52.8^{ab}	48.1^{a}	50.7^{b}	0.10	$P<0.05$	61.6^{d}	57.2^{cd}	52.7^{bc}	48.6^{ab}	47.3^{a}	0.10	$P<0.05$
*c*GP (%/h)	2.21^{a}	2.49^{b}	3.01^{bc}	3.70^{d}	4.08^{d}	0.17	$P<0.05$	2.21^{a}	2.34^{ab}	2.53^{ab}	2.34^{ab}	2.91^{b}	0.17	$P<0.05$
dOM (%)	49.9^{a}	50.6^{a}	53.0^{bc}	54.3^{c}	59.1^{d}	0.65	$P<0.05$	49.9^{ab}	50.3^{ab}	50.1^{ab}	48.1^{a}	50.8^{b}	0.65	$P<0.05$
ME (MJ/kg DM)	5.66^{a}	5.88^{a}	6.38^{b}	6.69^{b}	7.60^{c}	0.10	$P<0.05$	5.66^{ab}	5.77^{ab}	5.78^{ab}	5.52^{a}	6.02^{b}	0.10	$P<0.05$
NEL (MJ/kg DM)	2.97^{a}	3.11^{a}	3.45^{b}	3.66^{b}	4.31^{c}	0.07	$P<0.05$	2.91	3.04	3.04	2.83	3.18	0.07	NS

GP_{24}, Gas production at 24 h; *p*GP, Potential gas production; *c*GP; Gas production rate constant, dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; SEM, Standard error of the mean; NS, Not significance; $^{a-d}$ means with different superscripts in the same row are significantly different at ($P< 0.05$).

Table 4.8: CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM of different combinations of Ipil-ipil and *Gliricidia* with rice straw

Variables	Ipil-ipil (%)					SEM	Sig.	*Gliricidia* (%)					SEM	Sig.
	0	10	20	30	40			0	10	20	30	40		
CH_4 mL/120 mg DM	2.42	2.33	2.43	2.47	2.50	0.09	NS	2.42	2.34	2.30	2.11	2.16	0.08	NS
CH_4 conc. in GP (%)	15.1	14.7	15.4	15.2	14.0	0.42	NS	15.1	14.9	15.2	14.7	15.1	0.40	NS
L CH_4/kg dOM	40.4	38.9	38.2	37.9	35.4	1.45	NS	40.4	38.8	38.4	36.5	35.4	1.38	NS
L CH_4/kg DM	20.2	19.5	20.3	20.6	20.9	0.77	NS	20.2	19.5	19.2	17.6	17.9	0.74	NS

CH_4, Methane; CH_4 conc., Methane concentration; dOM, Digestibility of organic matter; DM, Dry matter; SEM, Standard error of the mean; NS, Not significance.

The associative effects calculated from the estimated and measured values of Ipil-ipil and *Gliricidia* with the ruminal fermentation characteristics and CH_4 reduction potential of rice straw are presented in Table 4.9. There were significant differences between the estimated and measured values of dOM, ME, and NEL for all mixed treatments ($p<0.05$), indicating a positive associative effect. The measured dOM, ME, and NEL values were consistently higher than the estimated values with the supplementation of rice straw with Ipil-ipil and *Gliricidia*. Adding 40% Ipil-ipil to rice straw, showed the maximum associative effect of dOM, ME, and NEL (13.3%, 17.6%, and 23.8%, respectively) among all treatments ($p<0.05$). There was no significant reduction in the estimated and measured values of CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM for all combinations of rice straw. However, the CH_4 concentration was reduced by 8.43% and had the highest CH_4 reduction potential among all treatments ($p>0.05$) when 40% Ipil-ipil was added.

Linear regression analysis was performed between the estimated values (calculated from the single feeds) and the measured values. The results of the linear regression analysis of dOM, ME, NEL, CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM are presented in Table 4.10. The measured dOM of Ipil-ipil differed (3.54 to 13.3%) from estimated values, and the estimated slope of regression was 2.43, which was associated with a large CI and R^2 value of 0.94 (Fig. 8) and is thus a reliable model for further forecasts. The regression line slopes of ME and NEL were 1.88 and 2.0, respectively, and regression equations showed higher R^2 values (0.95 for both ME and NEL, respectively) when Ipil-ipil was added (Fig. 9). However, the R^2 value was very low (0.09 and 0.08 for ME and NEL, respectively) when *Gliricidia* was added to the rice straw. The slopes of CH_4 production and CH_4/DM of Ipil-ipil were –1.63 and –1.73, and the R^2 value was close to 1 (0.88 and 0.94, respectively) (Fig. 10).

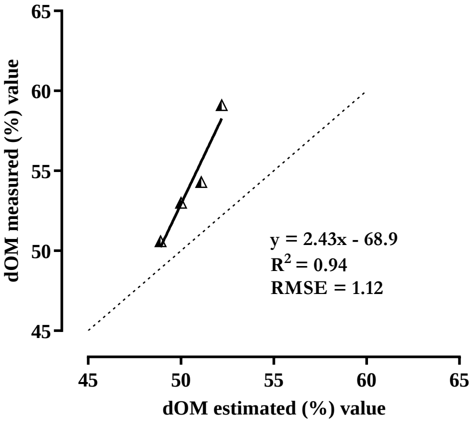

Fig. 8: Comparison of estimated and measured digestibility of organic matter (dOM) values of Ipil-ipil with rice straw using an *in vitro* ruminal fermentation technique. The dOM values of combind feeds were calculated from dOM values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

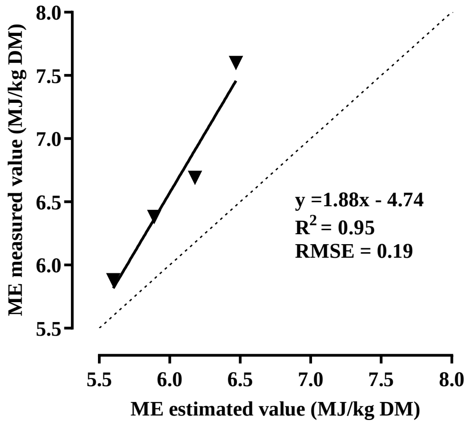

Fig. 9: Comparison of estimated and measured metabolisable energy (ME) values of Ipil-ipil with rice straw using an *in vitro* ruminal fermentation technique. The ME values of combind feeds were calculated from ME values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

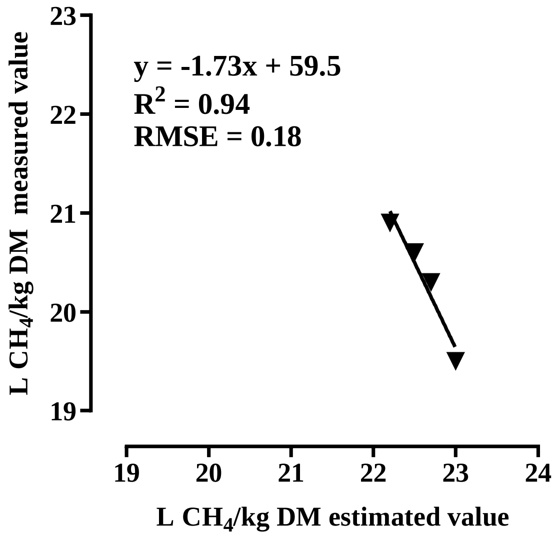

Fig. 10: Comparison of estimated and measured L CH_4/kg DM values of Ipil-ipil with rice straw using an *in vitro* ruminal fermentation technique. The L CH_4/kg DM value of combind feeds were calculated from L CH_4/kg DM values of single feeds.

Table 4.9: Associative effect of Ipil-ipil and *Gliricidia* on *in vitro* ruminal fermentation characteristics and CH_4 reduction potential of rice straw

Parameters	Ipil-ipil (%)				SEM	Sig.	*Gliricidia* (%)				SME	Sig.
	10	**20**	**30**	**40**			**10**	**20**	**30**	**40**		
dOM (%)												
Estimated*	48.9	50.0	51.1	52.2			47.7	47.6	47.5	47.4		
Measured	50.6	53.0	54.3	59.1			50.3	50.1	48.1	50.8		
Associative effect (%)	3.50^{c}	6.01^{bc}	6.13^{b}	13.3^{a}	0.99	P<0.05	5.50^{ab}	5.18^{ab}	1.24^{b}	7.24^{a}	1.52	P<0.05
ME (MJ/kg DM)												
Estimated*	5.60	5.89	6.18	6.47			5.35	5.38	5.42	5.46		
Measured	5.88	6.38	6.69	7.60			5.77	5.78	5.52	6.02		
Associative effect (%)	5.01^{b}	8.37^{b}	8.32^{b}	17.6^{a}	1.37	P<0.05	8.04^{a}	7.51^{a}	1.79^{b}	10.3^{a}	2.06	P<0.05
NEL (MJ/kg DM)												
Estimated*	2.91	3.10	3.29	3.48			2.73	2.75	2.76	2.78		
Measured	3.11	3.45	3.66	4.31			3.04	3.04	2.83	3.18		
Associative effect (%)	7.03^{b}	11.6^{b}	11.4^{b}	23.8^{a}	1.91	P<0.05	11.5^{a}	10.7^{ab}	2.56^{b}	14.8^{a}	3.13	P<0.05
CH4 production (mL)												
Estimated$^{\psi}$	2.76	2.73	2.70	2.66			2.67	2.56	2.44	2.32		
Measured	2.33	2.43	2.47	2.50			2.34	2.30	2.11	2.16		
Associative effect (%)	-15.2	-10.7	-8.10	-5.88	4.15	NS	-12.3	-9.68	-13.6	-7.06	5.86	NS
CH4 concentration (%)												
Estimated$^{\psi}$	15.6	15.5	15.4	15.3			16.0	16.2	16.5	16.7		
Measured	14.7	15.4	15.2	14.0			14.9	15.2	14.7	15.1		
Associative effect (%)	-5.67	-1.18	-1.55	-8.43	2.75	NS	-6.35	-6.65	-10.6	-10.1	5.10	NS
L CH4/kg dOM												
Estimated$^{\psi}$	47.3	45.9	44.6	43.2			46.7	44.7	42.7	40.7		
Measured	38.9	38.2	37.9	35.4			38.8	38.4	36.5	35.4		
Associative effect (%)	-18.6	-16.7	-14.7	-18.3	3.39	NS	-16.8	-14.0	-14.5	-13.1	5.62	NS
L CH4/kg DM												
Estimated$^{\psi}$	23.0	22.7	22.5	22.2			22.3	21.3	20.3	19.4		
Measured	19.5	20.3	20.6	20.9			19.5	19.2	17.6	17.9		
Associative effect (%)	-15.2	-10.7	-8.11	-5.89	4.12	NS	-12.3	-9.68	-13.6	-7.07	5.85	NS

dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH_4, Methane; SEM, Standard error of the mean; NS, Not significance; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p < 0.05$) *, The estimated values were calculated from single feeds that were determined by the equation of Menke and Steingass (1988); $^{\psi}$, The estimated values were calculated from single feeds.

Table 4.10: Results of linear regression of estimated and measured values of *in vitro* ruminal fermentation characteristics and CH_4 reduction of rice straw

Parameters	**Ipil-ipil (†estimated v. measured)**					RMSE	***Gliricidia* (†estimated v. measured)**					RMSE
	Slope	Slope CI	Intercept	Intercept CI	R^2		Slope	Slope CI	Intercept	Intercept CI	R^2	
dOM (%)	2.43	0.48 to 4.39	-68.9	-168 to 29.9	0.94	1.12	0.50	-27.4 to 28.4	26.1	-1302 to 1354	0.00	1.45
ME MJ/kg DM	1.88	0.61 to 3.15	-4.74	-12.4 to 2.92	0.95	0.19	1.31	-11.1 to 13.7	-1.31	-68.1 to 65.5	0.09	0.23
NEL MJ/kg DM	2.00	0.62 to 3.38	-2.76	-7.19 to 1.65	0.95	0.14	2.07	-18.6 to 22.7	-2.69	-59.7 to 54.3	0.09	0.17
CH_4 mL/120 mg DM	-1.63	-3.43 to 0.17	6.86	1.96 to 11.7	0.88	0.03	0.65	-0.53 to 1.84	0.59	-2.38 to 3.56	0.74	0.07
CH_4 conc. in GP (%)	2.30	-10.6 to 15.2	-20.7	-221 to 179	0.59	0.67	-0.13	-2.21 to 1.93	17.3	-16.6 to 51.1	0.04	0.25
L CH_4/kg dOM	0.73	-0.61 to 2.07	4.48	-56.2 to 65.2	0.73	0.94	0.61	0.18 to 1.02	10.8	-7.68 to 29.4	0.95	0.43
L CH_4/kg DM	-1.73	-3.05 to -0.4	59.5	29.8 to 89.3	0.94	0.17	0.63	0.47 to 1.74	5.33	-17.8 to 28.5	0.75	0.55

dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH_4 conc., Methane concentration; †The estimated values of CH_4 related traits were calculated from the single feeds and using the equation of Menke and Steingass (1988) for determining the ruminal fermentation characteristics (dOM, ME, and NEL) from single feeds, which consistently indicated X-variables.

4.2.2. Supplementation of Ipil-ipil and *Gliricidia* to German grass

No differences were observed in *in vitro* GP_{24}, and dOM increased with the addition of Ipil-ipil to German grass ($p>0.05$) (Table 4.11). Nevertheless, the *p*GP, *c*GP, ME, and NEL were significantly different ($p<0.05$) when Ipil-ipil was added to German grass. The value of ME and NEL was the highest (8.09 MJ ME/kg DM, 4.65 MJ NEL/kg DM, respectively) when 30% Ipil-ipil was added to the German grass and significantly differed from the control group ($p<0.05$), but there were no significant differences among 10%, 30%, and 40% Ipil-ipil additions to German grass. When greater proportions of *Gliricidia* were added to German grass, GP_{24}, *p*GP, *c*GP, dOM, ME, and NEL significantly decreased with respect to the treatment without the addition of *Gliricidia*. Improvement of more than 4% dOM was observed when 10% Ipil-ipil was added to the German grass, significantly ($p<0.05$) differing from the other combinations. However, ME and NEL did not differ significantly from the control (Fig. 11).

Interaction effects between the different combinations of German grass and incubation period were observed on the *in vitro* cumulative GP (Fig. 12), as it was presented before for rice straw. After 72 h of incubation, German grass with 10% Ipil-ipil produced the highest amount of gas (approximately 50 mL/200 mg DM) compared to other combinations.

The CH_4 concentration, CH_4/dOM, and CH_4/DM were not affected by the addition of Ipil-ipil and *Gliricidia* to German grass (Table 4.12). As the level of Ipil-ipil increased linearly, a significant ($p<0.05$) decline was found in CH_4 production compared to the control. The same trend was observed for CH_4/DM ($p<0.05$) when *Gliricidia* was added to German grass. Maximal reductions in CH_4 concentration and CH_4/dOM (more than 18%) was marked when 40% Ipil-ipil was added to the German grass ($p<0.05$) (Fig. 13).

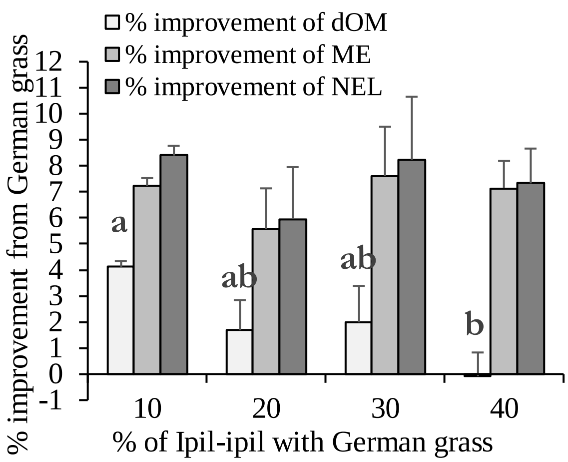

Fig. 11: % improvement of dOM, ME and NEL with the supplementation of Ipil-ipil to the German grass

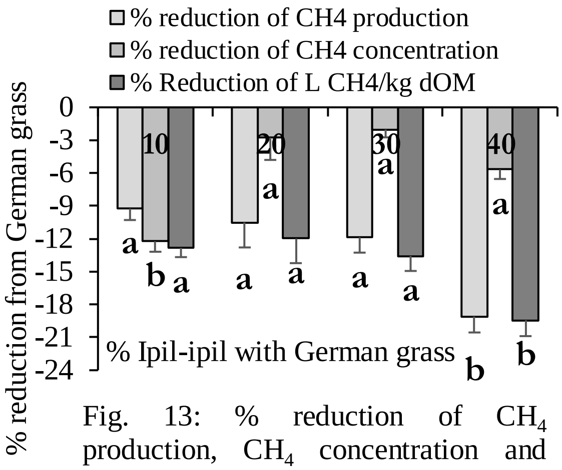

Fig. 13: % reduction of CH_4 production, CH_4 concentration and CH_4/dOM with the supplementation of Ipil-ipil to the German grass

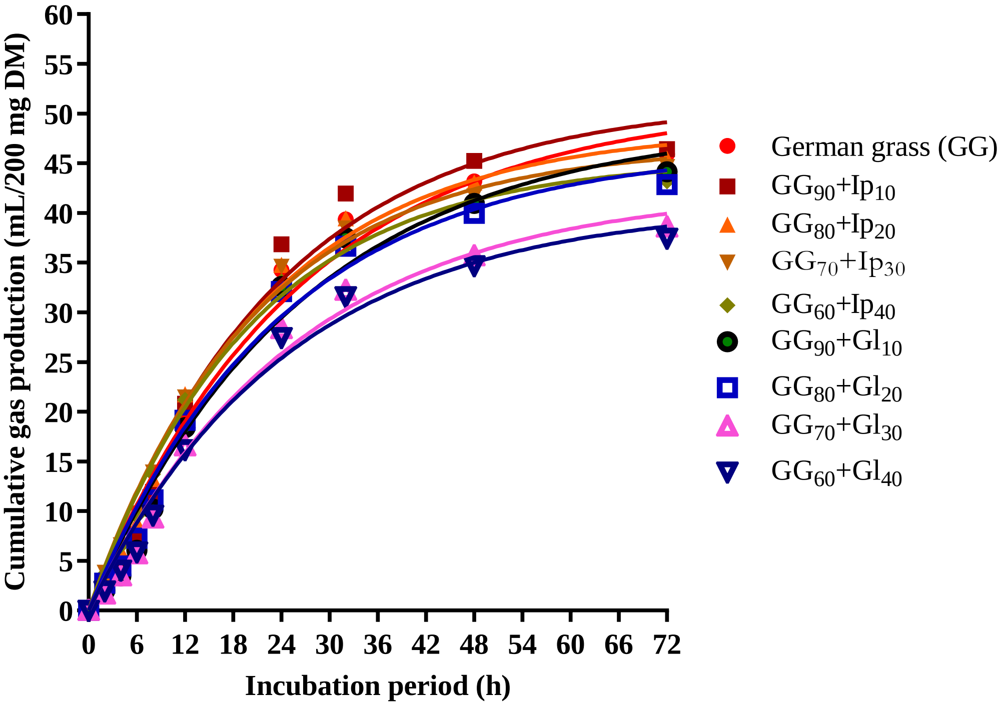

Fig.12: Cumulative gas production profile of different combinations of German grass. The *in vitro* gas production profile has been fitted using the equation $Y = p\text{GP}\cdot (1 - e^{-c\text{GP}\cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in Table 4.11.

Table 4.11: ***In vitro*** **ruminal fermentation characteristics of different combinations of Ipil-ipil and *Gliricidia* with German grass**

Variables	Ipil-ipil					SEM	Sig.	*Gliricidia*					SME	Sig.
	0	10	20	30	40			0	10	20	30	40		
GP_{24} (mL/200 mg DM)	34.2	36.8	34.8	34.6	33.1	0.71	NS	34.2^{b}	32.5^{b}	32.1^{b}	28.4^{a}	27.5^{a}	0.73	P<0.05
*p*GP (mL/200 mg DM)	51.2^{b}	51.5^{b}	48.7^{ab}	46.9^{a}	45.5^{a}	0.87	P<0.05	51.2^{d}	49.2^{cd}	46.5^{bc}	42.6^{ab}	40.9^{a}	0.87	P<0.05
*c*GP (%/h)	3.88^{a}	4.33^{ab}	4.64^{b}	4.93^{b}	4.98^{b}	0.16	P<0.05	3.88	3.79	4.24	3.90	4.04	0.17	P<0.05
dOM (%)	62.0	64.7	63.2	63.3	62.4	0.64	NS	62.0^{c}	60.6^{c}	60.4^{bc}	57.3^{ab}	56.7^{a}	0.64	P<0.05
ME (MJ/kg DM)	7.50^{a}	8.06^{b}	7.93^{ab}	8.09^{b}	8.05^{b}	0.10	P<0.05	7.50^{b}	7.34^{ab}	7.38^{ab}	6.93^{a}	6.90^{a}	0.11	P<0.05
NEL (MJ/kg DM)	4.26^{a}	4.66^{b}	4.55^{ab}	4.65^{b}	4.61^{b}	0.07	P<0.05	4.26^{b}	4.14^{ab}	4.16^{ab}	3.83^{a}	3.79^{a}	0.08	P<0.05

GP_{24}, Gas production at 24 h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; SEM, Standard error of the mean; NS, Not significant; $^{a\text{-}d}$ means with different superscripts in the same row are significantly different at (P< 0.05).

Table 4.12: CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM of different combinations of Ipil-Ipil-ipil and *Gliricidia* with German grass

Variables	Ipil ipil					SEM	Sig.	*Gliricidia*					SEM	Sig.
	0	10	20	30	40			0	10	20	30	40		
CH_4 mL/120 mg DM	3.12^{b}	2.91^{ab}	2.85^{ab}	2.80^{ab}	2.58^{a}	0.09	P<0.05	3.12^{b}	2.65^{a}	2.87^{ab}	2.56^{a}	2.47^{a}	0.09	P<0.05
CH_4 conc. in GP (%)	15.4	13.6	15.1	15.2	14.8	0.45	NS	15.4	14.7	15.4	15.0	15.6	0.43	NS
L CH_4/kg dOM	41.9	37.5	37.6	36.9	34.7	1.54	NS	41.9	36.4	39.5	37.4	36.4	1.47	NS
L CH_4/kg DM	26.0	24.3	23.7	23.4	21.6	0.78	NS	26.0^{b}	22.1^{ab}	23.9^{ab}	21.4^{a}	20.6^{a}	0.80	P<0.05

CH_4, Methane; CH_4 conc., Methane concentration; dOM, Digestibility of organic matter; DM, Dry matter; SEM, Standard error of the mean; NS, Not significant; $^{a,\,b}$means with different superscripts in the same row are significantly different at (p< 0.05).

No significant associative effects were observed with the addition of Ipil-ipil and *Gliricidia* to German grass on *in vitro* ruminal fermentation characteristics (Table 4.13). The measured values of dOM, ME, and NEL were consistently higher than the estimated values of Ipil-ipil and *Gliricidia* for German grass, which implied a positive associative effect. The associative effect of dOM, ME, and NEL that was calculated from the estimated and measured values, was the highest (5.69%, 7.61%, and 9.79%, respectively) when 10% Ipil-ipil was added to German grass, but this was not significant ($p>0.05$).

The estimated and measured values of CH_4 production, CH_4/dOM, and CH_4/DM did not differ significantly with Ipil-ipil addition. The greatest reduction in CH_4 concentration ($p<0.05$) among all treatments (9.15%) was obtained when 10% Ipil-ipil was added to the German grass. The same trend was observed for CH_4 production and CH_4/DM (13.1% for both CH_4 production and L CH_4/kg DM, respectively) when 10% *Gliricidia* was added to German grass ($p<0.05$).

The comparison between the estimated and measured dOM of Ipil-ipil-containing mixes showed a higher R^2 value (0.85 with an RMSE of 0.45 (Table 4.14). The results of regression analysis of estimated and measured ME, NEL, and CH_4 concentration values of Ipil-ipil-containing mixes showed the lowest accuracy (R^2 = 0.08, 0.01, and 0.01, respectively). However, the CH_4 concentration of Ipil-ipil was negatively correlated with the measured value, with a slope of 1.66 and RMSE value of 0.89. The R^2 values of L CH_4/kg dOM and L CH_4/kg DM were 0.78 and 0.86 respectively, indicating that this dataset was moderately accurate. The results of the regression analysis of dOM, ME, and NEL of *Gliricidia* were positively correlated between the estimated and measured values, with a slope of 1 and R^2 values of 0.87, 0.79, and 0.82, respectively.

Table 4.13: Associative effect of Ipil-ipil and *Gliricidia* on *in vitro* ruminal fermentation characteristics and CH_4 reduction potential of German grass

Parameters	Ipil-ipil (%)				SEM	Sig.	*Gliricidia* (%)				SME	Sig.
	10	**20**	**30**	**40**			**10**	**20**	**30**	**40**		
dOM (%)												
Estimated*	61.2	60.9	60.7	60.4			60.0	58.5	57.1	55.6		
Measured	64.7	63.1	63.3	62.4			60.6	60.4	57.3	56.7		
Associative effect (%)	5.69	3.66	4.42	3.29	1.01	NS	1.02	3.27	0.39	1.92	1.32	NS
ME (MJ/kg DM)												
Estimated*	7.49	7.57	7.65	7.73			7.24	7.07	6.90	6.73		
Measured	8.06	7.94	8.09	8.06			7.34	7.38	6.94	6.90		
Associative effect (%)	7.61	4.83	5.74	4.21	1.38	NS	1.39	4.44	0.53	2.60	1.79	NS
NEL (MJ/kg DM)												
Estimated*	4.25	4.29	4.33	4.38			4.07	3.94	3.80	3.67		
Measured	4.66	4.56	4.65	4.62			4.14	4.16	3.83	3.80		
Associative effect (%)	9.79	6.21	7.39	5.41	1.78	NS	1.80	5.81	0.70	3.47	2.36	NS
CH_4 production (mL)												
Estimated$^{\psi}$	3.14	3.07	2.99	2.92			3.06	2.90	2.74	2.58		
Measured	2.91	2.85	2.81	2.59			2.66	2.87	2.57	2.48		
Associative effect (%)	-7.31	-7.05	-6.20	-11.3	2.35	NS	-13.1^{b}	-0.93^{a}	-6.17^{ab}	-3.87^{ab}	3.60	$P<0.05$
CH_4 concentration (%)												
Estimated$^{\psi}$	15.0	15.0	15.0	14.9			15.4	15.7	16.0	16.3		
Measured	13.6	15.1	15.2	14.8			14.7	15.4	15.0	15.6		
Associative effect (%)	-9.15^{b}	0.43^{a}	1.41^{a}	-0.91^{a}	1.93	$P<0.05$	-4.41	-1.53	-6.27	-4.14	4.18	NS
L CH_4/kg dOM												
Estimated$^{\psi}$	42.7	41.9	41.0	40,2			42.1	40.7	39.2	37.7		
Measured	37.6	37.6	36.9	34,7			36.4	39.6	37.4	36.4		
Associative effect (%)	-12.1	10.2	-10.0	-13.6	2.05	NS	-13.5	-2.68	-4.66	-3.36	3.84	NS
L CH_4/kg DM												
Estimated$^{\psi}$	26.2	25.6	24.9	24.3			25.5	24.1	22.8	21.5		
Measured	24.3	23.8	23.4	21.6			22.1	23.9	21.4	20.6		
Associative effect (%)	-7.32	-7.05	-6.20	-11.3	2.35	NS	-13.1^{b}	-0.93^{a}	-6.18^{ab}	-3.87^{ab}	3.85	$P<0.05$

dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH4, Methane; SEM, Standard error of the mean; NS, Not significant; $^{a, b}$ means with different superscripts in the same row are significantly different at ($p< 0.05$); *, The estimated values were calculated from single feeds that were determined by the equation of Menke and Steingass (1988); $^{\psi}$, The estimated values were calculated from singles feeds.

Table 4.14: Results of linear regression of estimated and measured values of *in vitro* ruminal fermentation characteristics and CH_4 reduction of German grass

Parameters	**Ipil-ipil (†estimated v. measured)**					RMSE	***Gliricidia* (†estimated v. measured)**					RMSE
	Slope	Slope CI	Intercept	Intercept CI	R^2		Slope	Slope CI	Intercept	Intercept CI	R^2	
dOM (%)	2.64	-0.67 to 5.96	-97.6	-299 to 104	0.85	0.45	1.00	-0.17 to 2.19	0.46	-67.9 to 68.8	0.87	0.89
ME MJ/kg DM	0.18	-1.68 to 2.06	6.61	-7.65 to 20.9	0.08	0.08	1.03	-0.57 to 2.64	-0.09	-11.3 to 11.1	0.79	0.14
NEL MJ/kg DM	-0.06	-2.52 to 2.38	4.91	-5.67 to 15.5	0.01	0.05	1.01	-0.43 to 2.46	0.05	-5.56 to 5.68	0.82	0.10
CH_4 mL/120 mg DM	1.34	-0.39 to 3.08	-1.27	-6.55 to 3.99	0.85	0.06	0.52	-1.34 to 2.39	1.64	-4.12 to 6.45	0.42	0.15
CH_4 conc. in GP (%)	-1.66	-46.2 to 42.8	39.6	-627 to 706	0.01	0.89	0.76	-1.37 to 2.90	3.02	-30.9 to 36.9	0.54	0.33
L CH_4/kg dOM	1.11	-0.70 to 2.93	-9.56	-85.0 to 65.8	0.78	0.79	0.16	-2.20 to 2.53	30.9	-63.6 to 125.6	0.04	1.80
L CH_4/kg DM	1.32	-0.29 to 2.93	-10.0	-50.8 to 30.8	0.86	0.53	0.51	-1.42 to 2.45	9.90	-35.7 to 55.5	0.39	1.31

dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH_4 conc., Methane concentration; †The estimated values of CH_4 related traits were calculated from the single feeds and using the equation of Menke and Steingass (1988) for determining theruminal fermentation characteristics (dOM, ME, and NEL) from single feeds, which consistently indicated X-variables.

4.2.3. Supplementation of Ipil-ipil and *Gliricidia* to Napier silage

The *in vitro* ruminal fermentation characteristics of Ipil-ipil and *Gliricidia* with Napier silage are presented in Table 4.15. The increasing level of Ipil-ipil resulted in a gradual increase in GP_{24}, dOM, ME, and NEL, reaching the highest value (33.0 mL/200 mg DM, 56.5%, 7.55 MJ ME/kg DM, 4.28 MJ NEL/kg DM, respectively) when 30% Ipil-ipil was added to the Napier silage ($p<0.05$). With further addition of 40% Ipil-ipil, all values decreased. Interestingly, a combination of 30% Ipil-ipil and Napier silage showed the most significant improvement ($p<0.05$) compared to the control, resulting in a 15.7% increase in dOM, 23.0% ME, and 28.3% NEL (Fig. 14). However, the opposite trend was observed when *Gliricidia* was added to Napier silage, and Napier silage alone produced the highest volume of gas (28.5 mL/200 mg DM) compared to other combinations ($p<0.05$).

The cumulative gas production of different combinations of Ipil-ipil and *Gliricidia* with Napier silage during the incubation period is shown in Fig. 15. The same gas production pattern was recorded as described before for rice straw and German grass. With an increase in incubation period up to 72 h, addition of 30% Ipil-ipil to Napier silage produced the highest volume of gas (more than 45 mL/200 mg DM).

CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM showed no significant ($p>0.05$) variation among the treatments (Table 4.16). However, the lowest amount of CH_4 concentration and CH_4/dOM were observed (13.9 in GP (%), 36.6 L CH_4/kg dOM) among all treatments when 30% Ipil-ipil was added to the Napier silage. The highest percentage reduction of CH_4 concentration and CH_4/dOM were recorded (5.32% and 13.3%, respectively) when 30% Ipil-ipil was incorporated ($p<0.05$) (Fig. 16).

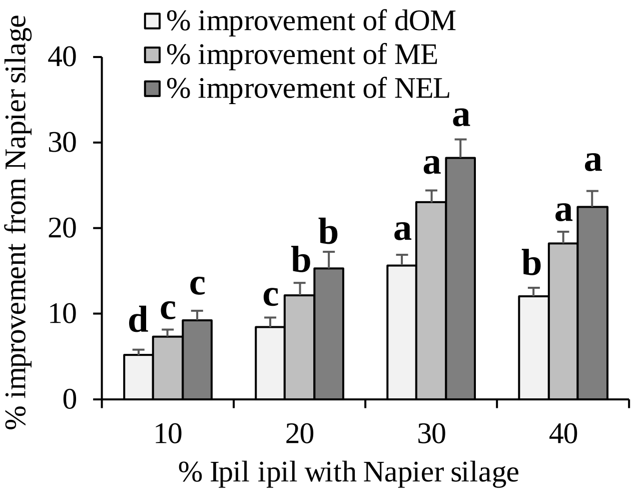

Fig.14: % improvement of dOM, ME and NEL with the supplementation of Ipil-ipil to the Napier silage

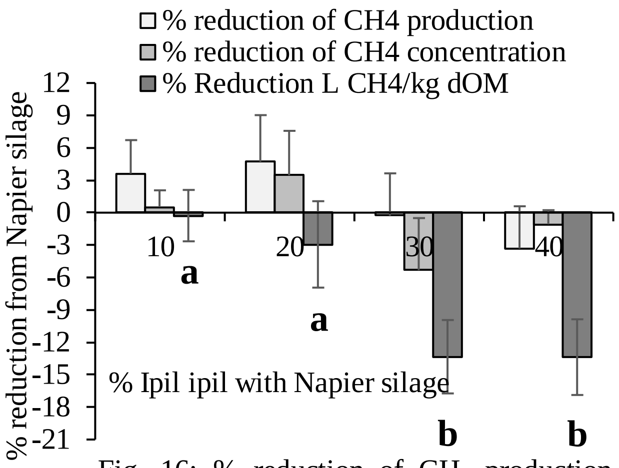

Fig. 16: % reduction of CH_4 production, CH_4 concentration, and CH_4/dOM with the supplementation of Ipil-ipil to the Napier sialge

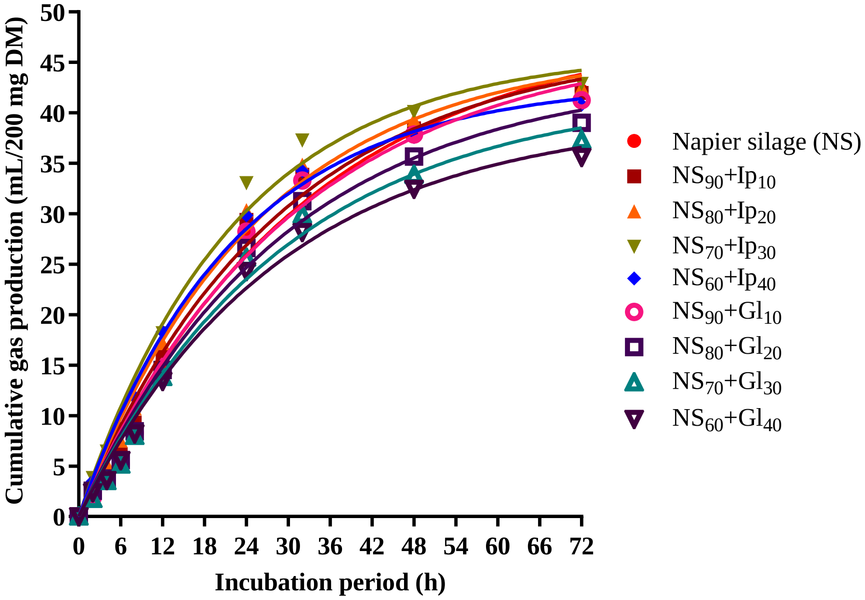

Fig.15: Cumulative gas production profile of different combinations of Napier silage. The *in vitro g*as production profile has been fitted to curves using the equation $Y = pGP\cdot (1- e^{-cGP\cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in Table 4.15.

Table 4.15: ***In vitro*** **ruminal fermentation characteristics of different combinations of Ipil-ipil and *Gliricidia* with Napier silage**

Variables	Ipil-ipil (%)					SEM	Sig.	*Gliricidia* (%)					SME	Sig.
	0	10	20	30	40			0	10	20	30	40		
GP_{24} (mL/200 mg DM)	28.5^{a}	29.3^{ab}	30.3^{a}	33.0^{b}	29.8^{a}	0.73	$P<0.05$	28.5^{b}	28.2^{b}	26.6^{ab}	25.6^{ab}	24.3^{a}	0.73	$P<0.05$
*p*GP (mL/200 mg DM)	49.1^{b}	47.1^{ab}	46.5^{ab}	46.2^{ab}	43.1^{a}	2.75	$P<0.05$	49.1^{d}	47.5^{cd}	44.1^{bc}	42.4^{ab}	39.8^{a}	2.75	$P<0.05$
*c*GP (%/h)	3.13^{a}	3.54^{ab}	3.92^{bc}	4.53^{c}	4.52^{c}	0.17	$P<0.05$	3.13	3.28	3.43	3.43	3.51	0.17	NS
dOM (%)	49.0^{a}	50.9^{ab}	52.9^{bc}	56.5^{d}	54.8^{cd}	0.65	$P<0.05$	49.0	49.8	49.3	49.3	49.1	0.65	NS
ME (MJ/kg DM)	6.23^{a}	6.56^{ab}	6.94^{bc}	7.55^{d}	7.31^{cd}	0.11	$P<0.05$	6.23	6.33	6.23	6.22	6.17	0.11	NS
NEL (MJ/kg DM)	3.36^{a}	3.59^{ab}	3.85^{b}	4.28^{c}	4.09^{c}	0.07	$P<0.05$	3.36^{a}	3.43	3.34	3.33	3.28	0.07	NS

GP_{24}, Gas production at 24 h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; SEM, Standard error of the mean; NS, Not significant; $^{a-d}$ means with different superscripts in the same row are significantly different at ($P< 0.05$).

Table 4.16: CH_4 production, CH_4 concentration, CH_4/dOM and CH_4/DM of different combinations of Ipil-ipil and *Gliricidia* with Napier silage

Variables	Ipil-ipil (%)					SEM	Sig.	*Gliricidia* (%)					SEM	Sig.
	0	10	20	30	40			0	10	20	30	40		
CH_4 mL/120 mg DM	2.50	2.57	2.60	2.47	2.40	0.08	NS	2.50	2.33	2.34	2.21	2.17	0.09	NS
CH_4 conc. in GP (%)	14.7	14.7	15.2	13.9	14.5	0.42	NS	14.7	14.6	14.9	14.0	14.7	0.41	NS
L CH_4/kg dOM	42.5	42.1	41.0	36.6	36.6	1.43	NS	42.5	39.1	39.7	37.3	36.8	1.42	NS
L CH_4/kg DM	20.8	21.5	21.7	20.6	20.0	0.76	NS	20.8	19.4	19.5	18.4	18.1	0.76	NS

CH_4, Methane; CH_4 conc., Methane concentration; dOM, Digestibility of organic matter; DM, Dry matter; SEM, Standard error of the mean; NS, Not significant.

silage to measure the ruminal fermentation characteristics (Table 4.17). Significant differences were observed between the estimated and measured values for dOM, ME, and NEL with the addition of Ipil-ipil to Napier silage. Maximal associative effects of dOM, ME, and NEL were obtained (9.49%,11.9% and 15.8%, respectively) when 30% Ipil-ipil was added to the Napier silage. Supplementation of *Gliricidia* to Napier silage did not significantly affect the differences between the estimated and measured values ($p>0.05$).

The reduction of CH_4 with the supplementation of Napier silage with Ipil-ipil and *Gliricidia* is also presented in Table 4.17. No significant differences were observed in CH_4 production, CH_4 concentration, CH_4/dOM, or CH_4/DM for all combinations of Napier silage.

The simple linear regression analysis of Ipil-ipil and *Gliricidia* between the estimated and measured values of Napier silage is shown in Table 4.18. The regression of dOM between the estimated and measured values of Ipil-ipil was positive, with a slope of 1.44 and *an* R^2 value of 0.67, but their RMSE was at 1.65, indicating that the dataset was only moderately accurate (Fig. 17). The regression line slopes of ME and NEL were 1.39 and 1.44, and their regression equations also showed high R^2 values (0.74 and 0.70, respectively), when Ipil-ipil was added to the Napier silage (Fig. 18). The CH_4 concentration and CH_4/DM were negatively correlated with the measured values of Ipil-ipil. Interestingly, the RMSE values were very low (0.36 and 0.39), indicating a good fit for the dataset (Fig. 19). The estimated values of *Gliricidia* were also positively correlated with the measured values of dOM, ME, and NEL, and the slopes were 1.34, 1.03, and 1.10, with high R^2 values (0.88, 0.88, and 0.93, respectively) (Tab. 4.18).

Table 4.17: Associative effect of Ipil-ipil and *Gliricidia* on *in vitro* ruminal fermentation characteristics and CH_4 reduction potential of Napier silage

Parameters	Ipil-ipil (%)				SEM	Sig.	*Gliricidia* (%)				SME	Sig.
	10	20	30	40			10	20	30	40		
dOM (%)												
Estimated*	49.5	50.5	51.6	52.6			48.3	48.1	48.0	47.8		
Measured	50.9	52.9	56.4	54.7			49.8	49.3	49.3	49.1		
Associative effect (%)	2.79^b	4.74^b	9.49^a	4.10^b	1.27	$P<0.05$	1.40	0.66	1.08	0.88	1.67	NS
ME (MJ/kg DM)												
Estimated*	6.34	6.55	6.76	6.96			6.09	6.05	6.00	5.96		
Measured	6.57	6.94	7.56	7.32			6.33	6.24	6.23	6.18		
Associative effect (%)	3.56^b	6.00^b	11.9^a	5.07^b	1.61	$P<0.05$	3.98	3.11	3.74	3.67	2.22	NS
NEL (MJ/kg DM)												
Estimated*	3.43	3.56	3.70	3.83			3.25	3.21	3.17	3.13		
Measured	3.59	3.85	4.28	4.09			3.43	3.35	3.33	3.29		
Associative effect (%)	4.80^b	8.03^b	15.8^a	6.72^b	2.21	$P<0.05$	5.43	4.27	5.16	5.09	3.12	NS
CH_4 production (mL)												
Estimated$^\psi$	2.68	2.66	2.63	2.61			2.59	2.48	2.38	2.27		
Measured	2.58	2.60	2.48	2.40			2.33	2.35	2.21	2.17		
Associative effect (%)	-3.81	-1.96	-5.83	-7.92	3.77	NS	-10.0	-5.58	-7.01	-4.32	4.59	NS
CH_4 concentration (%)												
Estimated$^\psi$	14.5	14.5	14.6	14.6			14.9	15.2	15.6	16.0		
Measured	14.8	15.2	13.9	14.5			14.6	14.9	14.0	14.7		
Associative effect (%)	1.71	4.51	-4.55	-0.44	3.68	NS	-1.51	-2.07	-10.1	-8.24	3.51	NS
L CH_4/kg dOM												
Estimated$^\psi$	45.3	44.2	43.0	41.9			44.7	42.9	41.2	39.4		
Measured	42.1	41.0	36.6	36.6			39.1	39.7	37.3	36.8		
Associative effect (%)	1.50	0.86	-7.00	-4.58	3.44	NS	-4.90	0.07	-2.80	-0.61	4.47	NS
L CH_4/kg DM												
Estimated$^\psi$	22.3	22.1	21.9	21.7			21.6	20.7	19.8	18.9		
Measured	21.5	21.7	20.7	20.0			19.4	19.6	18.4	18.1		
Associative effect (%)	-7.04	-7.19	-15.0	-12.7	3.65	NS	-12.6	-7.56	-9.39	-6.65	4.59	NS

dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH4, Methane; SEM, Standard error of the mean; NS, Not significance; $^{a, b}$ means with different superscripts in the same row are significantly different at ($p< 0.05$); *, The estimated values were calculated from single feeds that were determined by the equation of Menke and Steingass (1988); $^\psi$, The estimated value were calculated from single feeds.

Table 4.18: Results of linear regression of estimated and measured values of *in vitro* ruminal fermentation characteristics and CH_4 reduction of Napier silage

Parameters	**Ipil-ipil (†estimated v. measured)**					RMSE	***Gliricidia*** **(†estimated v. measured)**					RMSE
	Slope	Slope CI	Intercept	Intercept CI	R^2		Slope	Slope CI	Intercept	Intercept CI	R^2	
dOM (%)	1.44	-1.61 to 4.50	-20.0	-176 to 136	0.67	1.65	1.34	-0.16 to 2.85	-15.3	-87.7 to 57.1	0.88	0.12
ME MJ/kg DM	1.39	-1.14 to 3.93	-2.18	-19.1 to 14.7	0.74	0.27	1.03	-0.12 to 2.18	0.03	-6.90 to 6.97	0.88	0.03
NEL MJ/kg DM	1.44	-1.41 to 4.31	-1.30	-11.7 to 9.10	0.70	0.19	1.10	0.18 to 2.01	-0.15	-3.07 to 2.75	0.93	0.02
CH_4 mL/120 mg DM	2.73	-0.44 to 6.02	-4.87	-13.4 to 3.68	0.87	0.04	0.58	-0.28 to 1.45	0.85	-1.26 to 2.97	0.80	0.05
CH_4 conc. in GP (%)	-8.00	-23.5 to 7.51	131.0	-94.7 to 356.7	0.71	0.36	-0.16	-2.57 to 2.24	17.1	-20.0 to 54.3	0.04	0.46
L CH_4/kg dOM	10.4	-1.84 to 22.7	-215.9	-516 to 84.2	0.87	1.27	0.96	-0.69 to 2.63	16.4	-20.9 to 53.8	0.76	0.83
L CH_4/kg DM	-2.75	-1.06 to 6.56	-39.5	-123 to 44.5	0.83	0.39	0.56	-0.29 to 1.43	7.40	-10.1 to 24.9	0.80	0.40

dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH_4 conc., Methane concentration; †The estimated values of CH_4 related traits were calculated from the single feeds and using the equation of Menke and Steingass (1988) for determining the ruminal fermentation characteristics (dOM, ME, and NEL) from single feeds, which consistently indicated X-variables.

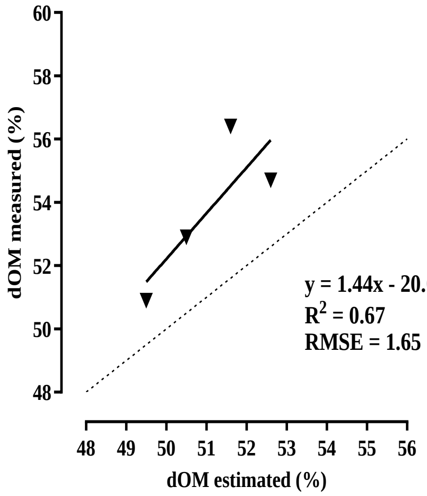

Fig. 17: Comparison of estimated and measured digestibility of organic matter (dOM) values of Ipil-ipil with Napier silage using an *in vitro* ruminal fermentation technique. The dOM values of combined feeds were calculated from dOM values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

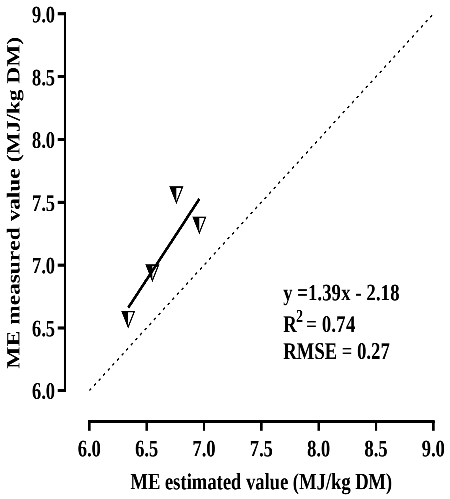

Fig. 18: Comparison of estimated and measured metabolisable energy (ME) values of Ipil-ipil with Napier silage an *in vitro* ruminal fermentation technique. The ME values of combined feeds were calculated from ME values of single feed that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

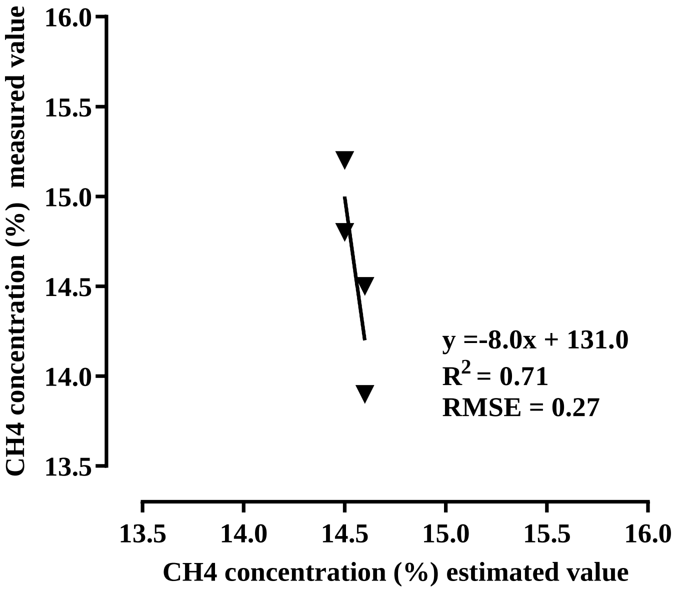

Fig. 19: Comparison of estimated and measured CH_4 concentration (%) values of Ipil-ipil with Napier silage using an *in vitro* ruminal fermentation technique. The CH_4 concentration values of combined feeds were calculated from CH_4 concentration values of single feeds.

4.2.4. Supplementation of Ipil-ipil and *Gliricidia* to maize silage

GP at 24 h did not differ significantly with addition of Ipil-ipil to maize silage (Tab. 4.19). However, increasing the Ipil-ipil level resulted in a gradual decrease in *p*GP ($p<0.05$). The dOM, ME, and NEL increased gradually with an increase in the Ipil-ipil level, and a maximum (58.7%, 7.90 MJ ME/kg DM, and 4.51 MJ NEL/kg DM, respectively) was reached with a 40% Ipil-ipil addition to maize silage ($p<0.05$) (Table 4.19). The most remarkable improvement was observed when 40% Ipil-ipil was added to the maize silage, resulting in 7.87% improvement in dOM, 14.8% improvement in ME, and 33.4% improvement in NEL (Fig. 20). The GP_{24}, dOM, ME, and NEL decreased gradually by adding *Gliricidia* to the maize silage; from 33.9 to 27.2 mL/200 mg DM, from 55.1 to 52.4% dOM, from 7.11 to 6.65 MJ/kg DM, and from 4.00 to 3.63 MJ/kg DM, respectively.

The interaction effect between different combinations of maize silage and incubation time on *in vitro* cumulative GP is shown in Fig. 21. The combination of Ipil-ipil and maize silage produced a higher amount of gas than with *Gliricidia*. Interestingly, the cumulative gas production of maize silage alone was the highest (> 50 mL/kg DM) at the end of 72 h of incubation.

CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM did not differ significantly ($p>0.05$) among the treatments (Tab. 4.20, Fig. 22).

Table 4.19: ***In vitro*** **ruminal fermentation characteristics of different combinations of Ipil-ipil and** ***Gliricidia*** **with maize silage**

Variables	Ipil-ipil (%)					SEM	Sig.	*Gliricidia* (%)					SME	Sig.
	0	10	20	30	40			0	10	20	30	40		
GP_{24} (mL/200 mg DM)	33.9	33.8	34.6	34.0	33.5	0.74	NS	33.9^{c}	31.9^{bc}	30.9^{bc}	28.9^{ab}	27.2^{a}	0.74	P<0.05
*p*GP (mL/200 mg DM)	53.0^{c}	50.9^{bc}	50.3^{bc}	48.5^{ab}	46.1^{a}	2.76	P<0.05	53.0^{d}	49.9^{cd}	47.8^{bc}	45.2^{ab}	42.5^{a}	2.76	P<0.05
*c*GP (%/h)	3.95^{a}	4.28^{ab}	4.63^{abc}	4.94^{bc}	5.43^{c}	0.17	P<0.05	3.95	4.00	4.14	3.04	3.99	0.17	NS
dOM (%)	55.1^{a}	56.0^{ab}	57.7^{ab}	58.2^{b}	58.7^{b}	0.65	P<0.05	55.1	54.1	54.1	53.2	52.4	0.65	NS
ME (MJ/kg DM)	7.11^{a}	7.31^{ab}	7.57^{bc}	7.77^{bc}	7.90^{c}	0.11	P<0.05	7.11	6.94	6.94	6.78	6.65	0.11	NS
NEL (MJ/kg DM)	4.00^{a}	4.13^{ab}	4.35^{bc}	4.43^{bc}	4.51^{c}	0.08	P<0.05	4.00^{b}	3.87^{ab}	3.86^{ab}	3.73^{ab}	3.63^{a}	0.08	P<0.05

GP_{24}, Gas production at 24 h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; SEM, Standard error of the mean; NS, Not significant; $^{a\text{-}d}$ means with different superscripts in the same row are significantly different at (P< 0.05).

Table 4.20: CH_4 production, CH_4 concentration, CH_4/dOM and CH_4/DM of different combinations of Ipil-ipil and ***Gliricidia*** **with maize silage**

Variables	Ipil-ipil (%)					SEM	Sig.	*Gliricidia* (%)					SEM	Sig.
	0	10	20	30	40			0	10	20	30	40		
CH_4 mL/120 mg DM	2.67	2.68	2.64	2.59	2.70	0.09	NS	2.67	2.59	2.65	2.43	2.33	0.09	NS
CH_4 conc. in GP (%)	13.5	14.0	13.9	13.8	13.6	0.43	NS	13.5	13.6	14.5	14.4	14.7	0.43	NS
L CH_4/kg dOM	40.5	39.9	38.2	37.2	39.0	1.46	NS	40.5	39.9	40.9	38.2	37.1	1.46	NS
L CH_4/kg DM	22.3	22.4	22.1	21.6	22.9	0.78	NS	22.3	22.1	23.1	20.3	19.4	0.78	NS

CH_4, Methane; CH_4 conc., Methane concentration; dOM, Digestibility of organic matter, DM, Dry matter; SEM, Standard error of the mean; NS, Not significance.

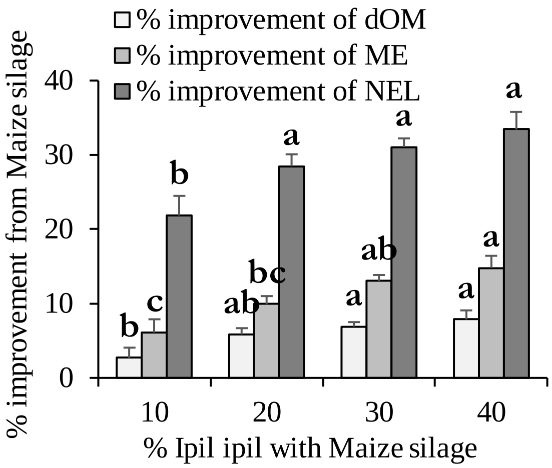

Fig. 20: % improvement of dOM, ME and NEL with the supplementation of Ipil-ipil to the maize silage

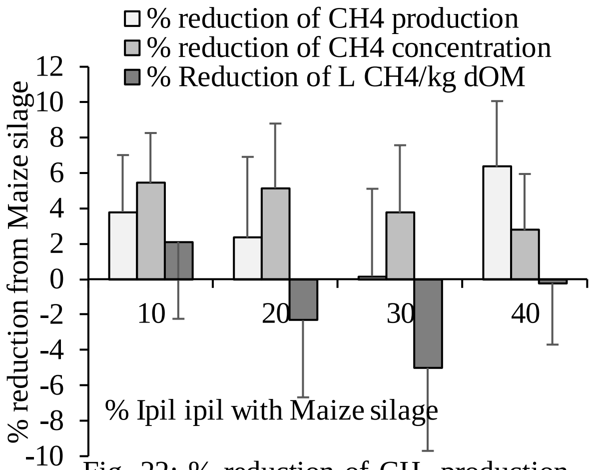

Fig. 22: % reduction of CH_4 production, CH_4 concentration, and CH_4/dOM with the supplementation of Ipil-ipil to the maize silage

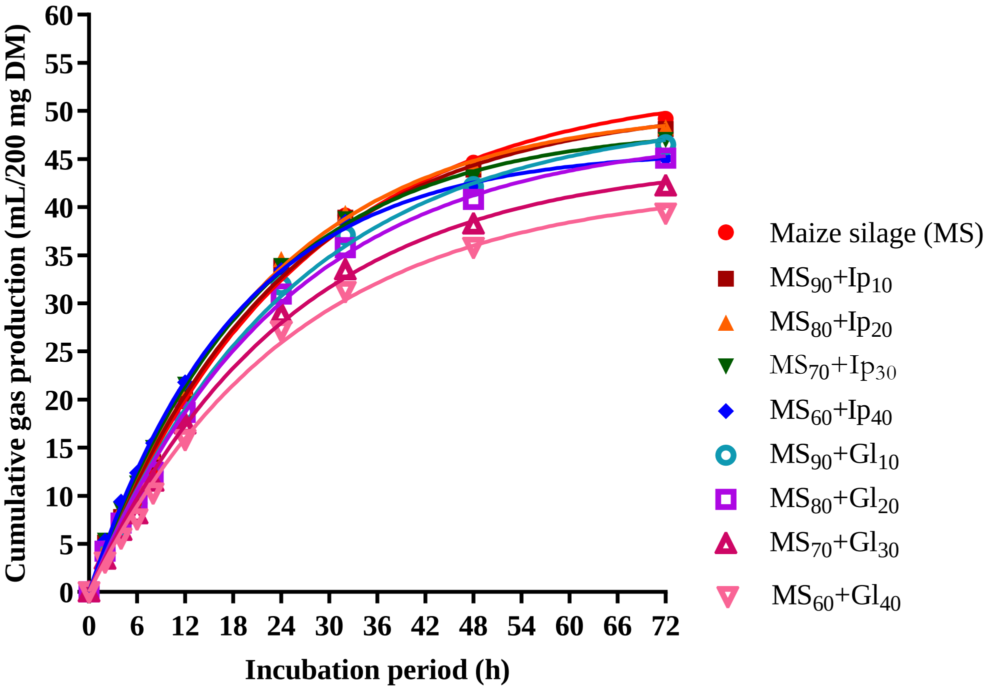

Fig. 21: Cumulative gas production profile of different combinations of maize silage. The *in vitro* gas production profile has been fitted using the equation $Y = p\mathrm{GP} \cdot (1- e^{-c\mathrm{GP} \cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in Table 4.19.

The differences between the estimated and measured values of dOM, ME, and NEL and their associative effect were not significant in any case ($p>0.05$) but showed a positive trend with the addition of Ipil-ipil and *Gliricidia* to the maize silage (Tab. 4.21). The differences tended to be higher when Ipil-ipil was added compared to *Gliricidia* ($p>0.05$). There was no significant associative effect on CH_4 reduction potential among all combinations of maize silage ($p>0.05$).

The linear regression of *in vitro* ruminal fermentation and CH_4 reduction potential between the estimated and measured values of Ipil-ipil and *Gliricidia* with maize silage are presented in Table 4.22. The results of linear regression of dOM and ME values showed that the estimated and measured values for Ipil-ipil addition were positively correlated (Fig. 23, 24). The CH_4 concentration was potentially reduced compared to the estimated values when Ipil-ipil was added to the maize silage, and its slope was –1.54 and the R^2 value was 0.97 (Fig. 25). *Gliricidia* did not show any negative relationship between the estimated and measured values of CH_4 related traits.

Table 4.21: Associative effect of Ipil-ipil and *Gliricidia* on *in vitro* ruminal fermentation characteristics and CH_4 reduction potential of maize silage

Parameters	Ipil-ipil (%)				SEM	Sig.	*Gliricidia* (%)				SME	Sig.
	10	**20**	**30**	**40**			**10**	**20**	**30**	**40**		
dOM (%)												
Estimated*	54.2	54.7	55.2	55.7			53.0	52.3	51.6	50.9		
Measured	56.0	57.7	58.2	58.7			54.1	54.1	53.1	52.4		
Associative effect (%)	3.22	5.41	5.30	5.27	1.40	NS	1.99	3.39	2.92	2.92	1.31	NS
ME (MJ/kg DM)												
Estimated*	7.02	7.16	7.29	7.42			6.77	6.65	6.53	6.41		
Measured	7.31	7.57	7.77	7.90			6.95	6.94	6.78	6.66		
Associative effect (%)	4.08	5.84	6.59	6.49	1.76	NS	2.55	4.36	3.78	3.80	1.70	NS
NEL (MJ/kg DM)												
Estimated*	3.92	4.00	4.08	4.16			3.75	3.65	3.55	3.46		
Measured	4.13	4.36	4.43	4.51			3.87	3.86	3.73	3.63		
Associative effect (%)	5.32	8.83	8.57	8.43	2.29	NS	3.37	5.80	5.06	5.13	2.28	NS
CH_4 production (mL)												
Estimated$^{\psi}$	2.91	2.86	2.81	2.76			2.82	2.69	2.56	2.42		
Measured	2.68	2.65	2.59	2.75			2.59	2.66	2.43	2.33		
Associative effect (%)	-7.71	-7.46	-7.88	-0.47	3.94	NS	-8.19	-1.25	-4.78	-3.68	3.72	NS
CH_4 concentration (%)												
Estimated$^{\psi}$	14.2	14.2	14.3	14.4			14.5	14.9	15.3	15.8		
Measured	14.0	14.0	13.8	13.7			13.6	14.4	14.4	14.7		
Associative effect (%)	-1.09	-1.83	-3.56	-4.90	3.91	NS	-6.18	-3.20	-5.97	-6.53	2.71	NS
L CH_4/kg dOM												
Estimated$^{\psi}$	44.8	43.7	42.6	41.5			44.2	42.5	40.8	39.1		
Measured	40.0	38.2	37.2	39.0			39.9	40.9	38.2	37.1		
Associative effect (%)	-10.8	-12.6	-12.8	-6.02	3.63	NS	-9.59	-3.69	-6.40	-4.99	3.61	NS
L CH_4/kg DM												
Estimated$^{\psi}$	24.2	23.8	23.4	23.0			23.5	22.4	21.3	20.2		
Measured	22.4	22.1	21.6	22.9			21.6	22.1	20.3	19.4		
Associative effect (%)	-7.71	-7.46	-7.88	-0.47	3.81	NS	-8.19	-1.25	-4.78	-3.68	3.71	NS

dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH_4, Methane; SEM, Standard error of the mean; NS, Not significance; *, The estimated values were calculated from single feeds that were determined by the equation of Menke and Steingass (1988); $^{\psi}$, The estimated values were calculated from single feeds.

Table 4.22: Results of linear regression of estimated and measured values of *in vitro* ruminal fermentation characteristics and CH_4 reduction of maize silage

Parameters	**Ipil-ipil ([†]estimated v. measured)**					RMSE	***Gliricidia* ([†]estimated v. measured)**					RMSE
	Slope	Slope CI	Intercept	Intercept CI	R^2		Slope	Slope CI	Intercept	Intercept CI	R^2	
dOM; %	1.72	-0.06 to 3.50	-36.8	-135.2 to 61.4	0.895	0.46	0.87	-0.01 to 1.75	8.15	-37.8 to 54.1	0.899	0.32
ME; MJ/kg DM	1.48	0.89 to 2.07	-3.08	-7.35 to 1.19	0.983	0.04	0.85	0.07 to 1.64	1.17	-4.02 to 6.37	0.916	0.04
NEL; MJ/kg DM	1.51	0.08 to 2.94	-1.75	-7.53 to 4.03	0.911	0.06	0.87	0.05 to 1.69	0.63	-2.33 to 3.59	0.912	0.04
CH_4 production (ml)	-0.30	-3.30 to 2.70	3.51	-4.98 to 2.70	0.084	0.07	0.76	-0.53 to 2.06	0.50	-2.91 to 3.92	0.760	0.08
CH_4 conc. (%)	-1.54	-2.32 to -0.76	35.9	24.7 to 47.1	0.973	0.03	0.75	-0.41 to 1.92	2.82	-14.8 to 20.4	0.797	0.26
CH4; L/kg dOM	0.36	-1.93 to 2.65	22.9	-76.1 to 122.0	0.188	1.31	0.65	-0.61 to 1.92	11.8	-41.0 to 64.7	0.710	1.12
CH4; L/kg DM	-0.25	-3.36 to 2.86	28.2	-45.4 to 101.7	0.056	0.64	0.76	-0.47 to 2.00	4.16	-22.9 to 31.3	0.778	0.71

dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH4 conc., Methane concentration; [†]The estimated values of CH_4 related traits were calculated from the single feeds and using the equation of Menke and Steingass (1988) for determining the ruminal fermentation characteristics (dOM, ME, and NEL) from single feeds, which consistently indicated X-variables.

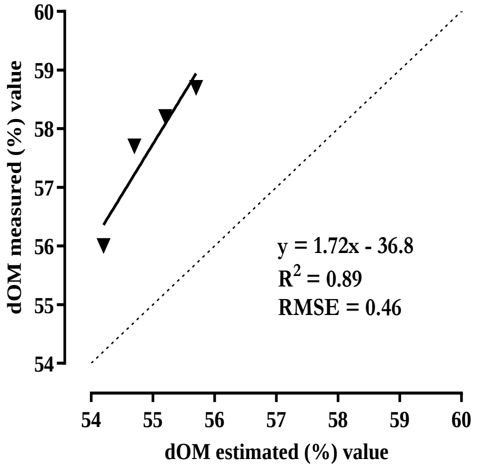

Fig. 23: Comparison of estimated and measured digestibility of organic matter (dOM) values of Ipil-ipil with maize silage using an *in vitro* ruminal fermentation technique. The dOM values of combined feeds were calculated from dOM values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

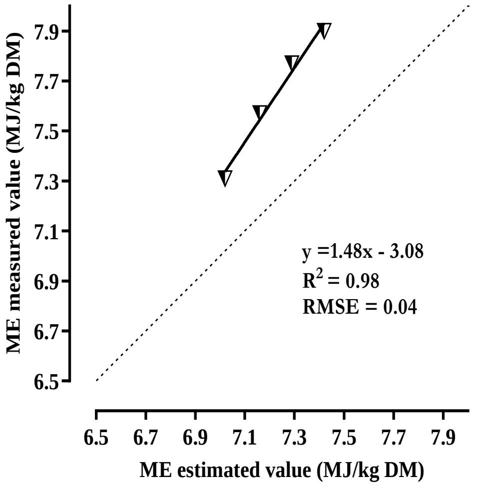

Fig. 24: Comparison of estimated and measured metabolisable energy (ME) values of Ipil-ipil with maize silage using an *in vitro* ruminal fermentation technique. The ME values of compound feeds were calculated from ME values of single feed that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

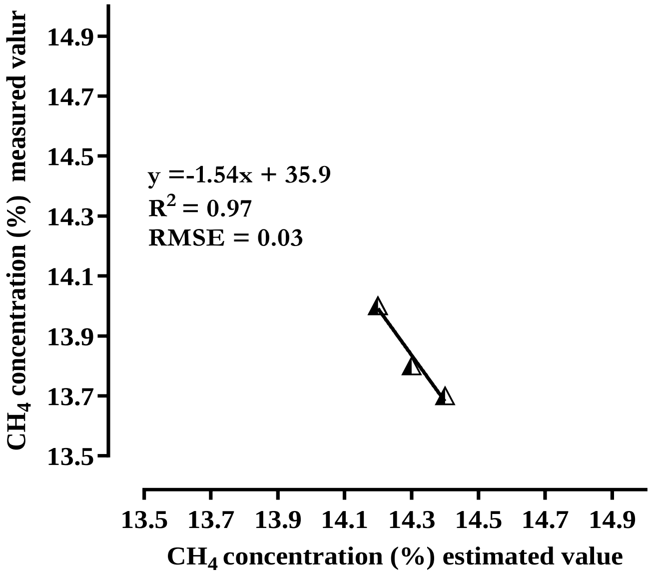

Fig. 25: Comparison of estimated and measured CH_4 concentration (%) values of Ipil-ipil with maize silage using an *in vitro* ruminal fermentation technique. The CH_4 concentration values of combined feeds were calculated from CH_4 concentration values of single feeds.

4.3. Study 3: Formulation of mixed feed with a single concentrate

4.3.1. Effects of single concentrates in rice straw–Ipil-ipil-based mixed feed (RS_{MF})

The results showed that the addition of different concentrates to the rice straw–Ipil-ipil mixed feed significantly affected the *in vitro* ruminal fermentation characteristics (Table 4.23). The addition of WB as a single concentrate to the RS_{MF} resulted in the highest GP_{24} and *c*GP (38.9 mL/200 mg DM and 6.95%/h, respectively), followed by RS_{MF}-KB (32.6 mL/200 mg DM and 3.88%/h, respectively) and RS_{MF}-RB (20.7 mL/200 mg DM and 4.53 %/h, respectively) ($p<0.05$). The RS_{MF}-RB feed had the lowest dOM (47.7%), ME (5.78 MJ ME/kg DM), and NEL (2.95 MJ NEL/kg DM); while those of the RS_{MF}-WB feed were ($p<0.05$) the highest (61.6% dOM**,** 8.34 MJ ME/kg DM and 4.85 MJ NEL/kg DM, respectively).

Table 4.23: *In vitro* ruminal fermentation characteristics of rice straw–Ipil-ipil-mixed feed (RS_{MF}) added with different concentrates

Parameters	**RS_{MF}**			**SEM**	**Sig.**
	RS_{MF}-RB	**RS_{MF}-WB**	**RS_{MF}-KB**		
GP_{24} (mL/200 mg DM)	20.7^a	38.9^c	32.6^b	1.28	P<0.05
*p*GP (mL/200 mg DM)	31.4^a	50.5^b	53.6^c	1.65	P<0.05
*c*GP (%/h)	4.53^b	6.95^c	3.88^a	0.11	P<0.05
dOM (%)	47.1^a	61.6^c	58.4^b	1.13	P<0.05
ME (MJ/kg DM)	5.78^a	8.34^c	7.56^b	0.18	P<0.05
NEL (MJ/kg DM)	2.95^a	4.85^c	4.27^b	0.13	P<0.05

RS_{MF}, Rice straw-based mixed feed (60% rice straw, 40% Ipil-ipil); GP_{24}, Gas production at 24 h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; RS_{MF}-RB, 60% RS_{MF} + 40% rice bran; RS_{MF}-WB, 60% RS_{MF} + 40% wheat bran; RS_{MF}-KB, 60% RS_{MF} + 40% kashari bran; SEM, Standard error of the mean; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$)

The interaction effect of the RS_{MF} and single concentrates was recorded by measuring the *in vitro* cumulative GP between the different concentrates added to RS_{MF} and incubation time (Fig. 26). The cumulative GP was lower from 0 h to 72 h of incubation when RB was added to the RS_{MF} than in the other two treatments.

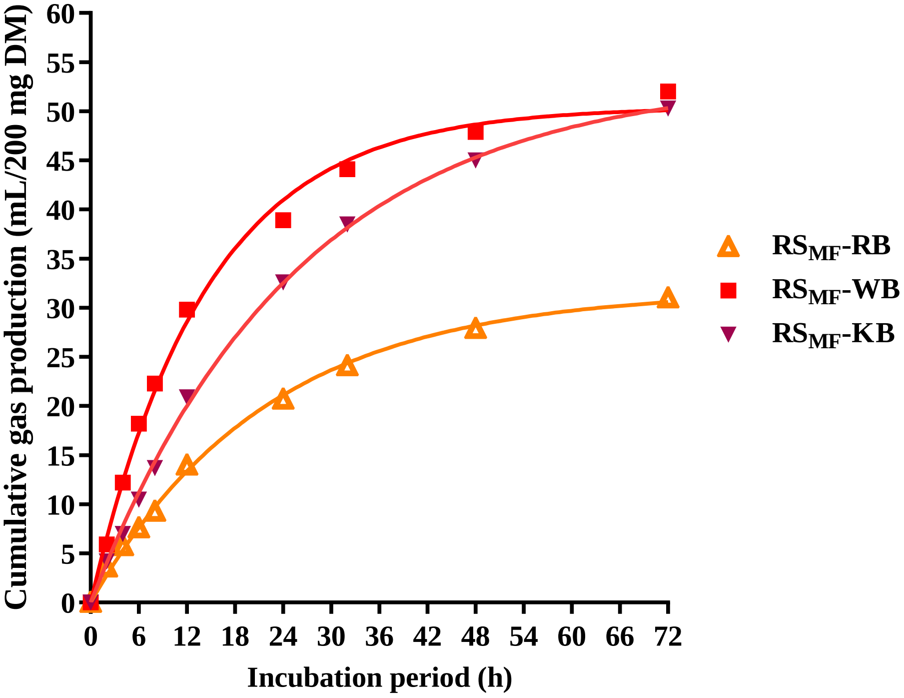

Fig. 26: Cumulative gas production profile of single concentrate with rice straw–Ipil-ipil-based mixed feed (RS_{MF}).The *in vitro* gas production profile has been fitted using the equation $Y= p\text{GP}\cdot (1-e^{-c\text{GP}\cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in table 4.23.

The CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM differed significantly among treatments (Table 4.24). CH_4 production, CH_4 concentration and CH_4/dOM were significantly ($p<0.05$) higher for RS_{MF}-WB (4.35 mL/120 mg DM, 17.7%, and 58.9 L CH_4/kg dOM, respectively) than for the other concentrates. The opposite was observed for RS_{MF}-RB, which had the lowest CH_4 production, CH_4 concentration, and CH_4/dOM (2.10 mL/120 mg DM, 16.1%, and 37.3 L CH_4/kg dOM, respectively) among all treatments ($p<0.05$).

Table 4.24: CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM measured in rice straw–Ipil-ipil-mixed feed (RS_{MF}) with different added concentrates

Parameters	RS_{MF}			SEM	Sig.
	RS_{MF}-RB	RS_{MF}-WB	RS_{MF}-KB		
CH_4 mL/120 mg DM	2.10^a	4.35^c	3.52^b	0.12	$P<0.05$
CH_4 conc. in GP (%)	16.1^a	17.7^b	16.6^{ab}	0.47	$P<0.05$
L CH_4/kg dOM	37.3^a	58.9^c	50.3^b	1.72	$P<0.05$
L CH_4/kg DM	17.6^a	36.3^c	29.4^b	1.00	$P<0.05$

RS_{MF}, Rice straw-based mixed feed (60% rice straw, 40% Ipil-ipil); CH_4, Methane; CH_4 conc., Methane concentration; dOM, Digestibility of organic matter; DM, Dry matter; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; RS_{MF}-RB, 60% RS_{MF} + 40% rice bran; RS_{MF}-WB, 60% RS_{MF} + 40% wheat bran; RS_{MF}-KB, 60% RS_{MF} + 40% kashari bran; SEM, Standard error of the mean; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$)

No significant differences were observed in the total VFA concentration (mmol/L) among all treatments *in vitro*, but were for butyrate and A:P. However, some changes were observed in the VFA profiles (Table 4.25). The concentration of butyrate significantly ($p<0.05$) increased (1.92 mmol/L) compared to the other treatments with the addition of WB to the RS_{MF}. The A:P ratio was the lowest for RS_{MF}-RB (2.40 mmol/L), slightly higher for RS_{MF}-KB (2.75 mmol/L), and the highest for RS_{MF}-WB (3.18 mmol/L) ($p<0.05$). No significant differences were observed in the concentration of NH_3–N among the treatments.

Table 4.25: Concentrations of VFA and NH_3–N after 24 h of incubation of rice straw–Ipil-ipil-mixed feed (RS_{MF}) with different added concentrates

Parameters	RS_{MF}			SEM	Sig.
	RS_{MF}-RB	RS_{MF}-WB	RS_{MF}-KB		
	VFA (mmol/L)				
Acetate (A)	5.49	8.48	7.44	1.07	NS
Propionate (P)	2.21	2.65	2.60	0.32	NS
Iso-butyrate	0.05	0.07	0.06	0.01	NS
Butyrate	0.67^{a}	1.92^{b}	1.22^{a}	0.27	$P<0.05$
Iso-valerate	0.05	0.02	0.06	0.02	NS
Valerate	0.09	0.18	0.11	0.03	NS
Total VFA	8.58	13.3	11.5	1.71	NS
A:P	2.40^{a}	3.18^{b}	2.75^{ab}	0.16	$P<0.05$
NH_3–N (mg/L) ¥	302	297	301	3.97	NS

RS_{MF}, Rice straw-based mixed feed (60% rice straw, 40% Ipil-ipil); VFA, volatile fatty acids; NH_3–N, ammonia–N; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; RS_{MF}-RB, 60% RS_{MF} + 40% rice bran; RS_{MF}-WB, 60% RS_{MF} + 40% wheat bran; RS_{MF}-KB, 60% RS_{MF} + 40% kashari bran; SEM, Standard error of the mean; NS, Not significant; $^{a, b}$ means with different superscripts in the same row are significantly different at ($p < 0.05$), ¥ blank syringe produced 307 mg/L NH_3–N

4.3.2. Effects of single concentrates in German grass–Ipil-ipil-based mixed feed (GG_{MF})

Significant differences in the *in vitro* ruminal fermentation characteristics were observed when single concentrates were added to the German grass–Ipil-ipil-based mixed feed (Table 4.26). The GP_{24} and *c*GP were significantly higher (43.6 mL/200 mg DM and 7.48%/h, respectively) for GG_{MF}-WB than for GG_{MF}-RB (24.2 mL/200 mg DM and 4.82%/h, respectively) and GG_{MF}-KB (35.9 mL/200 mg DM and 4.22%/h, respectively). When RB was added to the GG_{MF}, the dOM, ME, and NEL were the lowest (51.6%, 6.27 MJ ME/kg DM, and 3.32 MJ NEL/kg DM, respectively), whereas the highest concentration was recorded when WB was added to the GG_{MF} (67.3%, 9.00 MJ ME/kg DM, and 5.34 MJ NEL/kg DM, respectively) ($p<0.05$). With the addition of KB to GG_{MF}, all traits related to rumen fermentation were significantly ($p<0.05$) higher than those of GG_{MF}-RB except for *c*GP.

4.26: *In vitro* ruminal fermentation characteristics of German grass–Ipil-ipil-mixed feed (GG_{MF}) with different added concentrates

Parameters	**GG_{MF}**			**SEM**	**Sig.**
	GG_{MF}-RB	**GG_{MF}-WB**	**GG_{MF}-KB**		
GP_{24} (mL/200 mg DM)	24.2^a	43.6^c	35.9^b	1.28	$P<0.05$
*p*GP (mL/200 mg DM)	34.0^a	53.5^b	55.1^b	1.65	$P<0.05$
*c*GP (%/h)	4.82^b	7.48^c	4.22^a	0.12	$P<0.05$
dOM (%)	51.6^a	67.3^c	62.9^b	1.13	$P<0.05$
ME (MJ/kg DM)	6.27^a	9.00^c	8.04^b	0.18	$P<0.05$
NEL (MJ/kg DM)	3.32^a	5.34^c	4.62^b	0.13	$P<0.05$

GG_{MF}, German grass-based mixed feed (90% German grass, 10% Ipil-ipil); GP_{24}, Gas production at 24 h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; RB, Rice barn; WB, Wheat bran; KB, Kashari bran; GG_{MF}-RB, 60% GG_{MF} + 40% rice bran; GG_{MF}-WB, 60% GG_{MF} + 40% wheat bran; GG_{MF}-KB, 60% GG_{MF} + 40% kashari bran; SEM, Standard error of the mean; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$)

Interaction effects between the different single concentrates added to GG_{MF} and the incubation time were observed on the *in vitro* cumulative GP (Fig. 27), as presented previously for RS_{MF}. It can be seen that the gas production trend was the same as that of RS_{MF}-WB. After 72 h of incubation, GG_{MF}-WB produced the highest concentration of gas (approximately 55 mL/200 mg DM) among the different combinations.

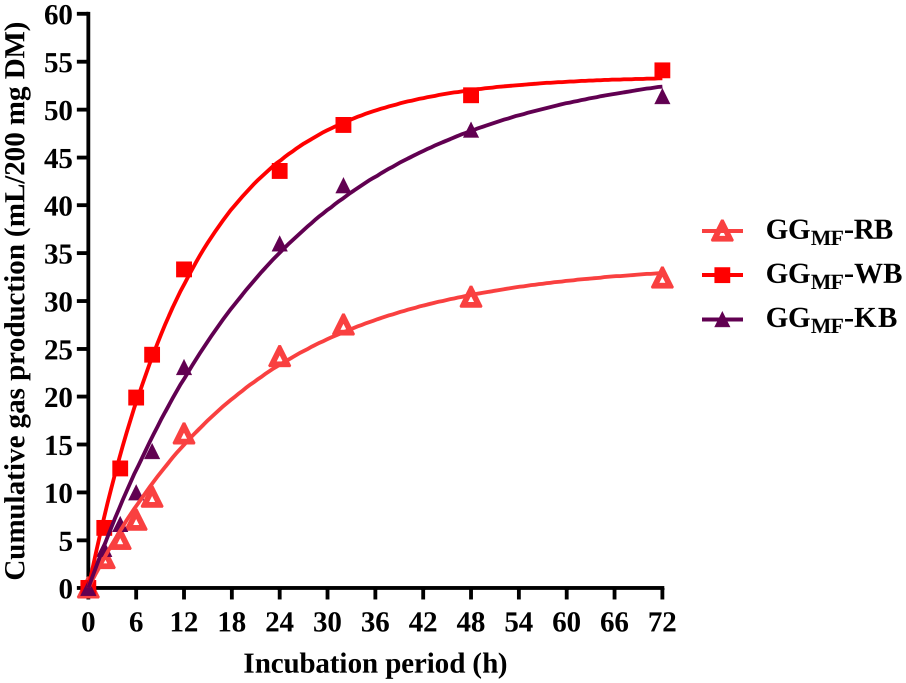

Fig. 27: Cumulative gas production profile of single concentrate with German grass–Ipil-ipil-based mixed feed (GG_{MF}). The *in vitro* gas production profile has been fitted using the equation $Y= p\text{GP}\cdot (1-e^{-c\text{GP}\cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in table 4.26.

The CH_4 production and related traits are presented in Table 4.27. There was no significant difference in the CH_4 concentrations among the treatments ($p>0.05$). The CH_4 production, CH_4/dOM, and CH_4/DM of GG_{MF}-RB were significantly lower (2.38 mL/120 mg DM, 38.5 L CH_4/kg dOM, and 19.9 L CH_4/kg DM, respectively) than those of the other concentrates. The opposite trend was observed for GG_{MF}-WB which produced the highest concentration of CH_4 and CH_4/DM (4.53 mL/120 mg DM and 37.8 L CH_4/kg DM, respectively) ($p<0.05$).

Table 4.27: CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM measured in German grass–Ipil-ipil-mixed feed (GG_{MF}) with different added concentrates

Parameters	GG_{MF}			SEM	Sig.
	GG_{MF}-RB	GG_{MF}-WB	GG_{MF}-KB		
CH_4 mL/120 mg DM	2.38^a	4.53^c	3.92^b	0.12	P<0.05
CH_4 conc. in GP (%)	16.2	16.8	16.20	0.47	NS
L CH_4/kg dOM	38.5^a	56.2^b	52.1^b	1.72	P<0.05
L CH_4/kg DM	19.9^a	37.8^c	32.7^b	1.00	P<0.05

GG_{MF}, German grass-based mixed feed (90% German grass, 10% Ipil-ipil); CH_4, Methane; CH_4 conc., Methane concentration; dOM, Digestibility of organic matter; DM, Dry matter; RB, Rice barn; WB, Wheat bran; KB, Kashari bran; GG_{MF}-RB, 60% GG_{MF} + 40% rice bran; GG_{MF}-WB, 60% GG_{MF} + 40% wheat bran; GG_{MF}-KB, 60% GG_{MF} + 40% kashari bran; SEM, Standard error of the mean; NS, Not significant; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p < 0.05$)

The concentrations of acetate and propionate in the rumen liquor were not affected ($p>0.05$) by the incorporation of a single concentrate into the GG_{MF}. The butyrate and valerate concentrations were significantly greater ($p<0.05$) in the treatment with GG_{MF}-WB (2.39 mmol/L and 0.21 mmol/L) than with GG_{MF}-RB, while GG_{MF}-KB did not differ significantly from the other treatments (Table 4.28). The lowest acetate-to-propionate ratio was obtained (2.48) when RB was added to the GG_{MF} and the highest ratio was found with the addition of WB (3.16). There were no significant differences in NH_3–N concentrations among the treatments.

Table 4.28: Concentrations of VFA and NH_3–N after 24 h of incubation of German grass–Ipil-ipil-mixed feed (GG_{MF}) with different added concentrates

Parameters	**GG_{MF}**			**SEM**	**Sig.**
	GG_{MF}-RB	**GG_{MF}-WB**	**GG_{MF}-KB**		
		VFA (mmol/L)			
Acetate (A)	6.55	10.5	10.8	1.07	NS
Propionate (P)	2.60	3.31	3.67	0.32	NS
Iso-butyrate	0.06	0.09	0.11	0.02	NS
Butyrate	0.81^{a}	2.39^{b}	1.89^{ab}	0.27	$P<0.05$
Iso-valerate	0.04	0.07	0.12	0.02	NS
Valerate	0.09^{a}	0.21^{b}	0.17^{ab}	0.03	$P<0.05$
Total VFA	10.2	16.6	16.7	1.71	NS
A:P	2.48^{a}	3.16^{c}	2.92^{b}	0.06	$P<0.05$
NH_3–N (mg/L) [¥]	298	296	303	3.36	NS

GG_{MF}, German grass-based mixed feed (90% German grass, 10% Ipil-ipil); VFA, volatile fatty acids; NH_3–N, ammonia-N; RB, Rice barn; WB, Wheat bran; KB, Kashari bran; GG_{MF}-RB, 60% GG_{MF} + 40% rice bran; GG_{MF}-WB, 60% GG_{MF} + 40% wheat bran; GG_{MF}-KB, 60% GG_{MF} + 40% kashari bran; SEM, Standard error of the mean; $^{a,\,b}$ means with different superscripts in the same row are significantly different at ($p<0.05$); [¥] blank syringe produced 307 mg/L NH_3–N.

4.3.3. Effects of single concentrates in Napier silage–Ipil-ipil-based mixed feed (NS_{MF})

The *in vitro* ruminal fermentation characteristics of the single concentrates with NS_{MF} are shown in Table 4.29. GP_{24} differed significantly among the treatments and produced the highest gas (40.5 mL/200 mg DM) when WB was used as a supplement to the NS_{MF}. No significant differences were observed between NS_{MF}-RB and NS_{MF}-KB with respect to *c*GP, whereas the *c*GP of NS_{MF}-WB was the highest ($p<0.05$). No significant difference was recorded for dOM between NS_{MF}-WB (61.6%) and NS_{MF}-KB (59.6%); however, it significantly differed from NS_{MF}-RB (46.2%). The ME and NEL of NS_{MF}-WB were significantly highest (8.58 MJ ME/kg DM and 5.03 MJ NEL/kg DM, respectively) followed by that of NS_{MF}-KB (8.00 MJ ME/kg DM and 4.59 MJ NEL/kg DM, respectively) and NS_{MF}-RB (5.88 MJ ME/kg DM and 3.03 MJ NEL/kg DM, respectively).

Table 4.29: *In vitro* ruminal fermentation characteristics of Napier silage–Ipil-ipil-mixed feed (NS_{MF}) with different added concentrates

Parameters	**NS_{MF}**			**SEM**	**Sig.**
	NS_{MF}-RB	**NS_{MF}-WB**	**NS_{MF}-KB**		
GP_{24} (mL/200 mg DM)	21.3^a	40.5^c	35.5^b	1.28	$P<0.05$
*p*GP (mL/200 mg DM)	30.5^a	50.2^b	53.4^c	1.65	$P<0.05$
*c*GP (%/h)	5.03^a	7.54^b	4.56^a	0.12	$P<0.05$
dOM (%)	46.2^a	61.6^b	59.6^b	1.13	$P<0.05$
ME (MJ/kg DM)	5.88^a	8.58^c	8.00^b	0.18	$P<0.05$
NEL (MJ/kg DM)	3.03^a	5.03^c	4.59^b	0.13	$P<0.05$

NS_{MF}, Napier silage-based mixed feed (70% Napier silage, 30% Ipil-ipil); GP_{24}, Gas production at 24 h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; RB, Rice barn; WB, Wheat bran; KB, Kashari bran; NS_{MF}-RB; 60% NS_{MF} + 40% rice bran; NS_{MF}-WB; 60% NS_{MF} + 40% wheat bran; NS_{MF}-KB; 60% NS_{MF} + 40% kashari bran; SEM: Standard error of the mean; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$)

The cumulative gas production of Napier silage–Ipil-ipil-based mixed feed with different added concentrates is shown in Fig. 28. The highest gas production was observed for NS_{MF}-WB from 0 h to 60 h. After this period**,** NS_{MF}-KB exceeded the NS_{MF}-WB gas production curve and reached the highest value (52 mL/200 mg DM) at 72 h of incubation.

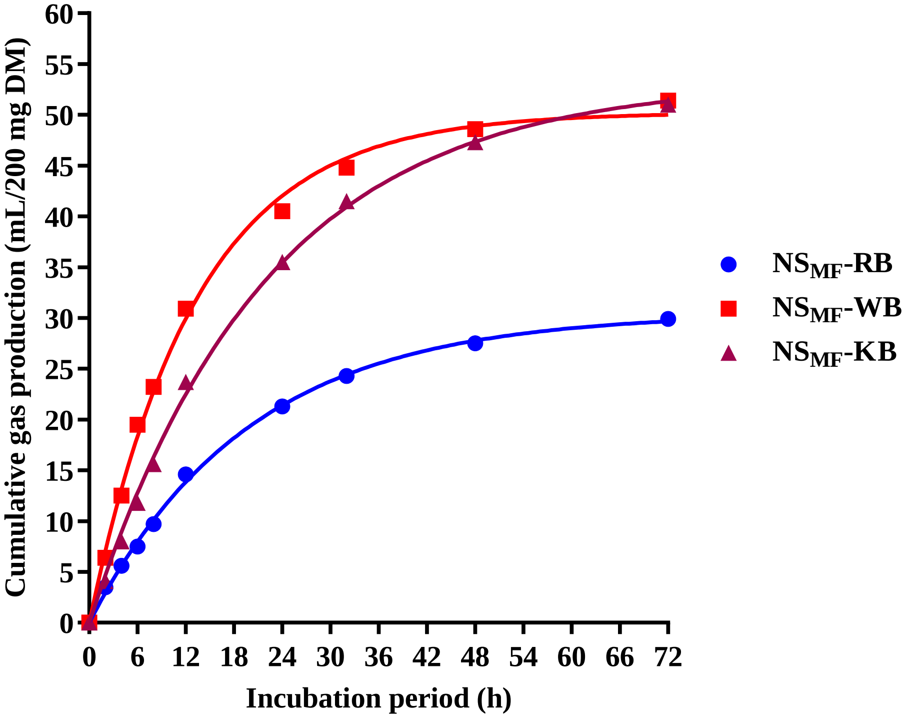

Fig. 28: Cumulative gas production profile of single concentrate with Napier silage–Ipil-ipil-based mixed feed (NS_{MF}). The *in vitro* gas production profile has been fitted using the equation $Y= p\text{GP}\cdot (1-e^{-c\text{GP}\cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in table 4.29.

No significant difference was observed in the CH_4 concentration among the treatments (Table 4.30). The highest CH_4 production, CH_4/dOM, and CH_4/DM were recorded (4.27 mL/120 mg DM, 57.8 L CH_4/kg dOM, and 35.6 L CH_4/kg DM, respectively) when WB was added to the NS_{MF} ($p<0.05$); the lowest value was observed with the addition of RB to the NS_{MF}.

Table 4.30: CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM measured in Napier silage–Ipil-ipil-mixed feed (NS_{MF}) with different added concentrates

Parameters	**NS_{MF}**			**SEM**	**Sig.**
	NS_{MF}-RB	**NS_{MF}-WB**	**NS_{MF}-KB**		
CH_4 mL/120 mg DM	2.15^a	4.27^c	3.65^b	0.12	$P<0.05$
CH_4 conc. in GP (%)	15.8	17.2	16.3	0.47	NS
L CH_4/kg dOM	38.7^a	57.8^c	51.1^b	1.72	$P<0.05$
L CH_4/kg DM	17.9^a	35.6^c	30.5^b	1.00	$P<0.05$

NS_{MF}, Napier silage-based mixed feed (70% Napier silage, 30% Ipil-ipil); CH_4, Methane; CH_4 conc., Methane concentration; dOM, Digestibility of organic matter; DM, Dry matter; RB, Rice barn; WB, Wheat bran; KB, Kashari bran; NS_{MF}-RB, 60% NS_{MF} + 40% rice bran; NS_{MF}-WB, 60% NS_{MF} + 40% wheat bran, NS_{MF}-KB, 60% NS_{MF} + 40% kashari bran; SEM, Standard error of the mean; NS, Not significant $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$)

The VFA concentrations in the three diets are presented in Table 4.31. Butyrate was the only VFA significantly affected. The lowest butyrate production was obtained (0.82 mmol/L) when RB was added to the NS_{MF} whereas NS_{MF}-WB produced the highest concentration of butyrate (2.17 mmol/L) ($p<0.05$). The ratio of acetate to propionate was highest for NS_{MF}-WB (3.15 mmol/L), followed by NS_{MF}-KB (2.84 mmol/L) and NS_{MF}-RB (2.41 mmol/L) ($p<0.05$). There were no significant differences in NH_3–N concentrations among treatments.

Table 4.31: Concentrations of VFA and NH_3–N after 24 h of incubation of Napier silage–Ipil-ipil-mixed feed (NS_{MF}) with different added concentrates

Parameters	**NS_{MF}**			**SEM**	**Sig.**
	NS_{MF}-RB	**NS_{MF}-WB**	**NS_{MF}-KB**		
	VFA (mmol/L)				
Acetate (A)	6.17	9.24	9.33	1.07	NS
Propionate (P)	2.54	2.93	3.28	0.32	NS
Iso-butyrate	0.07	0.08	0.10	0.02	NS
Butyrate	0.82^{a}	2.17^{b}	1.68^{ab}	0.27	$P<0.05$
Iso-valerate	0.06	0.05	0.11	0.02	NS
Valerate	0.11	0.20	0.16	0.03	NS
Total VFA	9.77	14.7	14.7	1.71	NS
A:P	2.41^{a}	3.15^{c}	2.84^{b}	0.05	$P<0.05$
NH_3–N (mg/L) $^{¥}$	295	301	339	3.92	NS

NS_{MF}, Napier silage-based mixed feed (70% Napier silage, 30% Ipil-ipil); VFA, volatile fatty acids; NH_3–N, ammonia–N; RB, Rice barn; WB, Wheat bran; KB, Kashari bran; NS_{MF}-RB, 60% NS_{MF} + 40% rice bran; NS_{MF}-WB, 60% NS_{MF} + 40% wheat bran; NS_{MF}-KB, 60% NS_{MF} + 40% kashari bran; SEM, Standard error of the mean; NS, Not significant; $^{a, b}$ means with different superscripts in the same row are significantly different at ($p< 0.05$); $^{¥}$ blank syringe produced 307 mg/L NH_3-N.

4.3.4. Effects of single concentrates in maize silage–Ipil-ipil-based mixed feed (MS_{MF})

The in vitro ruminal fermentation characteristics of MS_{MF} with different added concentrates are presented in Table 4.32. The GP_{24}, *c*GP, and dOM of MS_{MF}-WB were significantly higher (42.2 mL/200 mg DM, 7.88%/h and 63.6%, respectively) than those of the other two treatments. The same trend was obtained for ME and NEL (8.86 MJ ME/kg DM and 5.23 MJ NEL/kg DM, respectively) followed by **MS_{MF}-KB (**7.97 MJ ME/kg DM and 4.57 MJ NEL/kg DM, respectively) and **MS_{MF}-RB (**6.12 MJ ME/kg DM and 3.20 MJ NEL/kg DM, respectively).

Table 4.32: *In vitro* ruminal fermentation characteristics of maize silage–Ipil-ipil-mixed feed (MS_{MF}) with different added concentrates

Parameters	**MS_{MF}**			**SEM**	**Sig.**
	MS_{MF}-RB	**MS_{MF}-WB**	**MS_{MF}-KB**		
GP_{24} (mL/200 mg DM)	22.7^{a}	42.2^{c}	35.1^{b}	1.28	$P<0.05$
*p*GP (mL/200 mg DM)	31.8^{a}	52.2^{b}	53.3^{b}	1.65	$P<0.05$
*c*GP (%/h)	5.51^{b}	7.88^{c}	4.57^{a}	0.12	$P<0.05$
dOM (%)	47.9^{a}	63.6^{c}	59.7^{b}	1.13	$P<0.05$
ME (MJ/kg DM)	6.12^{a}	8.86^{c}	7.97^{b}	0.18	$P<0.05$
NEL (MJ/kg DM)	3.20^{a}	5.23^{c}	4.57^{b}	0.13	$P<0.05$

MS_{MF}, maize silage-based mixed feed (70% maize silage, 30% Ipil-ipil); GP_{24}, Gas production at 24h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; RB, Rice barn; WB, Wheat bran; KB, Kashari bran; MS_{MF}-RB, 60% MS_{MF} + 40% rice bran; MS_{MF}-WB, 60% MS_{MF} + 40% wheat bran; MS_{MF}-KB, 60% MS_{MF} + 40% kashari bran; SEM, Standard error of the mean; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

The interaction effect between the MS_{MF} with different added concentrates and the incubation time on the *in vitro* cumulative GP is shown in Fig. 29. The cumulative gas production of MS_{MF}-WB increased gradually with an increase in the incubation period and reached the highest value (53.6 mL/200 mg DM) at the end of 72 h of incubation compared to the other two treatments.

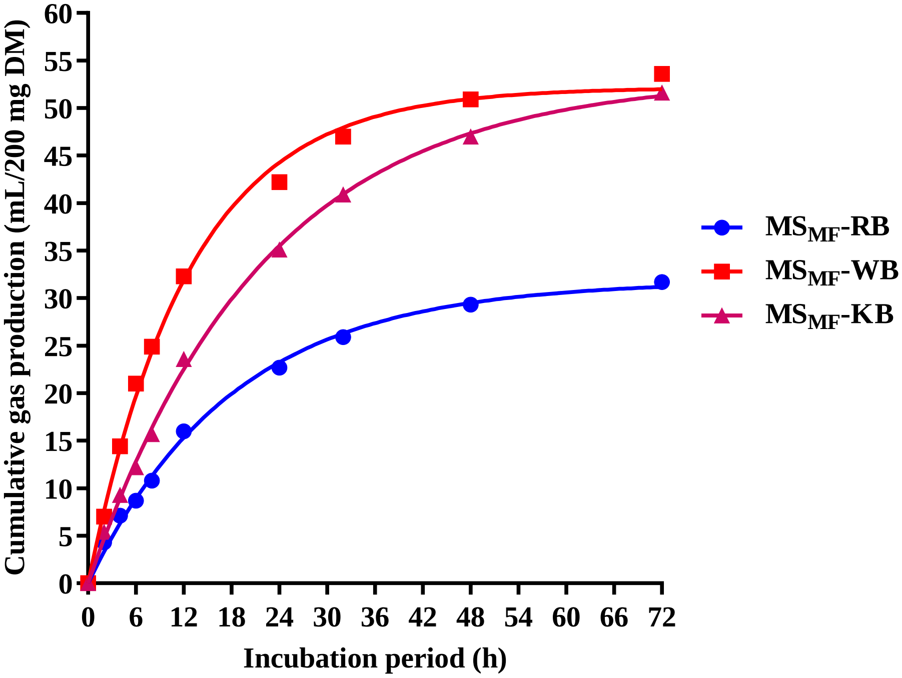

Fig. 29: Cumulative gas production profile of single concentrate with maize silage–Ipil-ipil-based mixed feed (MS_{MF}). The *in vitro* gas production profile has been fitted using the equation $Y= p\mathrm{GP}\cdot (1-e^{-c\mathrm{GP}\cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in table 4.32.

The CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM of MS_{MF} with different added concentrates are given in Table 4.33. MS_{MF}-RB had the lowest CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM (2.08 mL/120 mg DM, 14.5%, 36.2 L CH_4/kg dOM and 17.3 L CH_4/kg DM, respectively), followed by **MS_{MF}-KB (**3.80 mL/120 mg DM, 16.3%, 53.1 L CH_4/kg dOM, and 31.7 L CH_4/kg DM, respectively) and **MS_{MF}-WB (**4.46 mL/120 mg DM, 16.9%, 58.5 L CH_4/kg dOM, and 37.2 L CH_4/kg DM, respectively). There were no significant differences in the CH_4 concentration and CH_4/dOM between **MS_{MF}-WB and MS_{MF}-KB.**

Table 4.33: CH_4 production, CH_4 concentration, CH_4/dOM, and CH_4/DM measured in maize silage–Ipil-ipil-mixed feed (MS_{MF}) with different added concentrates

Parameters	MS_{MF}			SEM	Sig.
	MS_{MF}-RB	MS_{MF}-WB	MS_{MF}-KB		
CH_4 mL/120 mg DM	2.08^a	4.46^c	3.80^b	0.12	P<0.05
CH_4 conc. in GP (%)	14.5^a	16.9^b	16.3^b	0.47	P<0.05
L CH_4/kg dOM	36.2^a	58.5^b	53.1^b	1.72	P<0.05
L CH_4/kg DM	17.3^a	37.2^c	31.7^b	1.00	P<0.05

MS_{MF}, maize silage-based mixed feed (70% maize silage, 30% Ipil-ipil); CH_4, Methane; CH_4 conc., CH_4 concentration; dOM, Digestibility of organic matter; DM, Dry matter; RB, Rice barn; WB, Wheat bran; KB, Kashari bran; MS_{MF}-RB, 60% MS_{MF} + 40% rice bran; MS_{MF}-WB, 60% MS_{MF} + 40% wheat bran; MS_{MF}-KB, 60% MS_{MF} + 40% kashari bran; SEM, Standard error of the mean; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p < 0.05$)

No significant differences were observed in the VFA concentrations among the treatments (Table 4.34). The acetate-to-propionate ratio was significantly lower (2.21) in MS_{MF}-RB than in MS_{MF}-KB (2.59) and MS_{MF}-WB (2.71). NH_3–N concentration was not significantly different among the treatments.

Table 4.34: Concentrations of VFA and NH_3–N after 24 h of incubation of a maize silage–Ipil-ipil-mixed feed (MS_{MF}) with different added concentrates

Parameters	**MS_{MF}**			**SEM**	**Sig.**
	MS_{MF}-RB	**MS_{MF}-WB**	**MS_{MF}-KB**		
		VFA (mmol/L)			
Acetate (A)	6.24	8.08	9.39	1.07	NS
Propionate (P)	2.78	2.97	3.62	0.32	NS
Iso-butyrate	0.06	0.08	0.10	0.02	NS
Butyrate	0.79	1.95	1.70	0.27	NS
Iso-valerate	0.05	0.05	0.12	0.02	NS
Valerate	0.10	0.19	0.18	0.03	NS
Total VFA	10.0	13.3	15.1	1.71	NS
A:P	2.21^a	2.71^b	2.59^b	0.06	$P<0.05$
NH_3–N (mg/L) $^¥$	306	311	312	4.29	NS

MS_{MF}, maize silage-based mixed feed (70% maize silage, 30% Ipil-ipil); VFA, volatile fatty acids; NH_3–N, ammonia-N; RB, Rice barn; WB, Wheat bran; KB, Kashari bran; MS_{MF}-RB, 60% MS_{MF} + 40% rice bran; MS_{MF}-WB, 60% MS_{MF} + 40% wheat bran; MS_{MF}-KB, 60% MS_{MF} + 40% kashari bran; SEM, Standard error of the mean; NS, Not significant; $^{a, b}$ means with different superscripts in the same row are significantly different at ($p< 0.05$); $^¥$ blank syringe produced 307 mg/L NH_3-N.

4.4. Formulation of TMR

4.4.1. Rice straw-based TMR (RS_{TMR})

The *in vitro* ruminal fermentation characteristics of different RS_{TMR} are shown in Table 4.35. The total GP_{24} was affected by the different types of RS_{TMR} in all incubation periods ($p<0.05$); however, there were no significant variations among three of the RS_{TMR} treatments, namely, RS_{TMR}-3, RS_{TMR}-4, and RS_{TMR}-6. After 24 h of incubation, a greater amount of gas was produced in RS_{TMR}-3 and RS_{TMR}-6 ($p<0.05$) than in the other RS_{TMR} treatments. The same trend was observed for dOM, ME, and NEL, which were greatest for RS_{TMR}-3 and RS_{TMR}-6 followed by RS_{TMR}-2, RS_{TMR}-5, and RS_{TMR}-1, respectively. The *c*GP values of RS_{TMR}-3 and RS_{TMR}-6 were also significantly ($p<0.05$) higher than those of the other treatments.

Table 4.35: *In vitro* ruminal fermentation characteristics of RS_{TMR}

Parameters	(RS_{TMR})						SEM	Sig.
	RS_{TMR}-1	RS_{TMR}-2	RS_{TMR}-3	RS_{TMR}-4	RS_{TMR}-5	RS_{TMR}-6		
GP_{24} (mL/200 mg DM)	28.9^{c}	33.3^{b}	39.9^{a}	38.2^{a}	30.4^{c}	39.9^{a}	0.75	$P<0.05$
*p*GP (mL/200 mg DM)	42.0^{c}	49.9^{b}	52.2^{ab}	54.2^{a}	42.2^{c}	52.1^{ab}	0.69	$P<0.05$
*c*GP (%/h)	4.86^{bc}	4.50^{c}	6.43^{a}	5.17^{b}	5.45^{b}	6.34^{a}	0.26	$P<0.05$
dOM (%)	54.5^{c}	58.5^{b}	63.3^{a}	62.6^{a}	55.3^{c}	63.2^{a}	0.78	$P<0.05$
ME (MJ/kg DM)	6.99^{c}	7.63^{b}	8.53^{a}	8.34^{a}	7.17^{c}	8.53^{a}	0.12	$P<0.05$
NEL (MJ/kg DM)	3.85^{c}	4.32^{b}	4.98^{a}	4.84^{a}	3.98^{c}	4.98^{a}	0.09	$P<0.05$

RS_{MF}, Rice straw-based mixed feed (60% rice straw, 40% Ipil-ipil); GP_{24}, Gas production at 24h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; RS_{TMR}-1, 60% RS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); RS_{TMR}-2, 60% RS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); RS_{TMR}-3, 60% RS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); RS_{TMR}-4, 60% RS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); RS_{TMR}-5, 60% RS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); RS_{TMR}-6, 60% RS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of mean; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

The cumulative gas production of the different RS_{TMR} treatments during the incubation period is shown in Fig. 30. The highest gas production was observed for RS_{TMR}-3 and RS_{TMR}-6 from 0 h to 48 h. Subsequently, gas production in RS_{TMR}-4 exceeded both gas production curves and reached a maximum value (53.2 mL/200 mg DM) at 72 h of incubation.

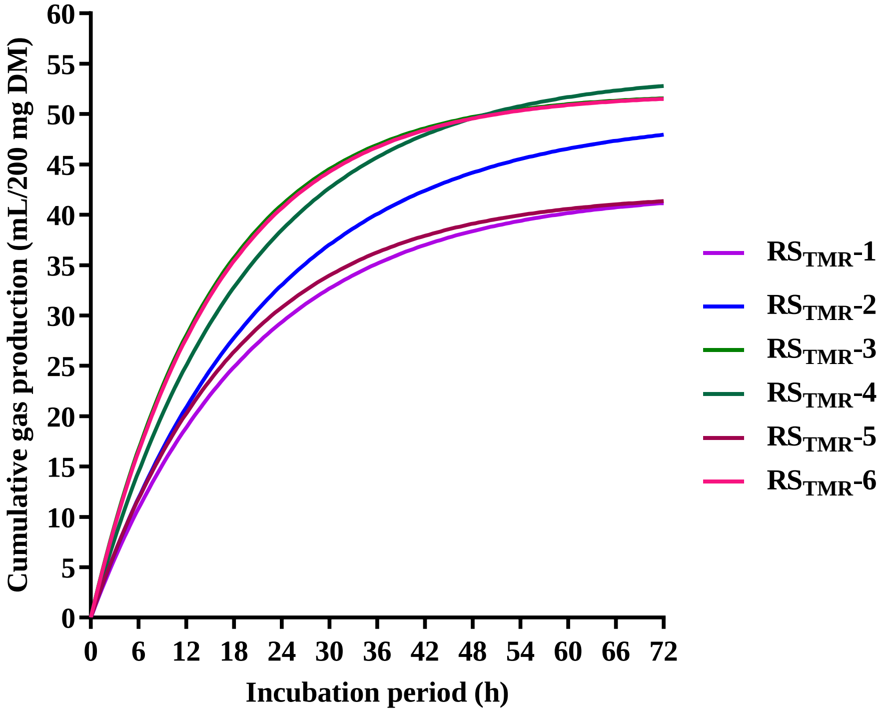

Fig. 30: Cumulative gas production profile of rice straw-based TMR. The *in vitro* gas production profile has been fitted using the equation $Y= pGP\cdot(1-e^{-cGP\cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in Table 4.35.

CH_4 production at 24 h and related traits during *in vitro* rumen fermentation are given in Table 4.36. The **RS_{TMR}-1 treatment** had the lowest CH_4 production (2.79 mL/120 mg DM), whereas the highest was acquired with **RS_{TMR}-6** (3.88 mL/120 mg DM). Remarkably, the CH_4 concentration of **RS_{TMR}-3** was significantly ($p<0.05$) lower (14.7% in GP) than those of RS_{TMR}-4 and RS_{TMR}-6; however, the opposite trend was recorded for **RS_{TMR}**-6 which produced the highest concentration of CH_4 (17.9% in GP). Therefore, considering CH_4/dOM, RS_{TMR}-1 produced the lowest concentration (42.9 L CH_4/kg dOM).

Table 4.36: CH_4 production, CH_4 concentration, and CH_4/dOM of RS_{TMR}

Parameters	RS_{TMR}						SEM	Sig.
	RS_{TMR}-1	RS_{TMR}-2	RS_{TMR}-3	RS_{TMR}-4	RS_{TMR}-5	RS_{TMR}-6		
CH_4 mL/120 mg DM	2.79^{c}	3.20^{bc}	3.53^{ab}	3.88^{a}	2.90^{c}	3.95^{a}	0.22	$P<0.05$
CH_4 conc. in GP (%)	15.9^{ab}	16.5^{ab}	14.7^{b}	17.1^{a}	16.5^{ab}	17.9^{a}	0.58	$P<0.05$
L CH_4/kg dOM	42.9^{c}	45.6^{abc}	46.5^{abc}	51.6^{ab}	43.8^{bc}	52.0^{a}	3.06	$P<0.05$

RS_{MF}, Rice straw-based mixed feed (60% rice straw, 40% Ipil-ipil); CH_4, Methane; CH_4 conc., Methane concentration; dOM, Digestibility of organic matter DM; Dry matter, RB; Rice bran; WB, Wheat bran; KB, Kashari bran; RS_{TMR}-1, 60% RS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); RS_{TMR}-2, 60% RS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); RS_{TMR}-3, 60% RS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); RS_{TMR}-4, 60% RS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); RS_{TMR}-5, 60% RS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); RS_{TMR}-6, 60% RS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of the mean; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

The concentrations of the VFAs for the different types of RS_{TMR} are shown in Table 4.37. No significant differences were observed among the treatments ($P >0.05$). Nevertheless, it was observed that RS_{TMR}-3 produced the highest concentration of propionate, butyrate, and total VFA (2.59 mmol/L, 1.59 mmol/L and 12.3 mmol/L).

Table 4.37: Volatile fatty acids (VFA) production of RS_{TMR}

Parameters	RS_{TMR}						SEM	Sig.
	RS_{TMR}-1	RS_{TMR}-2	RS_{TMR}-3	RS_{TMR}-4	RS_{TMR}-5	RS_{TMR}-6		
	VFA (mmol/L)							
Acetate (A)	6.38	6.96	7.87	7.56	6.58	7.69	1.70	NS
Propionate (P)	2.37	2.49	2.59	2.58	2.38	2.58	0.52	NS
Iso-butyrate	0.06	0.06	0.07	0.06	0.06	0.06	0.02	NS
Butyrate	0.96	1.13	1.59	1.38	1.10	1.52	0.40	NS
Iso-valerate	0.02	0.02	0.01	0.02	0.02	0.02	0.13	NS
Valerate	0.11	0.12	0.16	0.14	0.13	0.16	0.04	NS
Total VFA	9.90	10.8	12.3	11.7	10.3	12.0	2.78	NS
A:P	2.60	2.70	3.00	2.87	2.71	2.96	0.14	NS

RS_{MF}, Rice straw-based mixed feed (60% rice straw, 40% Ipil-ipil); VFA, Volatile fatty acids; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; RS_{TMR}-1, 60% RS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); RS_{TMR}-2, 60% RS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); RS_{TMR}-3, 60% RS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); RS_{TMR}-4, 60% RS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); RS_{TMR}-5, 60% RS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); RS_{TMR}-6, 60% RS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); NS, Not significant.

The associative effects calculated from the estimated and measured values of different RS_{TMR} treatments on rumen fermentation characteristics and CH_4 reduction are given in Table 4.38. The table shows the significant differences between the estimated and measured dOM, ME, and NEL values, which demonstrated a positive associative effect. Although RS_{TMR}-6 showed the most significant ($p<0.05$) interaction between dOM, ME, and NEL (10.1%, 12.5%, and 16.1%, respectively), it did not show any significant reduction in CH_4 concentration. In contrast, the RS_{TMR}-3 showed a remarkable reduction in CH_4 production, CH_4 concentration, and CH_4/dOM (–8.63%, –9.63%, and –8.41%, respectively), which was significant ($p<0.05$).

The results of the linear regression analysis between the estimated and measured values of the *in vitro* rumen fermentation characteristics and CH_4 reduction are presented in Table 4.39. The measured values of dOM and ME were positively correlated with estimated values (slopes = 1.24 and 1.26 and R^2 values = 0.91 and 0.93, respectively) (Fig. 31 and 32). The CH_4 concentration was negatively correlated with the estimated values (slope = –0.17 and RMSE = 1.2), indicating that the dataset was moderately accurate.

Table 4.38: Associative effect on *in vitro* ruminal fermentation and CH_4 reduction potential of RS_{TMR}

Parameters	**RS_{TMR}**						**SEM**	**Sig.**
	RS_{TMR}-1	RS_{TMR}-2	RS_{TMR}-3	RS_{TMR}-4	RS_{TMR}-5	RS_{TMR}-6		
dOM (%)								
Estimated*	52.5	56.3	60.0	59.3	52.9	57.4		
Measured	54.5	58.5	63.3	62.6	55.3	63.2		
Associative effect (%)	3.87^{b}	3.98^{b}	5.59^{b}	5.59^{b}	4.46^{b}	10.1^{a}	0.82	$P<0.05$
ME (MJ/kg DM)								
Estimated*	6.66	7.27	7.98	7.79	6.79	7.58		
Measured	6.99	7.63	8.53	8.34	7.17	8.53		
Associative effect (%)	4.91^{b}	5.05^{b}	6.88^{b}	6.97^{b}	5.70^{b}	12.5^{a}	1.04	$P<0.05$
NEL (MJ/kg DM)								
Estimated*	3.61	4.06	4.58	4.45	3.70	4.29		
Measured	3.85	4.32	4.98	4.84	3.98	4.98		
Associative effect (%)	6.68^{b}	6.59^{b}	8.73^{b}	8.91^{b}	7.62^{b}	16.1^{a}	1.35	$P<0.05$
CH_4 production (mL)								
Estimated$^{\psi}$	2.71	3.21	3.86	3.67	2.84	3.53		
Measured	2.79	3.20	3.53	3.88	2.90	3.95		
Associative effect (%)	3.01^{ab}	-0.15^{ab}	-8.63^{b}	5.71^{ab}	2.23^{ab}	11.8^{a}	7.26	$P<0.05$
CH_4 concentration (%)								
Estimated$^{\psi}$	15.3	15.7	16.3	16.1	15.4	16.0		
Measured	15.9	16.5	14.7	17.1	16.5	17.9		
Associative effect (%)	4.16^{ab}	5.08^{a}	-9.63^{b}	6.15^{a}	7.40^{a}	11.9^{a}	4.92	$P<0.05$
L CH_4/kg dOM								
Estimated$^{\psi}$	39.9	44.7	50.8	49.1	41.0	47.6		
Measured	42.9	45.6	46.5	51.6	43.8	52.0		
Associative effect (%)	7.52^{a}	1.99^{ab}	-8.41^{b}	5.11^{ab}	6.76^{a}	9.35^{a}	7.58	$P<0.05$

RS_{MF}, Rice straw-based mixed feed (60% rice straw, 40% Ipil-ipil); dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH_4, Methane; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; RS_{TMR}-1, 60% RS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); RS_{TMR}-2, 60% RS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); RS_{TMR}-3, 60% RS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); RS_{TMR}-4, 60% RS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); RS_{TMR}-5, 60% RS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); RS_{TMR}-6, 60% RS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of the mean; $^{a,\ b}$ means with different superscripts in the same row are significantly different at ($p< 0.05$); * The estimated values were calculated from single feeds that were determined by the equation of Menke and Steingass (1988); $^{\psi,}$ The estimated values were calculated from single feeds.

Table 4.39: Results of linear regression of estimated and measured values of *in vitro* ruminal fermentation characteristics and CH_4 reduction of RS_{TMR}

Parameters	RS_{TMR} (†Estimated vs. Measured)					RMSE
	Slope	Slope CI	Intercept	Intercept CI	R^2	
dOM (%)	1.24	0.69 to 1.78	-10.5	-41.4 to 20.4	0.91	1.93
ME MJ/kg DM	1.26	0.76 to 1.76	-1.42	-5.08 to 2.22	0.93	0.21
NEL MJ/kg DM	1.25	0.76 to 1.74	-0.67	-2.70 to 1.34	0.93	0.15
CH_4 production (ml)	0.91	0.18 to 1.64	0.35	-2.06 to 2.77	0.75	0.27
CH_4 conc. (%)	-0.17	-3.93 to 3.58	19.2	-40.2 to 78.6	0.00	1.21
L CH_4/kg dOM	0.63	-0.19 to 1.46	18.1	-19.8 to 55.9	0.53	2.95

TMR, Total mixed ration; RS_{TMR}, Rice straw-based TMR; dOM, digestibility of organic matter; ME, metabolisable energy; NEL, net energy lactation; DM, dry matter; CH_4 conc., methane concentration. †The estimated values of CH_4-related traits were calculated from the single feeds using the equation of Menke and Steingass (1988) for determining the ruminal fermentation characteristics (dOM, ME, and NEL) from single feeds, which consistently indicated X-variables.

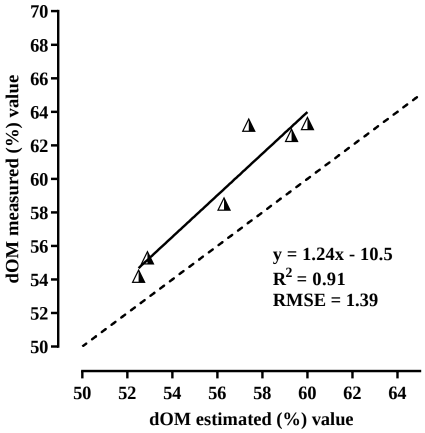

Fig. 31: Comparison of estimated and measured digestibility of organic matter (dOM) values of different combinations of rice straw-based TMR(RS_{TMR}) using an *in vitro* ruminal fermentation technique. The dOM values of compound feeds were calculated from dOM values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

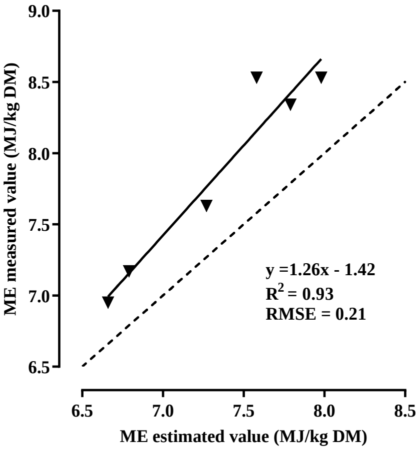

Fig. 32: Comparison of estimated and measured metabolisable energy (ME) values of different combinations of rice straw-based TMR (RS_{TMR}) using an *in vitro* ruminal fermentation technique. The ME values of compound feeds were calculated from ME values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

4.4.2. German grass-based TMR (GG_{TMR})

The rumen fermentation characteristics of GG_{TMR} are presented in Table 4.40. GG_{TMR}-3 produced the highest volume of GP_{24} (44.3 mL/200 mg DM), whereas the *c*GP of GG_{TMR}-6 showed the same trend as the GP_{24} of GG_{TMR}-3 (6.62 %/h), which was significantly ($p<0.05$) higher than those GG_{TMR}-1, GG_{TMR}-2, and GG_{TMR}-4. The highest dOM was recorded for GG_{TMR}-3 (68.8%), which was significantly higher than those of other treatments ($p<0.05$), except those of GG_{TMR}-4 and GG_{TMR}-6. The opposite trend was recorded for GG_{TMR}-1 and GG_{TMR}-5, which produced the lowest concentration of ME and NEL (7.85 MJ ME/kg DM and 4.48 MJ NEL/kg DM, and 7.76 MJ ME/kg DM and 4.42 MJ NEL/kg DM, respectively), while the GG_{TMR}-3 was the highest (9.16 MJ ME/kg DM and 5.45 MJ NEL/kg DM).

Table 4.40: *In vitro* ruminal fermentation characteristics of GG_{TMR}

Parameters	**GG_{TMR}**						**SEM**	**Sig.**
	GG_{TMR}-1	GG_{TMR}-2	GG_{TMR}-3	GG_{TMR}-4	GG_{TMR}-5	GG_{TMR}-6		
GP_{24} (mL/200 mg DM)	35.0^{d}	37.2^{cd}	44.3^{a}	40.1^{bc}	34.6^{d}	41.1^{ab}	0.87	P<0.05
*p*GP (mL/200 mg DM)	45.9^{c}	50.8^{b}	55.7^{a}	53.2^{ab}	44.2^{c}	51.5^{b}	0.68	P<0.05
*c*GP (%/h)	5.59^{bc}	5.10^{c}	6.38^{a}	5.52^{bc}	6.02^{ab}	6.62^{a}	0.26	P<0.05
dOM (%)	61.3^{cd}	63.5^{bc}	68.8^{a}	65.8^{ab}	60.5^{d}	65.8^{ab}	0.77	P<0.05
ME (MJ/kg DM)	7.85^{d}	8.18^{cd}	9.16^{a}	8.59^{bc}	7.76^{d}	8.69^{ab}	0.12	P<0.05
NEL (MJ/kg DM)	4.48^{d}	4.73^{cd}	5.45^{a}	5.03^{bc}	4.42^{d}	5.10^{ab}	0.09	P<0.05

GG_{MF}, German grass-based mixed feed (90% German grass, 10% Ipil-ipil); GP_{24},Gas production at 24 h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; GG_{TMR}-1, 60% GG_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); GG_{TMR}-2, 60% GG_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); GG_{TMR}-3, 60% GG_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); GG_{TMR}-4, 60% GG_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); GG_{TMR}-5, 60% GG_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); GG_{TMR}-6, 60% GG_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of mean; $^{a\text{-}d}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

Interaction effects between the different combinations of German grass-based TMR and the incubation period were observed on the *in vitro* cumulative GP (Fig. 33). The gas production trend of GG_{TMR}-3 was the highest with an increase in the incubation period from 0 h to 72 h with a maximum value of 54.6 mL/200 mg DM.

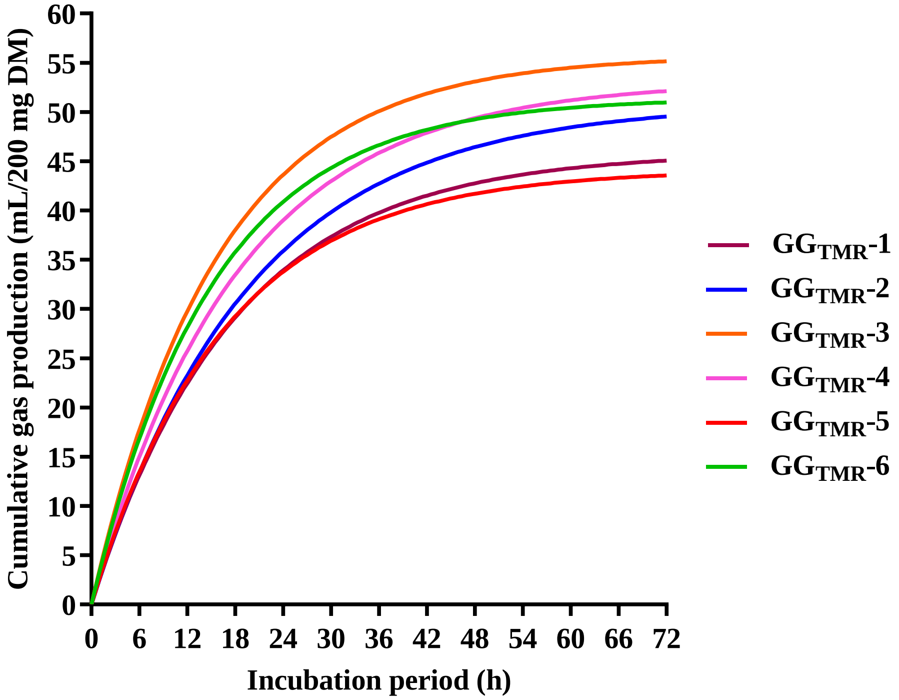

Fig. 33: Cumulative gas production profile of German grass-based TMR. The *in vitro* gas production profile has been fitted using the equation $Y= pGP\cdot (1-e^{-cGP\cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in Table 4.40.

CH_4 production and its related traits are listed in Table 4.41. The GG_{TMR}-4 and GG_{TMR}-6 treatments produced the highest concentration of CH_4 (4.21 mL/120 mg DM and 4.07 mL/120 mg DM, respectively), whereas the GG_{TMR}-1 showed the lowest ($p<0.05$) production of CH_4 (3.33 mL/120 mg DM); however, there was no significant difference among GG_{TMR}-2, GG_{TRM}-3, and GG_{TMR}-5. Surprisingly, the opposite trend was obtained for GG_{TMR}-3, which produced the lowest concentration of CH_4 (14.5%) among all treatments. No significant differences for CH_4/dOM were observed among the treatments.

Table 4.41: CH_4 production, CH_4 concentration, and CH_4/dOM of GG_{TMR}

Parameters	**GG_{TMR}**						**SEM**	**Sig.**
	GG_{TMR}-1	GG_{TMR}-2	GG_{TMR}-3	GG_{TMR}-4	GG_{TMR}-5	GG_{TMR}-6		
CH_4 mL/120 mg DM	3.33^{b}	3.79^{ab}	3.72^{ab}	4.21^{a}	3.64^{ab}	4.07^{a}	0.22	$P<0.05$
CH_4 conc. in GP (%)	16.8^{a}	16.7^{a}	14.5^{b}	16.9^{a}	16.9^{a}	16.6^{a}	0.58	$P<0.05$
L CH_4/kg dOM	45.3	49.7	45.0	53.4	50.2	51.6	3.07	NS

GG_{MF}, German grass-based mixed feed (90% German grass, 10% Ipil-ipil); CH_4, Methane; CH_4 conc., Methane concentration; dOM, Digestibility of organic matter; RB;, Rice bran; WB, Wheat bran; KB, Kashari bran; GG_{TMR}-1, 60% GG_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); GG_{TMR}-2, 60% GG_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); GG_{TMR}-3, 60% GG_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); GG_{TMR}-4, 60% GG_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); GG_{TMR}-5, 60% GG_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); GG_{TMR}-6, 60% GG_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); NS, Not significant; SEM, Standard error of the mean; $^{a,\,b}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

The production of individual VFA and total VFA in this study is presented in Table 4.42. From Table 4.42, it can be seen that all treatments had significant effects ($p<0.05$) on total VFA production, acetate, butyrate, valerate production, and the acetate to propionate ratio; there was no significant effect on propionate production. The highest acetate and total VFA concentration were observed both in GG_{TMR}-3 and GG_{TMR}-4 (10.2 mmol/L, 10.3 mol/L, and 16.0 mmol/L, respectively), which was significantly ($p<0.05$) higher than those of GG_{TMR}-1 and GG_{TMR}-5. Interestingly, GG_{TMR}-3 also produced the highest concentration of butyrate (2.09 mmol/L). In contrast, the highest valerate production was recorded for GG_{TMR}-6 (0.20 mmol/L).

Table 4.42: Volatile fatty acids (VFA) production of GG$_{\text{TMR}}$

Parameters	GG$_{\text{TMR}}$						SEM	Sig.
	GG$_{\text{TMR}}$-1	GG$_{\text{TMR}}$-2	GG$_{\text{TMR}}$-3	GG$_{\text{TMR}}$-4	GG$_{\text{TMR}}$-5	GG$_{\text{TMR}}$-6		
	VFA (mmol/L)							
Acetate (A)	8.20^{b}	9.47^{ab}	10.2^{a}	10.3^{a}	8.15^{b}	9.05^{ab}	0.61	$P<0.05$
Propionate (P)	2.99	3.32	3.35	3.46	2.92	3.06	0.22	NS
Iso-butyrate	0.08	0.09	0.10	0.10	0.08	0.12	0.01	NS
Butyrate	1.29^{c}	1.62^{abc}	2.09^{a}	1.93^{ab}	1.39^{bc}	1.84^{ab}	0.18	$P<0.05$
Iso-valerate	0.07	0.09	0.09	0.10	0.06	0.10	0.02	NS
Valerate	0.12^{c}	0.15^{abc}	0.19^{ab}	0.18^{abc}	0.13^{bc}	0.20^{a}	0.02	$P<0.05$
Total VFA	12.8^{b}	14.7^{ab}	16.0^{a}	16.0^{a}	12.7^{b}	14.4^{ab}	0.99	$P<0.05$
A:P	2.73^{d}	2.85^{bc}	3.04^{a}	2.96^{ab}	2.78^{cd}	2.95^{ab}	0.04	$P<0.05$

GG$_{\text{MF}}$, German grass-based mixed feed (90% German grass, 10% Ipil-ipil); VFA, Volatile fatty acids; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; GG$_{\text{TMR}}$-1, 60% GG$_{\text{MF}}$ + 40% concentrates (60% RB + 10% WB + 30% KB); GG$_{\text{TMR}}$-2, 60% GG$_{\text{MF}}$ + 40% concentrates (30% RB + 10% WB + 60% KB); GG$_{\text{TMR}}$-3, 60% GG$_{\text{MF}}$ + 40% concentrates (10% RB + 60% WB + 30% KB); GG$_{\text{TMR}}$-4, 60% GG$_{\text{MF}}$ + 40% concentrates (10% RB + 30% WB + 60% KB); GG$_{\text{TMR}}$-5, 60% GG$_{\text{MF}}$ + 40% concentrates (60% RB + 30% WB + 10% KB); GG$_{\text{TMR}}$-6, 60% GG$_{\text{MF}}$ + 40% concentrates (30% RB + 60% WB + 10% KB); NS, Not significant; SEM, Standard error of mean; $^{a–d}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

The associative effects between the estimated and measured values of the rumen fermentation characteristics and CH_4 reduction of different GG$_{\text{TMR}}$ treatments are given in Table 4.43. A positive associative effect was observed between the estimated and measured values of GG$_{\text{TMR}}$. The GG$_{\text{TMR}}$-1 and GG$_{\text{TMR}}$-3 showed the highest associative effects ($p<0.05$) for dOM and ME (5.96% and 7.87%, and 5.23% and 6.52%, respectively) among all GG$_{\text{TMR}}$. Surprisingly, GG$_{\text{TMR}}$-3 resulted in the highest reductions in percentage for CH_4 production (–10.3%), CH_4 concentration (–9.90%), and CH_4/dOM (–11.4%), which were significantly ($p<0.05$) higher than in the other treatments.

Table 4.43: Associative effect on *in vitro* ruminal fermentation and CH_4 reduction of GG$_{TMR}$

Parameters	**GG$_{TMR}$**						**SEM**	**Sig.**
	GG$_{TMR}$-1	GG$_{TMR}$-2	GG$_{TMR}$-3	GG$_{TMR}$-4	GG$_{TMR}$-5	GG$_{TMR}$-6		
dOM (%)								
Estimated*	57.9	61.7	65.4	64.7	58.3	62.8		
Measured	61.3	63.5	68.8	65.8	60.5	65.8		
Associative effect (%)	5.96^{a}	2.97^{ab}	5.23^{a}	1.71^{b}	3.76^{ab}	4.79^{ab}	1.15	$P<0.05$
ME (MJ/kg DM)								
Estimated*	7.28	7.88	8.60	8.41	7.40	8.19		
Measured	7.85	8.18	9.16	8.59	7.76	8.69		
Associative effect (%)	7.87^{a}	3.81^{ab}	6.52^{a}	2.16^{b}	4.86^{ab}	6.01^{a}	1.60	$P<0.05$
NEL (MJ/kg DM)								
Estimated*	4.07	4.51	5.04	4.90	4.16	4.74		
Measured	4.48	4.73	5.45	5.03	4.42	5.10		
Associative effect (%)	10.3^{a}	4.85^{ab}	8.10^{ab}	2.70^{b}	6.30^{ab}	7.57^{ab}	1.88	$P<0.05$
CH_4 production (mL)								
Estimated$^{\psi}$	2.99	3.50	4.15	3.96	3.12	3.82		
Measured	3.33	3.75	3.72	4.21	3.64	4.07		
Associative effect (%)	11.2^{a}	8.43^{a}	-10.3^{b}	6.39^{a}	16.6^{a}	6.66^{a}	4.52	$P<0.05$
CH_4 concentration (%)								
Estimated$^{\psi}$	15.1	15.6	16.1	15.9	15.2	15.8		
Measured	16.8	16.7	14.5	16.9	16.9	16.6		
Associative effect (%)	11.3^{a}	7.17^{a}	-9.90^{b}	6.52^{a}	11.3^{a}	5.29^{a}	2.68	$P<0.05$
L CH_4/kg dOM								
Estimated$^{\psi}$	39.9	44.7	50.8	49.1	41.0	47.6		
Measured	45.3	49.7	45.0	53.4	50.2	51.6		
Associative effect (%)	13.6^{a}	11.1^{a}	-11.4^{b}	8.79^{a}	22.4^{a}	8.50^{a}	4.69	$P<0.05$

GG$_{MF}$, German grass-based mixed feed (90% German grass, 10% Ipil-ipil); dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH_4, Methane; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; GG$_{TMR}$-1, 60% GG$_{MF}$ + 40% concentrates (60% RB + 10% WB + 30% KB); GG$_{TMR}$-2, 60% GG$_{MF}$ + 40% concentrates (30% RB + 10% WB + 60% KB); GG$_{TMR}$-3, 60% GG$_{MF}$ + 40% concentrates (10% RB + 60% WB + 30% KB); GG$_{TMR}$-4, 60% GG$_{MF}$ + 40% concentrates (10% RB + 30% WB + 60% KB); GG$_{TMR}$-5, 60% GG$_{MF}$ + 40% concentrates (60% RB + 30% WB + 10% KB); GG$_{TMR}$-6, 60% GG$_{MF}$ + 40% concentrates (30% RB + 60% WB + 10% KB); NS, Not significant; SEM, Standard error of mean; $^{a, b}$ means with different superscripts in the same row are significantly different at ($p< 0.05$); *, The estimated values were calculated from single feeds that were determined by the equation of Menke and Steingass (1988); $^{\psi;}$ The estimated values were calculated from single feeds.

Table 4.44: Results of linear regression of *in vitro* ruminal fermentation and CH_4 reduction of GG_{TMR}

Parameters	GG_{TMR} ([†]Estimated vs. Measured)					RMSE
	Slope	Slope CI	Intercept	Intercept CI	R^2	
dOM (%)	0.94	0.53 to 1.35	5.84	-19.3 to 30.9	0.91	1.03
ME MJ/kg DM	0.96	0.55 to 1.36	0.73	-2.46 to 3.93	0.92	0.17
NEL MJ/kg DM	0.96	0.56 to 1.36	0.46	-1.35 to 2.28	0.92	0.12
CH_4 production (ml)	0.48	-0.17 to 1.13	2.06	-0.31 to 4.43	0.51	0.24
CH_4 conc. (%)	-1.42	-3.83 to 0.98	38.6	1.10 to 76.2	0.40	0.77
L CH_4/kg dOM	0.23	-1.21 to 1.67	37.3	-28.8 to 103	0.05	5.15

TMR, total mixed ration; GG_{TMR}, German grass-based TMR; dOM, digestibility of organic matter; ME, metabolisable energy; NEL, net energy lactation; DM, dry matter; CH_4 conc., methane concentration. [†]The estimated values of CH_4 related traits were calculated from the single feeds using the equation of Menke and Steingass (1988) to determine ruminal fermentation characteristics (dOM, ME, and NEL) from single feeds, which consistently indicated X variables.

The linear regression results between the estimated and measured values of the different GG_{TMR} are presented in Table 4.44. The measured value of dOM was positively correlated with the estimated value, with a slope of 0.94 and R^2 value of 0.91 (Fig. 34). The line slope of ME was 0.96, and this regression equation showed a higher R^2 value of 0.92 and a low RMSE of 0.17, indicating that the dataset is moderately accurate (Fig. 35). The opposite trend was observed for CH_4 concentration, which was negatively correlated with estimated values that indicated a reduction in the CH_4 concentration in the diet, with a slope of –1.42 and R^2 value of 0.77 (Fig. 36).

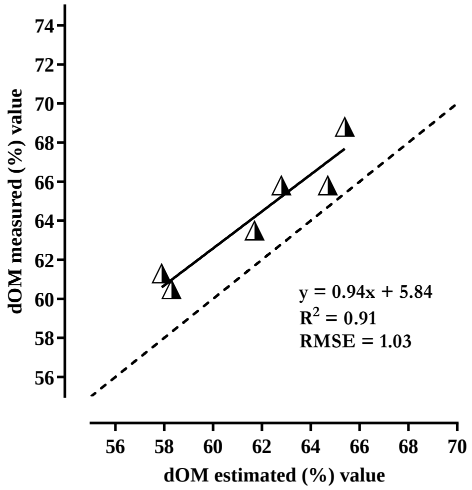

Fig. 34: Comparison of estimated and measured digestibility of organic matter (dOM) values of different combinations of German grass-based TMR(GG_{TMR}) using an *in vitro* ruminal fermentation technique. The dOM values of compound feeds were calculated from dOM values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

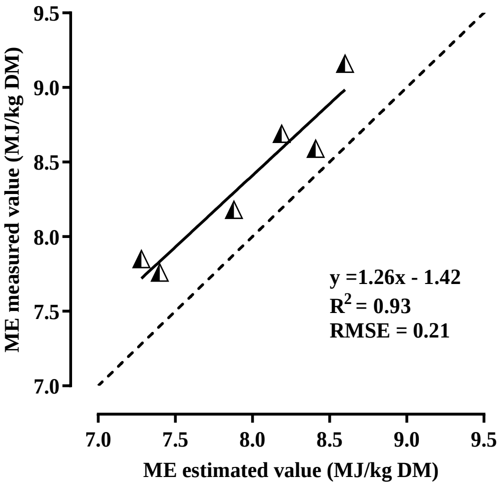

Fig. 35: Comparison of estimated and measured metabolisable energy (ME) values of different combinations of German grass-based TMR(GG_{TMR}) using an *in vitro* ruminal fermentation technique. The ME values of compound feeds were calculated from ME values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

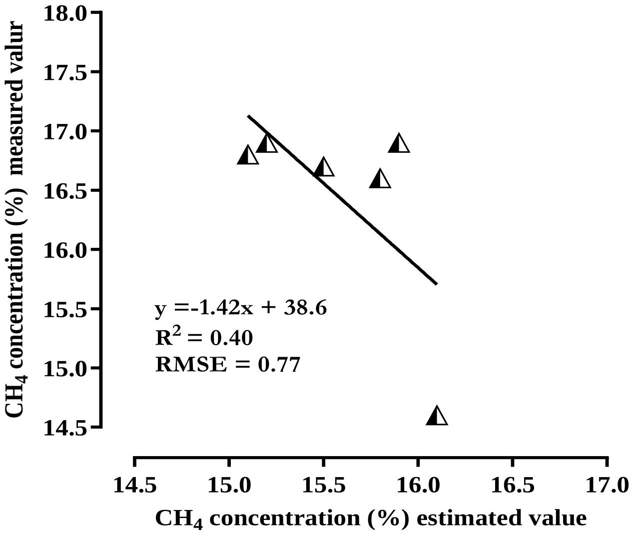

Fig. 36: Comparison of estimated and measured CH_4 concentration (%) values of different combinations of German grass-based TMR using an *in vitro* ruminal fermentation technique. The CH_4 concentration values of compound feeds were calculated from CH_4 concentration values of single feeds.

4.4.3. Napier silage-based TMR (NS$_{TMR}$)

The rumen fermentation characteristics of NS$_{TMR}$ are shown in Table 4.45. NS$_{TMR}$-3 produced the highest concentration of gas (39.9 mL/200 mg DM), which was significantly ($p<0.05$) higher than that of the other treatments except NS$_{TMR}$-4, however, no significant difference was recorded between NS$_{TMR}$-3 and NS$_{TMR}$-6. The opposite trend was observed for NS$_{TMR}$-1, which produced the lowest concentration of gas (29.0 mL/200 mg DM). No significant differences were seen between NS$_{TMR}$-3 and NS$_{TMR}$-6 for dOM, ME, and NEL; however, these were significantly ($p<0.05$) different from that of the other treatments.

Table 4.45: *In vitro* ruminal fermentation characteristics of NS$_{TMR}$

Parameters	**NS$_{TMR}$**						**SEM**	**Sig.**
	NS$_{TMR}$-1	NS$_{TMR}$-2	NS$_{TMR}$-3	NS$_{TMR}$-4	NS$_{TMR}$-5	NS$_{TMR}$-6		
GP$_{24}$ (mL/200 mg DM)	29.0^{d}	34.8^{b}	39.9^{a}	37.9^{ab}	31.3^{c}	38.9^{a}	0.87	P<0.05
*p*GP (mL/200 mg DM)	40.3^{b}	48.1^{a}	50.9^{a}	51.4^{a}	40.9^{b}	48.6^{a}	0.68	P<0.05
*c*GP (%/h)	5.16^{d}	5.15^{d}	6.67^{ab}	5.46^{cd}	6.01^{bc}	7.15^{a}	0.26	P<0.05
dOM (%)	53.2^{c}	58.5^{b}	62.0^{a}	60.9^{ab}	54.7^{c}	61.0^{ab}	0.77	P<0.05
ME (MJ/kg DM)	7.01^{c}	7.86^{b}	8.55^{a}	8.29^{ab}	7.31^{c}	8.39^{a}	0.12	P<0.05
NEL (MJ/kg DM)	3.87^{c}	4.49^{b}	5.00^{a}	4.81^{ab}	4.09^{c}	4.88^{a}	0.09	P<0.05

NS$_{MF}$, Napier silage-based mixed feed (70% Napier silage, 30% Ipil-ipil); GP$_{24}$, Gas production at 24 h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; NS$_{TMR}$-1, 60% NS$_{MF}$ + 40% concentrates (60% RB + 10% WB + 30% KB); NS$_{TMR}$-2, 60% NS$_{MF}$ + 40% concentrates (30% RB + 10% WB + 60% KB); NS$_{TMR}$-3, 60% NS$_{MF}$ + 40% concentrates (10% RB + 60% WB + 30% KB); NS$_{TMR}$-4, 60% NS$_{MF}$ + 40% concentrates (10% RB + 30% WB + 60% KB); NS$_{TMR}$-5, 60% NS$_{MF}$ + 40% concentrates (60% RB + 30% WB + 10% KB); NS$_{TMR}$-6, 60% NS$_{MF}$ + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of the mean; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

Interaction effects between the different NS_{TMR} combinations and the incubation period on *in vitro* cumulative gas production is shown in Fig. 37. NS_{TMR}-3 produced the highest concentration of gas from 0 h to 72 h (51.0 mL/200 mg DM).

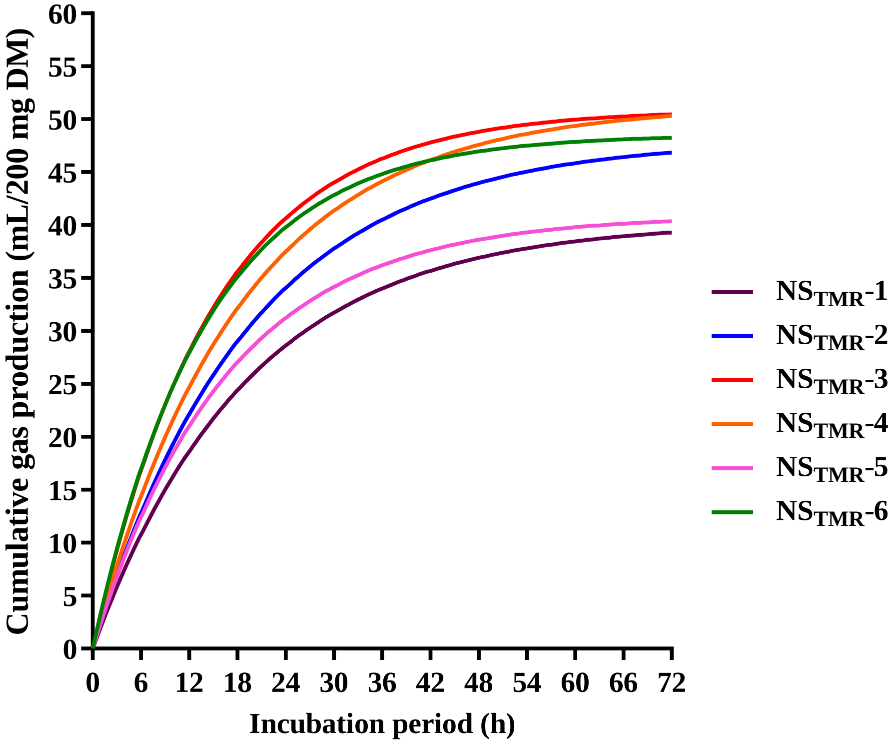

Fig. 37: Cumulative gas production profile of Napier silage-based TMR. The *in vitro* gas production profile has been fitted using the equation $Y = p\mathrm{GP} \cdot (1-e^{-c\mathrm{GP} \cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in Table 4.45.

The CH_4 production, CH_4 concentration, and CH_4/dOM of NS_{TMR} are presented in Table 4.46. The lower CH_4 production was observed for NS_{TMR}-1 (2.86 mL/120 mg DM) and NS_{TMR}-5 (3.13 mL/120 mg DM) from the rest of the NS_{TMR} ($p<0.05$). The CH_4 concentration did not differ significantly among the NS_{TMR} treatments. Additionally, NS_{TMR}-1 also produced the lowest concentration of CH_4/dOM (44.9 L CH_4/kg dOM) compared to the other NS_{TMR} treatments ($p<0.05$).

Table 4.46: CH_4 production, CH_4 concentration, and CH_4/dOM of NS_{TMR}

Parameters	NS_{TMR}						SEM	Sig.
	NS_{TMR}-1	NS_{TMR}-2	NS_{TMR}-3	NS_{TMR}-4	NS_{TMR}-5	NS_{TMR}-6		
CH_4 mL/120 mg DM	2.86^{c}	3.35^{bc}	3.85^{ab}	3.88^{ab}	3.13^{c}	3.98^{a}	0.22	$P<0.05$
CH_4 conc. in GP (%)	16.0	16.0	15.8	16.2	16.3	17.2	0.58	NS
L CH_4/kg dOM	44.9^{b}	47.8^{ab}	51.7^{ab}	53.2^{ab}	47.7^{ab}	54.4^{a}	3.07	$P<0.05$

NS_{MF}, Napier silage-based mixed feed (70% Napier silage, 30% Ipil-ipil); CH_4, Methane; CH_4 conc., Methane concentration; DOM, Digestibility of organic matter; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; NS_{TMR}-1, 60% NS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); NS_{TMR}-2, 60% NS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); NS_{TMR}-3, 60% NS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); NS_{TMR}-4, 60% NS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); NS_{TMR}-5, 60% NS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); NS_{TMR}-6, 60% NS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of the mean; NS, Not significant; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

The VFA in the different NS_{TMR} treatments are presented in Table 4.47. NS_{TMR}-3 produced the highest concentration of total VFA (14.2 mmol/L), which was significantly ($p<0.05$) higher than that of NS_{TMR}-1 and NS_{TMR}-5. The same trend was observed for the production of valerate (0.18 mmol/L) and butyrate (1.89 mmol/L), which were significantly ($p<0.05$) higher than those of NS_{TMR}-1 and NS_{TMR}-5. No significant differences were observed in the production of propionate; however, NS_{TMR}-3 and NS_{TMR}-4 produced the highest concentrations of acetate (8.96 mmol/L and 8.97 mmol/L, respectively) among the treatments.

Table 4.47: Volatile fatty acids (VFA) production of NS_{TMR}

Parameters	**NS_{TMR}**						**SEM**	**Sig.**
	NS_{TMR}-1	NS_{TMR}-2	NS_{TMR}-3	NS_{TMR}-4	NS_{TMR}-5	NS_{TMR}-6		
VFA (mmol/L)								
Acetate (A)	7.42^{b}	8.37^{ab}	8.96^{a}	8.97^{a}	7.38^{b}	8.33^{ab}	0.47	$P<0.05$
Propionate (P)	2.80	3.02	3.00	3.09	2.72	2.85	0.13	NS
Iso-butyrate	0.08	0.09	0.09	0.09	0.07	0.08	0.01	NS
Butyrate	1.21^{c}	1.47^{abc}	1.89^{a}	1.73^{ab}	1.30^{bc}	1.71^{ab}	0.15	$P<0.05$
Iso-valerate	0.08^{ab}	0.09^{a}	0.07^{ab}	0.08^{ab}	0.05^{b}	0.06^{b}	0.01	$P<0.05$
Valerate	0.14^{b}	0.15^{ab}	0.18^{a}	0.17^{ab}	0.14^{b}	0.17^{ab}	0.01	$P<0.05$
Total VFA	11.7^{bc}	13.2^{abc}	14.2^{a}	14.1^{ab}	11.6^{c}	13.2^{abc}	0.71	$P<0.05$
A:P	2.64^{b}	2.76^{b}	3.00^{a}	2.90^{a}	2.71^{b}	2.92^{a}	0.04	$P<0.05$

NS_{MF}, Napier silage-based mixed feed (70% Napier silage, 30% Ipil-ipil); VFA, Volatile fatty acids; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; NS_{TMR}-1, 60% NS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); NS_{TMR}-2, 60% NS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); NS_{TMR}-3, 60% NS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); NS_{TMR}-4, 60% NS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); NS_{TMR}-5, 60% NS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); NS_{TMR}-6, 60% NS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of mean; NS, Not significant; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

The associative effects between the estimated and measured values of rumen fermentation characteristics and CH_4 reduction are shown in Table 4.48. The measured values of dOM, ME, and NEL of different NS_{TMR} combinations were consistently higher than the estimated values, indicating a positive associative effect. NS_{TMR}-6 illustrated the treatments' highest positive associative effect on dOM, ME, and NEL (6.88%, 8.30%, and 10.6%, respectively). However, the concentrations of CH_4 production and CH_4/dOM were also higher than the estimated values, implying that CH_4 reduction did not occur in the treatments. Surprisingly, NS_{TMR}-3 showed the highest reduction in CH_4 concentration (–0.32%) from the estimated values among the treatments, which significantly differed from that of NS_{TMR}-6.

The linear regression results between the estimated and measured values of *in vitro* rumen fermentation and CH_4 reduction of the NS_{TMR} treatments are shown in Table 4.49. The linear regressions of dOM and ME were positively correlated with the estimated values, and their slopes were 1.12 and 1.14, and R^2 values were 0.95 (Fig. 38 and 39). No negative interaction in CH_4 production was observed between the estimated and measured values.

Table 4.48: Associative effect on *in vitro* ruminal fermentation and CH_4 reduction potential of NS_{TMR}

Parameters	NS_{TMR}						SEM	Sig.
	NS_{TMR}-1	NS_{TMR}-2	NS_{TMR}-3	NS_{TMR}-4	NS_{TMR}-5	NS_{TMR}-6		
dOM (%)								
Estimated*	52.1	55.9	59.6	58.9	52.5	57.1		
Measured	53.2	58.5	62.0	60.9	54.7	61.0		
Associative effect (%)	2.06^{b}	4.58^{ab}	4.04^{ab}	3.36^{b}	4.08^{ab}	6.88^{a}	1.05	P<0.05
ME (MJ/kg DM)								
Estimated*	6.84	7.44	8.15	7.97	6.96	7.75		
Measured	7.01	7.86	8.55	8.29	7.31	8.39		
Associative effect (%)	2.57^{b}	5.63^{ab}	4.84^{ab}	4.07^{b}	5.04^{ab}	8.30^{a}	1.28	P<0.05
NEL (MJ/kg DM)								
Estimated*	3.74	4.18	4.71	4.57	3.83	4.42		
Measured	3.87	4.49	5.00	4.81	4.09	4.88		
Associative effect (%)	3.43^{b}	7.30^{ab}	6.10^{ab}	5.17^{b}	6.68^{ab}	10.6^{a}	1.64	P<0.05
CH_4 production (mL)								
Estimated$^{\psi}$	2.69	3.19	3.84	3.65	2.82	3.51		
Measured	2.86	3.35	3.85	3.88	3.13	3.98		
Associative effect (%)	6.48	5.01	0.08	6.24	11.2	13.4	4.92	NS
CH_4 concentration (%)								
Estimated$^{\psi}$	14.8	15.3	15.8	15.7	14.9	15.5		
Measured	16.0	16.0	15.8	16.5	16.3	17.2		
Associative effect (%)	8.11^{ab}	4.81^{ab}	-0.32^{b}	4.69^{ab}	9.83^{ab}	11.1^{a}	3.34	P<0.05
L CH_4/kg dOM								
Estimated$^{\psi}$	39.5	44.4	50.4	48.7	40.7	47.2		
Measured	44.9	47.8	51.7	53.2	47.7	54.4		
Associative effect (%)	13.6	7.76	2.56	9.21	17.4	15.3	5.20	NS

NS_{MF}, Napier silage-based mixed feed (70% Napier silage, 30% Ipil-ipil); dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH_4, Methane; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; NS_{TMR}-1, 60% NS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); NS_{TMR}-2, 60% NS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); NS_{TMR}-3, 60% NS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); NS_{TMR}-4, 60% NS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); NS_{TMR}-5, 60% NS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); NS_{TMR}-6, 60% NS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of mean; NS, Not significance; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p < 0.05$); *, The estimated values were calculated from single feeds that were determined by the equation of Menke and Steingass (1988); $^{\psi}$, The estimated values were calculated from single feeds.

Table 4.49: Results of simple linear regression of *in vitro* ruminal fermentation and CH_4 reduction potential of NS_{TMR}

Parameters	NS_{TMR} (†Estimated vs. Measured)					RMSE
	Slope	Slope CI	Intercept	Intercept CI	R^2	
dOM (%)	1.12	0.76 to 1.48	-4.62	-24.9 to 16.7	0.95	0.92
ME MJ/kg DM	1.14	0.79 to 1.48	-0.67	-3.29 to 1.94	0.95	0.14
NEL MJ/kg DM	1.13	0.80 to 1.46	-0.28	-1.68 to 1.10	0.96	0.10
CH_4 production (ml)	0.96	0.49 to 1.44	0.34	-1.21 to 1.91	0.89	0.17
CH_4 conc. (%)	0.09	-1.58 to 1.77	14.8	-10.9 to 40.6	0.01	0.55
L CH_4/kg dOM	0.42	-0.58 to 1.44	29.2	-16.8 to 75.3	0.26	3.60

TMR, Total mixed ration; NS_{TMR}, Napier silage-based TMR; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH_4 concentration, Methane concentration. †The estimated values of CH_4 related traits were calculated from the single feeds using the equation of Menke and Steingass (1988) for determining the ruminal fermentation characteristics (dOM, ME, and NEL) from single feeds, which consistently indicated X-variables.

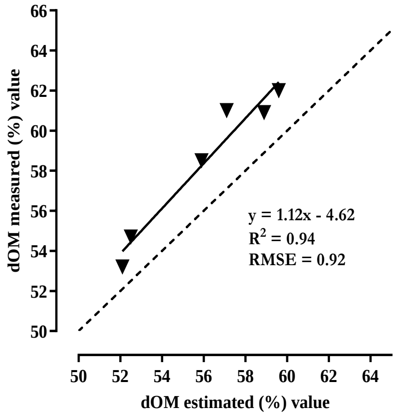

Fig. 38: Comparison of estimated and measured digestibility of organic matter (dOM) values of different combinations of Napier silage-based TMR(NS_{TMR}) using an *in vitro* ruminal fermentation technique. The dOM values of compound feeds were calculated from dOM values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

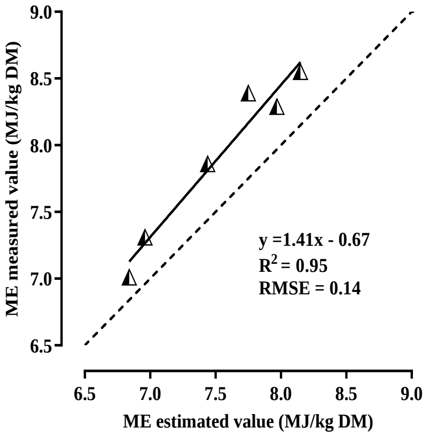

Fig. 39: Comparison of estimated and measured metabolisable energy (ME) values of different combinations of Napier silage-based TMR (NS_{TMR}) using an *in vitro* ruminal fermentation technique. The ME values of compound feeds were calculated from ME values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

4.4.4. Maize silage-based TMR (MS_{TMR})

The *in vitro* rumen fermentation characteristics of MS_{TMR} are presented in Table 4.50. The highest GP_{24} and *c*GP were recorded for MS_{TMR}-3 (45.6 mL/200 mg DM and 7.17%/h), whereas these were the lowest for MS_{TMR}-1 (32.9 mL/200 mg DM and 5.57%/h). In addition, MS_{TMR}-3 also produced the highest concentrations of dOM, ME, and NEL (67.6%, 9.42 MJ ME/kg DM and 5.63 MJ NEL/kg DM), which were significantly ($p<0.05$) higher than those of the other treatments. On the contrary, MS_{TMR}-1 and MS_{TMR}-5 showed the lowest production performance among the treatments based on dOM, ME, and NEL (57.1%, 7.61 MJ ME/kg DM and 4.30 MJ NEL/kg DM, and 57.1%, 7.66 MJ ME/kg DM and 4.34 MJ NEL/kg DM, respectively) ($p<0.05$).

Table 4.50: *In vitro* ruminal fermentation characteristics of MS_{TMR}

Parameters	**MS_{TMR}**						**SEM**	**Sig.**
	MS_{TMR}-1	MS_{TMR}-2	MS_{TMR}-3	MS_{TMR}-4	MS_{TMR}-5	MS_{TMR}-6		
GP_{24} (mL/200 mg DM)	32.9^{d}	36.9^{c}	45.6^{a}	41.0^{b}	33.4^{d}	40.0^{bc}	0.87	$P<0.05$
*p*GP (mL/200 mg DM)	44.3^{c}	50.7^{b}	57.4^{a}	54.4^{a}	43.3^{c}	49.9^{b}	0.68	$P<0.05$
*c*GP (%/h)	5.57^{d}	5.49^{d}	7.17^{ab}	6.03^{cd}	6.52^{bc}	7.31^{a}	0.26	$P<0.05$
dOM (%)	57.1^{d}	60.9^{c}	67.6^{a}	64.2^{b}	57.1^{d}	62.4^{bc}	0.77	$P<0.05$
ME (MJ/kg DM)	7.61^{d}	8.22^{c}	9.42^{a}	8.79^{b}	7.66^{d}	8.58^{bc}	0.13	$P<0.05$
NEL (MJ/kg DM)	4.30^{d}	4.75^{c}	5.63^{a}	5.17^{b}	4.34^{d}	5.02^{bc}	0.09	$P<0.05$

MS_{MF}, Maize silage-based mixed feed (70% Maize silage, 30% Ipil-ipil); GP_{24},Gas production at 24 h; *p*GP, Potential gas production; *c*GP, Gas production rate constant; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; MS_{TMR}-1, 60% MS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); MS_{TMR}-2, 60% MS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); MS_{TMR}-3, 60% MS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); MS_{TMR}-4, 60% MS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); MS_{TMR}-5, 60% MS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); MS_{TMR}-6, 60% MS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of the mean; $^{a-d}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

The interaction effects between different MS_{TMR} combinations and incubation time on the *in vitro* cumulative GP is shown in Fig. 40. The cumulative gas production of MS_{TMR}-3 increased gradually with the increase in the incubation period and reached a maximum (57.8 mL/200 mg DM) after 72 h of incubation.

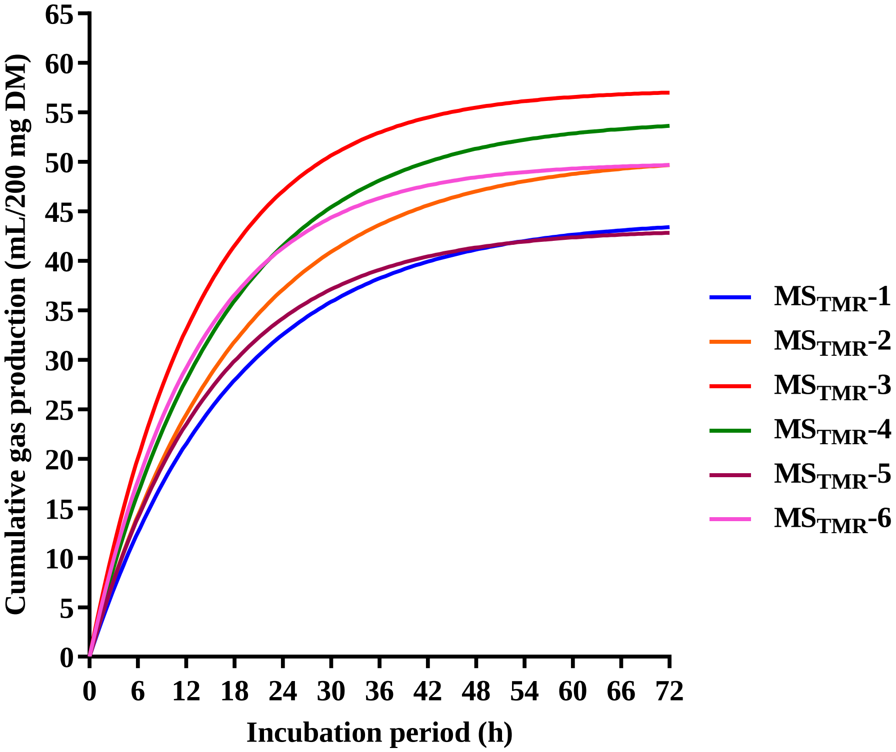

Fig. 40: Cumulative gas production profile of maize silage-based TMR. The *in vitro* gas production profile has been fitted using the equation $Y= pGP\cdot(1-e^{-cGP\cdot 0.01t})$. Estimated parameters of the nonlinear functions are presented in Table 4.50.

CH_4 production and related traits are shown in Table 4.51. It can be seen that the CH_4 production of MS_{TMR}-1 and MS_{TMR}-5 were significantly lower ($p<0.05$) than those of the other treatments. No significant differences were observed in the production of CH_4 and CH_4/dOM among all MS_{TMR} ($p>0.05$).

Table 4.51: CH_4 production, CH_4 concentration, and CH_4/dOM of MS_{TMR}

Parameters	**MS_{TMR}**						**SEM**	**Sig.**
	MS_{TMR}-1	MS_{TMR}-2	MS_{TMR}-3	MS_{TMR}-4	MS_{TMR}-5	MS_{TMR}-6		
CH_4 mL/120 mg DM	3.10^{b}	3.18^{ab}	3.76^{a}	3.77^{a}	3.10^{b}	3.76^{a}	0.22	$P<0.05$
CH_4 conc. In GP (%)	15.2	15.7	14.4	16.2	15.8	16.2	0.58	NS
L CH_4/kg dOM	45.3	43.6	46.4	48.9	45.3	50.2	3.06	NS

MS_{MF}, Maize silage-based mixed feed (70% Maize silage, 30% Ipil-ipil); CH_4, Methane; CH_4 conc., Methane concentration; dOM, Digestibility of organic matter; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; MS_{TMR}-1, 60% MS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); MS_{TMR}-2, 60% MS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); MS_{TMR}-3, 60% MS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); MS_{TMR}-4, 60% MS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); MS_{TMR}-5, 60% MS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); MS_{TMR}-6, 60% MS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of mean; NS, Not significant; $^{a, b}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

The VFA production of different MS_{TMR} treatments is presented in Table 4.52. No significant differences were observed in acetate, propionate, or total VFA production among the treatments. The butyrate and valerate production of MS_{TMR}-3 were significantly higher (1.75 mmol/L and 0.21 mmol/L, respectively) than those of MS_{TMR}-1 and MS_{TMR}-5. The opposite trend was observed for the ratio of acetate to propionate in MS_{TMR}-1 and MS_{TMR}-5 compared to other treatments, except for MS_{TMR}-2.

Table 4.52: Volatile fatty acids (VFA) production of MS_{TMR}

Parameters	MS_{TMR}						SEM	Sig.
	MS_{TMR}-1	MS_{TMR}-2	MS_{TMR}-3	MS_{TMR}-4	MS_{TMR}-5	MS_{TMR}-6		
	VFA (mmol/L)							
Acetate (A)	7.37	8.31	8.07	8.55	7.10	7.65	0.47	NS
Propionate (P)	3.05	3.30	3.08	3.29	2.92	2.97	0.15	NS
Iso-butyrate	0.08^{ab}	0.09^{ab}	0.11^{a}	0.09^{ab}	0.07^{b}	0.07^{b}	0.01	$P<0.05$
Butyrate	1.18^{c}	1.45^{abc}	1.75^{a}	1.67^{ab}	1.23^{bc}	1.58^{abc}	0.14	$P<0.05$
Iso-valerate	0.07	0.09	0.10	0.09	0.06	0.05	0.02	NS
Valerate	0.14^{b}	0.16^{ab}	0.21^{a}	0.17^{ab}	0.13^{b}	0.16^{ab}	0.05	$P<0.05$
Total VFA	11.9	13.4	13.3	13.9	11.5	12.5	0.77	NS
A:P	2.40^{b}	2.51^{ab}	2.62^{a}	2.59^{a}	2.42^{b}	2.57^{a}	0.03	$P<0.05$

MS_{MF}, Maize silage-based mixed feed (70% Maize silage, 30% Ipil-ipil); VFA, Volatile fatty acids; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; MS_{TMR}-1, 60% MS_{MF} + 40% concentrates (60% RB + 10% WB + 30% KB); MS_{TMR}-2, 60% MS_{MF} + 40% concentrates (30% RB + 10% WB + 60% KB); MS_{TMR}-3, 60% MS_{MF} + 40% concentrates (10% RB + 60% WB + 30% KB); MS_{TMR}-4, 60% MS_{MF} + 40% concentrates (10% RB + 30% WB + 60% KB); MS_{TMR}-5, 60% MS_{MF} + 40% concentrates (60% RB + 30% WB + 10% KB); MS_{TMR}-6, 60% MS_{MF} + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of mean; NS, Not significant; $^{a-c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$).

The associative effects between the estimated and measured values of different MS_{TMR} combinations on *in vitro* rumen fermentation and CH_4 reduction are presented in Table 4.53. It can be seen that the interaction between estimated and measured values of dOM, ME, and NEL differed significantly and also indicated a positive associative effect. Interestingly, MS_{TMR}-3 had the highest percentage of associative effects for dOM, ME, and NEL (9.34%, 11.2%, and 14.0%, respectively) among the treatments ($p<0.05$). However, the opposite trend was observed for the production of CH_4 and which indicates a negative associative effect. MS_{TMR}-3 also had the highest reduction percentages for CH_4 production, CH_4 concentration, and CH_4/dOM from the estimated values, which were –4.81%, –8.02%, and –7.63%, respectively ($p<0.05$).

Table 4.54 shows that the estimated values of dOM and ME were positively correlated with the measured values (slope = 1.26 and 1.26 and R^2 values close to 1) (Fig. 41 and 42). The opposite trend was observed for the production of CH$_4$, which was negatively correlated with the measured values, indicating that CH$_4$ reduction occurred (slope = –0.20 and R^2 value = 0.70).

Table 4.53: Associative effect on *in vitro* ruminal fermentation and CH$_4$ reduction potential of MS$_{TMR}$

Parameters	MS$_{TMR}$						SEM	Sig.
	MS$_{TMR}$-1	MS$_{TMR}$-2	MS$_{TMR}$-3	MS$_{TMR}$-4	MS$_{TMR}$-5	MS$_{TMR}$-6		
dOM (%)								
Estimated*	54.3	58.1	61.8	61.1	54.8	59.3		
Measured	57.1	60.9	67.6	64.2	57.1	62.4		
Associative effect (%)	5.10^{b}	4.79^{b}	9.34^{a}	5.05^{b}	4.28^{b}	5.31^{b}	0.98	$P<0.05$
ME (MJ/kg DM)								
Estimated*	7.15	7.76	8.47	8.29	7.28	8.07		
Measured	7.61	8.22	9.42	8.79	7.66	8.58		
Associative effect (%)	6.34^{b}	5.88^{b}	11.2^{a}	6.10^{b}	5.28^{b}	6.38^{b}	1.72	$P<0.05$
NEL (MJ/kg DM)								
Estimated*	3.97	4.41	4.94	4.80	4.06	4.65		
Measured	4.30	4.75	5.63	5.17	4.34	5.02		
Associative effect (%)	8.34^{b}	7.53^{b}	14.0^{a}	7.67^{b}	6.90^{b}	8.08^{b}	1.56	$P<0.05$
CH$_4$ production (mL)								
Estimated$^{\psi}$	2.80	3.30	3.95	3.76	2.93	3.62		
Measured	3.10	3.18	3.76	3.77	3.10	3.76		
Associative effect (%)	10.9^{a}	-3.57^{b}	-4.81^{b}	0.24^{ab}	6.11^{ab}	3.95^{ab}	4.71	$P<0.05$
CH$_4$ concentration (%)								
Estimated$^{\psi}$	14.7	15.1	15.7	15.5	14.8	15.35		
Measured	15.2	15.7	14.4	16.2	15.8	16.2		
Associative effect (%)	3.49^{a}	4.11^{a}	-8.02^{b}	4.16^{a}	7.16^{a}	5.53^{a}	2.99	$P<0.05$
L CH$_4$/kg dOM								
Estimated$^{\psi}$	39.3	44.2	50.2	48.5	40.5	46.99		
Measured	45.3	43.6	46.4	48.9	45.3	50.2		
Associative effect (%)	15.3^{a}	-1.27^{bc}	-7.63^{c}	0.84^{abc}	12.0^{ab}	6.88^{abc}	4.96	$P<0.05$

MS$_{MF}$, Maize silage-based mixed feed (70% Maize silage, 30% Ipil-ipil); dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH$_4$, Methane; RB, Rice bran; WB, Wheat bran; KB, Kashari bran; MS$_{TMR}$-1, 60% MS$_{MF}$ + 40% concentrates (60% RB + 10% WB + 30% KB); MS$_{TMR}$-2, 60% MS$_{MF}$ + 40% concentrates (30% RB + 10% WB + 60% KB); MS$_{TMR}$-3, 60% MS$_{MF}$ + 40% concentrates (10% RB + 60% WB + 30% KB); MS$_{TMR}$-4, 60% MS$_{MF}$ + 40% concentrates (10% RB + 30% WB + 60% KB); MS$_{TMR}$-5, 60% MS$_{MF}$ + 40% concentrates (60% RB + 30% WB + 10% KB); MS$_{TMR}$-6, 60% MS$_{MF}$ + 40% concentrates (30% RB + 60% WB + 10% KB); SEM, Standard error of mean; NS, Not significance; $^{a, b, c}$ means with different superscripts in the same row are significantly different at ($p< 0.05$) *, The estimated values were calculated from single feeds that were determined by the equation of Menke and Steingass (1988), $^{\psi}$, The estimated values were calculated from single feeds.

Table 4.54: Results of simple linear regression of *in vitro* ruminal fermentation and CH_4 reduction potential of MS_{TMR}

Parameters	MS_{TMR} (†Estimated vs. Measured)					RMSE
	Slope	Slope CI	Intercept	Intercept CI	R^2	
dOM (%)	1.26	0.87 to 1.66	-12.2	-35.3 to 10.9	0.95	1.00
ME MJ/kg DM	1.26	0.88 to 1.64	-1.53	-4.49 to 1.42	0.96	0.16
NEL MJ/kg DM	1.26	0.90 to 1.62	-0.79	-2.41 to 0.83	0.96	0.11
CH_4 production (ml)	0.70	0.33 to 1.07	1.05	-0.20 to 2.32	0.87	0.13
CH_4 conc. (%)	-0.20	-2.39 to 1.99	18.6	-14.7 to 51.9	0.02	0.70
L CH_4/kg dOM	0.31	-0.34 to 0.96	32.7	3.22 to 62.2	0.30	2.31

TMR, Total mixed ration; MS_{TMR}, Maize silage-based TMR; dOM, Digestibility of organic matter; ME, Metabolisable energy; NEL, Net energy lactation; DM, Dry matter; CH_4 concentration, Methane concentration. †The estimated values of CH_4 related traits were calculated from the single feeds using the equation of Menke and Steingass (1988) for determining the ruminal fermentation characteristics (dOM, ME, and NEL) from single feeds, which consistently indicated X-variables.

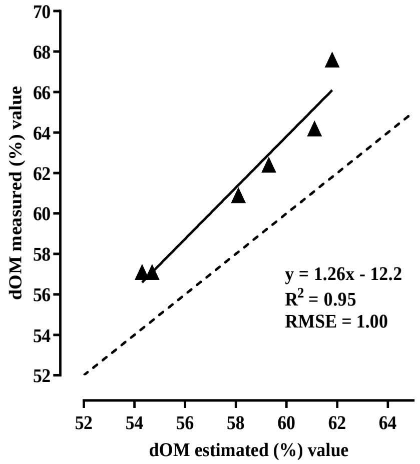

Fig. 41: Comparison of estimated and measured digestibility of organic matter (dOM) values of different combinations of Maize silage-based TMR(MS_{TMR}) using an *in vitro* ruminal fermentation technique. The dOM values of compound feeds were calculated from dOM values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

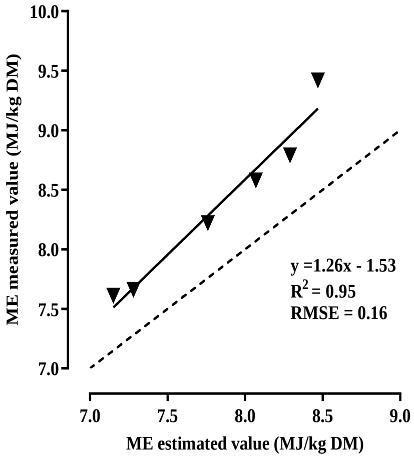

Fig. 42: Comparison of estimated and measured metabolisable energy (ME) values of different combinations of Maize silage-based TMR(MS_{TMR}) using an *in vitro* ruminal fermentation technique. The ME values of compound feeds were calculated from ME values of single feeds that were determined by the equation of Menke and Steingass (1988). The dotted line represents the angle bisector.

5. GENERAL DISCUSSION

5.1. Nutrient composition of all single feedstuffs

Crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) are crucial factors for evaluating the nutritional quality of diverse feedstuffs. Most feedstuffs used in tropical countries, such as crop residues, crop by-products, and common grasses, are deficient in CP, have high cell wall content, and are used for extensive livestock production. Overall, the CP content of the roughages varied between 44.3 g/kg and 314.0 g/kg. (Table 4.1). Ipil-ipil and rice straw had the highest and lowest CP concentrations, respectively. Therefore, CP is a fundamental factor in measuring the overall nutritional value of any feed. A threshold value of 7.0% CP has been recommended for good roughage quality; however, the microbial fermentation of roughages may be limited due to a lack of N, and the protein requirements of animals are not satisfied (Arelovich et al. 2008, Hariyadi and Santoso 2010).

In this research, all of the test roughages had a CP level higher than the threshold of 7.0%, except crop residues, Napier silages, and common grasses. Particularly, the high CP content of Ipil-ipil (314.0 g/kg DM) and *Gliricidia* (239.6 g/kg DM) suggests that these species can be utilised as a supplement for ruminants where local forages are deficient in CP (Givens et al. 2000, Waghorn and Clark 2004, Lopez et al. 2005, Njidda et al. 2017, Giridhar et al. 2018). These leguminous fodders effectively contribute to nutrient supply, which may enhance DM intake as well as the digestion of roughages of lower digestibility (Albores-Moreno et al. 2019, Rodriguez-Villanueva et al. 2020, Yusuf et al. 2020). Rice straw's CP content of 44.3 g/kg DM is below the cut-off; however, crop leftovers likely still make up a significant portion of ruminant feeds in many parts of developing world.

NDF and ADF content are also important factors for assessing feed quality. Roughages with an NDF content below 35.5% are considered to be of high quality, while those with an NDF level above 46.0% are deemed to be of low quality (Harper and McNeill 2015). Only Ipil-ipil exhibited an NDF value around the threshold in this investigation. In addition to having a lower ADF concentration of Ipil-ipil (186.9 g/kg DM), which can have a favourable effect on forage quality because lower ADF concentration is indicative of greater digestion of the feed (Dasci and Comakli 2010). When compared to other feed ingredients, Ipil-ipil had the lowest NDF and ADF concentrations (380.2 g/kg DM and 186.9 g/kg DM, respectively), while Napier silage and rice straw had the highest (770.9 g/kg DM and 559.3 g/kg DM, and 743.4 g/kg DM and 547.4 g/kg DM, respectively). The lowest ADL value was measured in UMS (28.6 g/kg DM) and the highest in *Gliricidia* (300.9 g/kg DM) (Table 4.1). Many factors may affect cell wall (NDF and ADF) content, such as the stage of maturity, variety or species, growth environment, and soil type (Sanz-Saez et al. 2012, Polley et al. 2013).

Most of the roughages used in this research had a high cell wall content (NDF, ADF, and ADL), which inhibited microbial activity by limiting the accessibility of readily fermentable carbohydrates and was inversely associated with GP (Njidda and Nasiru 2010). This can also cause higher energy loss, which increases CH_4 production by reducing the efficiency of animal production (Frei et al. 2013).

5.2. *In vitro* ruminal fermentation characteristics of single feedstuffs

The production of more gas with the rapid fermentation of available carbohydrates (CHO) through ruminal microorganisms is a good indicator for extending digestion (Getachew et al. 2004). Variations in the chemical components of the feedstuffs may account for differences in cumulative gas and CH_4 output. Total GP was highest after 24 h of *in vitro* fermentation for all concentrates used in this investigation except rice bran (Table 4.2). Conversely, after 24 h of *in vitro* fermentation, the average total GP of protein-rich leguminous and fibrous feeds was lower. According to these findings, consistent with those of Singer et al. (2008), cereals have more readily fermentable CHO and a higher degree of ruminal fermentation than fibrous, protein-rich legumes and forages. The increased dOM of cereals compared to the other categories of feedstuffs is a direct reflection of the exclusive ruminal fermentation that occurs due to their high starch and lower undegradable CHO contents.

Additionally, the leguminous fodder of Ipil-ipil and *Gliricidia* produced lower amounts of gas at 24 h of 27.0 mL/200 mg DM and 15.2 mL/200 mg DM, respectively. Protein-rich diets release amino acids, peptides, and ammonia, eventually converted to microbial proteins by microbial degradation. Although dietary soluble proteins ferment rapidly like NSC, less gas is produced during protein fermentation than in CHO fermentation (Singh et al. 2012). These findings also indicate that secondary metabolites like tannins and saponins may have an impact on overall volume of gas produced during fermentation (Jayanegara et al. 2014, Kondo et al. 2014). However, the existence of secondary metabolites was not evaluated in this study. Rice straw and forage both contained a greater level of cell walls; however, the total GP of forages was considerably ($p<0.05$) higher than that of rice straw when expressed in mL/200 mg DM incubated in the current investigation. These findings can be understood by considering the distinctive characteristics of the CHO found in rice straw and forage. Starch, sugar, and NDF-containing feeds ferment faster than ADF-containing feeds; nevertheless, lignin cannot be decomposed by cellulolytic rumen microbes (Singh et al. 2012). Consequently, the forages used in this study had a lower lignin content, which led to a higher total gas production from each gram of substrate that was incubated as well as a higher dOM than that achieved with rice straw.

The concentrate feeds were found to have the highest digestibility value, ME, and NEL concentrations compared to the other feedstuffs. The crushed maize and wheat exhibited higher dOM, ME, and NEL content (90.3%, 13.8 MJ/kg DM, 8.83 MJ/kg DM and 87.8%, 14.4 MJ/kg DM, 9.12 MJ/kg DM, respectively). Feed that has a dOM that is more than 50% has a good chance of having a high potential to deliver the ME and NEL that are necessary to support animal production (Evitayani et al. 2004).

5.3. *In vitro* CH_4 production and related traits of single feedstuffs

Anaerobic decomposition of cells and cell walls containing slowly fermentable carbohydrates of cellulose and hemicellulose in feedstuffs is associated with higher *in vitro* CH_4 production (Santoso and Hariadi 2009). The major methanogenic bacteria in the rumen utilise H_2 as a significant energy source to reduce CO_2 to CH_4. Therefore, CO_2 and H_2 are positively correlated with CH_4 during microbial fermentation of feed in the rumen (Janssen 2010). During the 24 h incubation period, the CH_4 output (mL/200 mg DM) and its concentration (%) from the examined feedstuffs varied substantially ($p<0.05$). The CH_4 production of concentrates following 24 h *in vitro* incubation was the highest in crushed wheat, kashari bran, crushed maize, mustard oil cake, and wheat bran, respectively, and the lowest in rice bran at 0.94 mL/120 mg DM ($p<0.05$). The relatively high CH_4 production of concentrates, with the exception of wheat bran and rice bran, could be attributed to the high amount of fermentable starch, sugars, or hemicelluloses as substrates for rumen microorganisms. These soluble carbohydrates enhance ciliate protozoa and accelerate their hydrogen transfer to methanogens, leading to a significant increase in CH_4 emission (Molina-Botero et al. 2020). Rice bran has the maximum amount of unsaturated fatty acids (Czerkawski et al. 1966) that can be hydrogenated by rumen microorganisms. This process results in a decreased pressure of H_2, which is a prerequisite for reducing CH_4 output. In addition, fat (47.3 g/kg DM) is thought to decrease CH_4 formation by accelerating propionate production and inhibiting protozoa activity, as well as suppressing cellulolytic bacteria and feed digestion in the rumen, according to the current study.

The CH_4 concentration of the produced gas can be considered an indicator of a plant or forage's ability to inhibit CH_4 release *in vitro* (Lopez et al. 2010, Bhatta et al. 2012). A low CH_4 concentration implies that a candidate would be a more effective rumen modulator for CH_4 reduction than a high yield percentage. Ipil-ipil demonstrated a consistently decreased CH_4 percentage in the current study, making it a promising species for lowering CH_4 production in ruminants in the study area. It is also crucial to mention that Ipil-ipil had a relatively higher CP concentration (Table 4.1), making it an ideal protein supplement source for ruminant feed. Furthermore, most of the high CP containing leguminous fodder have a promising reduction potential for CH_4 owing to the lower production of H_2 and CO_2 of protein than CHO (Jayanegara et al. 2009, Melesse et al. 2017, Yonas Berhanu et al. 2019). Another important factor is that a large portion of CH_4 produced by leguminous fodder can be associated with the presence of secondary metabolites, which can also inhibit CH_4 emissions (Tavendale et al. 2005, Patra 2010, Rira et al. 2019). The loss of gross energy (GE) as CH_4 (0.50–4.45%) varied in different feedstuffs depending on the concentrations of fibre and lignin (Table 4.1) and digestibility (Minson 1990, Van Soest 1994).

5.4. Association between CH_4 production, chemical constituents, and fermentation characteristics of single feedstuffs

This study offered direct information for predicting the CH_4 production of feedstuffs as per gram of DM consumed by ruminants based on the link between CH_4 production, chemical ingredients, and fermentation characteristics (Santoso and Hariadi 2009, Navarro-Villa et al. 2011). This will be beneficial for future usage in formulating diets with reduced CH_4 production and for comparison with future *in vivo* trials on ruminants.

In the present investigation, correlation analysis of the findings determined from all feedstuffs revealed that the presence of more CP and ADL had a negative influence on *in vitro* CH_4 output per unit of incubated DM (Table 4.5). This result is consistent with that reported by Singh et al. (2012), who indicated that increasing protein and non-degradable cell wall fractions would decrease *in vitro* CH_4 emission. In contrast, increasing gas production and dOM were positively linked with CH_4 production because feedstuffs contained more fermentable substrates (Navarro-Villa et al. 2011). Despite these discrepancies, a significant connection was shown between CH_4 production and NDF content for all feedstuffs, suggesting that an increase in the content of degradable cell-wall fractions is responsible for the high CH_4 production, as cellulolytic bacteria favour acetate production and thus produce more H_2 that can be used in methanogenesis (Christophersen et al. 2008, Janssen 2010). In the present research, despite considerable diversity in the chemical contents of the feedstuffs (Table 4.1), the association between CH_4 emission and the majority of chemical parameters was not statistically significant (Table 4.5). Forage containing secondary metabolites (condensed tannins and saponins) or starch may be responsible for modifying rumen methanogenesis and reducing CH_4 emission. For instance, condensed tannin-containing forages, particularly legumes, are believed to decrease CH_4 formation *in vitro* by inhibiting methanogens directly and indirectly by lowering methanogenesis due to the lack of H_2 (Tavendale et al. 2005, Patra 2010). However, they may also impair livestock output by reducing feed intake and digestibility (Beauchemin et al. 2008).

In order to formulate diets for ruminants or to anticipate *in vitro* and *in vivo* CH_4 emission of feedstuffs, many researchers have derived prediction equations (Hindrichsen et al. 2004, Singh et al. 2012, Ramin and Huhtanen 2013). The majority of these proposed equations for CH_4 prediction were created for the same categories of feedstuffs with excellent precision (Hindrichsen et al. 2004, Singh et al. 2012). In the present study, the equation using NDF and CP had an $R^2=0.76$ ($p<0.002$), whereas equations using protein and NDF individually had $R^2=0.72$ ($p<0.001$) and 0.45 ($p<0.01$), respectively. CH_4 output in each category of feedstuffs could be correctly predicted by their chemical composition (CP and NDF) as indicated by the regression coefficient of the equations in this study. Santoso and Hariadi (2009), and Singh et al. (2012) reached a similar conclusion, stating that carbohydrate fractions (NDF and ADF) are stronger predictors of CH_4 than feed components.

5.5. Supplementation of Ipil-ipil and *Gliricidia* with roughages on *in vitro* ruminal fermentation characteristics

Nutrient imbalance directly affects rumen function owing to low energy and protein intake. In third-world countries, low-quality roughage is a less expensive source of metabolisable energy to support animals than supplements. Rice (*Oryza sativa*) straw and Napier (Pennisetum purpureum) silage, which are low in CP (44.0 g/kg and 94.0 g/kg) and high in NDF and ADF (743 g/kg and 547 g/kg and 771 g/kg and 559 g/kg, respectively; Study 1), are frequent in tropical ruminant feeding. This basal feed often produces low animal performance, as expressed by milk production, growth, or reproductive rates. Therefore, dietary supplementation to increase roughage intake and digestibility is desirable to achieve a high production performance.

The use of legume trees and shrubs as most promising supplements for low-quality roughages has been highlighted as one of the better possibilities that have been explored (Hegarty et al. 1964, Jones 1979, Reed et al. 1990, Siaw et al. 1993, Richards et al. 1994, Irawan et al. 2020), as demonstrated by the use of Ipil-ipil (*Leucaena leucocephala*) as a supplement instead of *Gliricidia* in the present studies. Ipil-ipil is a good source of protein, amino acids, vitamins, minerals, β-carotene (D'Mello and Thomas 1977), calcium, and phosphorus (Vietmeyer et al. 1977, Ter Meulen et al. 1979, Brewbaker et al. 1985, Scapinello et al. 2000, Figueredo et al. 2019, Lou et al. 2019); however, it is generally deficient in sodium (Brewbaker et al. 1985, Garcia et al. 1996, Figueredo et al. 2019). These stimulate rumen function and activate bacteria, especially cellulolytic bacteria, which help break down low-quality fibre (Guo et al. 2008, Barros-Rodríguez et al. 2012, Ningrat et al. 2019, Albores-Moreno et al. 2019). In addition, *in vitro* dOM has been widely utilised to assess the nutritional value of roughage since it is closely linked with *in vivo* digestibility (Getachew et al. 2004). In the current study, the supplementation of rice straw and Napier silage with 40% and 30% Ipil-ipil significantly increased the dOM, ME, and NEL compared to all other treatment conditions involving Ipil-ipil and *Gliricidia*. This could be attributed to the increased availability of nutrients for rumen microbes. Ipil-ipil also contains sulfur and phosphorus (not identified in the Study), which are vital for cellulolytic bacteria and fungi to increase digestibility (Aregheore 1999). Moreover, adding Ipil-ipil to poor-quality roughages increased the amount of protein in the diet comparable to the threshold values of CP of good-quality roughages (7.0 %) (Arelovich et al. 2008, Hariyadi and Santoso 2010). It is converted to ammonia and forms a protein complex in the presence of CT. This combination increases the efficacy of microbial N formation

(Anantasook et al. 2016), which in turn enhances the delivery of non-degradable protein to the small intestine, improving the post-rumen protein digestibility and absorption rate (Soltan et al. 2012).

Furthermore, a high CP/ME ratio is necessary for optimising the growth efficiency of rumen microbes which helps animals absorb maximum nutrients from the diet (Leng 1990). In the current study, the CP/ME ratio of 40% Ipil-ipil with rice straw and 30% Ipil-ipil with Napier silage was

20.0 g/MJ and 21.0 g/MJ, respectively, indicating optima; microbial fermentation and protein synthesis. The minimal requirement for the CP/ME ratio was 12.0 g/MJ (Menke and Huss 1987).

However, no significant interaction was observed between Ipil-ipil and good-quality roughages such as German grass and maize silage, resulting in excess dietary protein or carbohydrates. This may lead to an imbalance in the ratio, suggesting a lack of efficient utilisation of dietary nutrients and poor microbial protein synthesis (Nocek and Russell 1988, Makkar 2003). In addition, presence of tannins and other phenolic substances as well as mimosine (3,4 dihydroxy pyridine), which forms complexes with Zn, Cu, and Fe (Ghosh and Samiran 2007), may also contribute to the suppression of microbial fermentation (Patra 2010, Patra et al. 2017). Therefore, adding Ipil-ipil to German grass and maize silage had no noticeable impact on the rumen fermentation characteristics of the diet. Evidently, dOM, ME, and NEL concentrations were comparable across all combinations.

5.6. Supplementation of Ipil-ipil and *Gliricidia* with roughages on *in vitro* CH_4 production

One of the key goals of this study was to evaluate the combination effects of Ipil-ipil and *Grilicidia* on CH_4 emissions. CH_4 production, CH_4 concentration, and CH_4/dOM differed among treatments; however, it was lower in rice straw and Napier silage than with the other roughages. Notably, when the data were expressed in terms of CH_4 reduction, 40% Ipil-ipil with rice straw diminished the CH_4 concentration by 6.89% and CH_4/dOM by 11.7% (Fig. 7). When 30% Ipil-ipil was added to Napier silage, CH_4 concentration and CH_4/dOM were reduced by 5.32% and 13.3%, respectively (Fig. 16) with respect to the control, thereby underscoring its biological and environmental significance (Figs. 7 and 16). In Mexico, supplementing 30% foliage of Ipil-ipil to Napier grass (*Pennisetum purpureum*) with basal diet reduced CH_4 production by 30.0% of dairy cattle and improved energy efficiency (Albores-Moreno et al. 2019). In Australia, the productivity of herds increased with the reduction of greenhouse gas emissions when animals were grazing on Ipil-ipil compared to those grazing on native pasture (Taylor et al. 2016). This result was supported by Piñeiro-Vázquez et al. (2018), who observed a significant decrease in CH_4 production by 30.0% with the supplementation of Ipil-ipil, which contained 23.0 mg/kg DM of CT. Notably, CT-containing animal feed can modify the microbial communities in the rumen (Albores et al. 2017, Piñeiro-Vázquez et al. 2018, Valencia et al. 2018). It may increase the populations of *Propionibacterium* (BMI210), *Ruminococcus albus* (Krause et al. 2004), and *Selenomonas ruminantium* (Wang et al. 2009, Patra et al. 2012), which are responsible for the synthesis of propionic acid that absorbs the available H^+ in the succinate-propionate pathway (Newbold et al. 2005) and reduces CH_4 production (Patra et al. 2017). Additionally, the reduction in the ruminal CH_4 concentration may be due to the presence of equivalent reducers such as H^+ and NADH during anabolic decomposition that fixes $NH4^+$ in the carbon chains for amino acid synthesis (Ungerfeld et al. 2003). Consequently, microbial anabolism is stimulated, and microbial biomass production increases, functioning as an H^+ sink (Ungerfeld 2015). Similarly, Soltana et al. (2013) reported that Ipil-ipil (6.52 mg/g DM of CT) in the ruminant diet reduced CH_4 production by 14.1%. Tan et al. (2011) also observed that an increased level of Ipil-ipil (up to 15.0 mg of CT per 500 mg of DM) in the ruminant diet led to the highest reduction in CH_4 production (47.0%), with only a 7.0% reduction in the degradation of feed DM. Synchronisation of nitrogen and energy in the diet is crucial for microbial utilisation, which enhances CH_4 reduction by forage legumes (Rodríguez et al. 2010). This phenomenon was observed in the current study in the case of rice straw and Napier silage. Collectively, these results indicate that forage legumes improved microbial biomass synthesis by adding ruminally degraded dOM into the microbial cell while decreasing the CH_4 concentration.

The presence of CT and HT of Ipil-ipil has caused by a reduction in the number of protozoa populations in the rumen, which is an effective approach to reducing CH_4 gas production (Tan et al. 2011, Nguyen et al. 2017). In the current study, the CH_4 concentration reduction occurred in the presence of Ipil-ipil for poor-quality roughages, such as rice straw and Napier silage. This result is supported by Retnani et al. (2010), who observed that 27.0% Ipil-ipil together with Napier grass (P. *purpureum*) reduced CH_4 concentration by 15.6% without affecting nutrient digestibility in sheep. This result may provide fundamental information to reduce the protozoa population and decrease the number of methanogenic bacteria, thereby reducing CH_4 gas production (Beauchemin et al. 2007, Jayanegara et al. 2012). The tannins of Ipil-ipil also inhibited methanogenesis and may also reduce fibre digestion. Consequently, tannins also indirectly reduced the production of H_2, which is a precursor of CH_4 production (Tavendale et al. 2005).

5.7. Associative effects between Ipil-ipil and *Gliricidia,* and roughages of *in vitro* ruminal fermentation characteristics

In 1967, Minson and Milford observed that substituting white clover or lucerne for mature pangola in a sheep's diet enhanced the feed consumption and digestion of DM and CP. This result indicated that legumes compensated for N deficiency in low-quality roughages and had a positive associative effect. Legumes also have favourable impacts on fibre degradation and the consumption of poor-quality roughages (Paterson et al. 1982, Ndlovu and Buchanan Smith 1985, Brandt and Klopfenstein 1986, Hunt et al. 1988, Atwell et al. 1991, Haddad 2000). On the subject of neutral detergent fibre digestion (NDFD), *in vitro* studies have shown that the presence of tropical grasses and legumes has a favorable associative impact (Brown et al. 1991). In the present study, the measured values of dOM, ME, and NEL were significantly higher than the estimated values ($p<0.05$), except for German grass and maize silage, demonstrating a significant positive associative effect on rumen fermentation characteristics. This result suggests that the addition of rapidly fermentable cellulose and hemicellulose from Ipil-ipil to the diet enhanced the quantity of fibrolytic microbes, hence enhancing the digestibility of other less degradable fibres, such as rice straw and Napier silage. Straw with the optimal combination of lucerne has a positive associative effect of 25% (Hunt et al. 1988, Atwell et al. 1991, Haddad 2000). Adding 40% Ipil-ipil to the rice straw exhibited the maximum positive associative effect (Table 4.9) on dOM, ME, and NEL (8.95%, 10.9%, and 14.3%, respectively) compared to that in other treatment ratios of Ipil-ipil and *Gliricidia*. The same trend was observed with the addition of 30% Ipil-ipil to the Napier silage (Table 4.17). Using protein-rich fodder trees or shrubs to supplement poor roughage sources can positively affect DMD and NDFD (Liu et al. 2002, Doran et al. 2007) as well as voluntary feed intake (Norton 1994). Notably, a mixture of tree leaves with poor roughage synchronised the fermentation rates of the different components of a mixture, depending on the fermentation capacity of the chemical constituents, such as proteins, sugars, and cellulose (Rosales 1996).

5.8. Associative effects between Ipil-ipil and *Gliricidia,* and roughages of *in vitro* CH_4 reduction potential

Some plants, especially most legumes, have secondary metabolites that directly or indirectly affect intake and the rumen environment (Rochfort et al. 2008). Their defining characteristics and the concentrations to which they are added to animal feed can confer both nutritional and environmental benefits and drawbacks. Owing to the ban on the use of antibiotics as growth promoters in animal feed by the European Union in 2006 and the vision for developing sustainable animal production, efforts are being made to identify plants that positively affect digestive efficiency and reduce CH_4 emissions to the environment (Makkar et al. 2007).

Tannins are generally considered to have antinutritional properties. Dietary-CT, particularly in legumes such as Ipil-ipil, bind proteins and that inhibits degradation in the rumen (Soltan et al. 2012, Zain et al. 2019, Irawan et al. 2020). Their capacity to lower CH_4 emission is well-documented (Martin et al. 2009) and is likely due to a direct effect on ruminal methanogens and an indirect effect on H^+ production as a result of decreased feed degradation (Tavendale et al. 2005). The CH_4/dOM of rice straw, German grass, and Napier silage decreased by 18.3% (Table 4.9), 12.1% (Table 4.13), and 7.0% (Table 4.17) upon supplementation with 40%, 10%, and 30% Ipil-ipil, respectively, indicating that these interactions were negatively associated with CH_4/dOM. These three combinations increased dOM, resulting in faster fermentation, and may produce a significant proportion of propionate in VFA (Pacheco et al. 2014, Van Lingen et al. 2016). This is associated with a reduction in enteric CH_4 production, given the negative relationship between propionate formation and enteric CH_4 emissions. This association is also corroborated by the current study, where the increase in CP content was positively associated with the dOM of low-quality roughages and negatively associated with the CH_4 yield. However, the increased dOM of low-quality roughages suggests that an increase in CP is often associated with a decrease in NDF content. Overall, the results indicate that adding Ipil-ipil with low-quality roughages (particularly by increased dOM and ME) consistently decreases CH_4 production and may be a potent CH_4 reduction strategy.

5.9. Effects of single concentrate addition to Ipil-ipil-based Mixed feed (MF) on *in vitro* ruminal fermentation

Ruminants are mainly fed on low-quality roughages with a small quantum of concentrates in the tropics. Among the concentrates are brans, which are traditionally combined to produce mixed feed (MF) on farms and supplied to animals (Sagar et al. 2013). In the current study, MF was formulated based on different types of commonly available bran, such as rice bran (RB), wheat bran (WB), and kashari bran (KB) in Bangladesh, to identify the type of bran best suited for rumen fermentation. The extent of GP of feeds depends on the chemical composition, rate, and degradability of the feedstuff (Blümmel et al. 1999). Variable GP was observed among single concentrates with Ipil-ipil-based MF in this study, suggesting that substantial differences in the CHO fractions of the feeds may influence the fermentation rate in the rumen (Deaville and Givens 2001). When RB was added to MF, the gas production of the fermentable substrate decreased with increasing incubation time. Similar results were observed by Hungate (1966) and Jayanegara and Sofyan (2008). However, the opposite trend was observed for WB and KB, owing to their high protein content, readily available carbohydrates, and low fibre content (Table 4.1).

Furthermore, the ash content of RB was significantly higher than that of WB (14.3% vs. 4.16%, respectively; Table 4.1), resulting in higher silica content of the mixed feed. Silica and lignocellulose are not readily degraded in the rumen (Fonnesbeck et al. 1981, Muhammad et al. 2013, Quispe et al. 2017), thereby reducing the total GP and dOM. On the other hand, soluble CHO and CP were found to have a positive connection with potential GP, GP rate, and dOM. The digestibility of nutrients improves with an increase in the CHO and CP content of the diet; however, it is inversely connected with an increase in the lignin content of the diet (Fonnesbeck et al.1981, De Boever et al. 2005). Additionally, Sari et al. (2018) and Fidriyanto et al. (2019) observed that increasing the fibre content of the diet decreased the DMD and dOM of the feed. As a result, incorporating rice bran into the diet has the potential to lower the dietary value, as well as *in vitro* gas output and rumen digestibility. The opposite trend was observed upon adding WB or KB as a single concentrate to the Ipil-ipil-based MF.

5.10. Effects of single concentrate addition to Ipil-ipil-based MF on *in vitro* CH_4 production

In this study, it was demonstrated that supplementation of 40% RB to the Ipil-ipil-based MF significantly decreased CH_4 production and concentration compared to that with WB and KB. Shinoda et al. (2007) and Cao et al. (2009) both made observations that were very similar to one another. They found that the addition of RB to the diet reduced the amount of CH_4 that was produced during both *in vitro* and *in vivo* rumen fermentation. This may be attributed to the high-fat concentration in RB, which likely inhibits CH_4 production by stimulating propionate and reducing acetate production, thereby adversely affecting protozoan activity, cellulolytic bacteria, and feed digestion in the rumen (Czerkawski et al. 1966).

Based on the reports of Johnson and Jonnson (1995), the CH_4 emissions were highest during the fermentation of fibrous carbohydrates, followed by the fermentation of soluble sugars and starch. Structural carbohydrate, NFC, and starch contents were lower in RB than in WB. The most notable distinction between the two diets was the RB diet's higher lipid content compared to WB (4.73% vs 1.87%, respectively), which was primarily composed of unsaturated fatty acids (USFA) like oleic (C18:1) and linoleic (C18:2) acids (Lugay and Juliano 1964). The supplementation of MF with RB is regarded as a CH_4 mitigation strategy because of the reduction in CH_4-producing bacteria and biohydrogenation of USFA (Hook et al. 2010, Shibata and Terada 2010). These USFA have been shown to have a harmful effect on ciliate protozoa (Dohme et al. 2001), and they are also responsible for an 18% reduction in CH_4 emissions (Matsui et al. 2013). Dohme et al. (2001) used the rumen simulation technique (RUSITEC) to document those individual fatty acids have a detrimental effect on ruminal methanogenesis. They also observed that supplementation with linoleic acid suppressed methanogen density and CH_4 production.

Additionally, KB resulted in a lower CH_4 concentration than WB ($P < 0.05$). KB tended to decrease CH_4 production with increasing CP (16.3% vs 13.1%; Table 4.1). This is consistent with the view that proteolytic microbes (bacteria and protozoa) are responsible for the fermentation of feed proteins in the rumen. The proteolytic enzymes that these microbes create, such as proteases, peptidases, and deaminases, are responsible for the breakdown of proteins into amino acids and peptides, which results in the production of ammonia when they are further degraded (Chuzaemi et al. 1990). The formation of NH_4, when compared with substrates contributing to CO_2 and CH_4, results in a reduction in the production of CH_4 (Getachew et al. 1998). Kurihara et al. (1997) also reported that increasing the CP of the diet consequently decreased CH_4 production in cattle.

5.11. Effects of single concentrate with Ipil-ipil-based MF on *in vitro* VFA and NH_3-N production

The status of VFA production in the rumen is a good indicator of feed quality. Optimum VFA production is required to reflect the digestibility of feed and support ruminant productivity (Tampoebolon et al. 2019, Niu et al. 2018). Acetate, propionate, and butyrate contribute 70% of the energy required for ruminant animals (Bergman 1990). In this 3rd study, VFA concentration (mmol/L) did not differ among most treatments except with butyrate and valerate. WB with Ipil-ipil-based MF resulted in the highest concentration of VFA, especially butyrate and valerate ($p<0.05$), instead of KB and RB. The high organic matter (OM) content is the predominant signal for WB rather than for KB and RB, and it raises VFA levels mostly through the microbial fermentation of carbohydrates (France and Siddons 1993). In addition, WB contained a significantly lower amount of ADL than either RB (4.43% vs. 14.4; Table 4.1) or their mixture, and the amount of ADL had a negative effect on the formation of VFA *in vitro* (France and Siddons 1993).

The amount of protein consumed, the rate of protein breakdown in the rumen, and the amount of time that had passed since the previous feeding all had an effect on the concentration of NH_3-N (Hungate 1966). Increased NH_3-N concentration indicated that highly degradable proteins in the rumen subsequently stimulated the growth of rumen microbes. However, in the current study, the different combinations of single concentrate with Ipil-ipil-based MF did not affect NH_3-N levels *in vitro* ($p>0.05$). This was probably because all treatments contained Ipil-ipil, which contains the CT and can bind polymers, particularly proteins, to form tannin-protein complexes, leading to a reduction in NH_3-N concentration (Bhatta et al. 2012). Another possibility is that a fraction of nitrogen in the fungal cell was incorporated into chitin; this chitin and its derivative could reduce the NH_3 concentration in the rumen fluid (Niu et al. 2018). Furthermore, no significant differences were observed in NH_3-N production between treatments and the blank. Despite the variation in the CP content of a single concentrate between different treatments, the composition of non-N and N-containing proteins may remain the same.

5.12. *In vitro* rumen fermentation characteristics of different roughage-based TMR

The energy- and protein-rich feedstuffs evaluated in this study can help in meeting the nutritional requirements of animals when supplemented to a basal feed of poor-quality roughages. The nutrient composition, fermented rumen parameters, and CH_4 production of the selected feedstuffs recorded in Study 1 are consistent with that in previous reports (Lee et al. 2003, Chung-Nan Chen et al. 2016, Bhatta et al. 2017). High protein-containing leguminous fodder, gross energy in the cereal grain and its by-products, and lipid-containing RB indicate that these feedstuffs are highly digestible and high in metabolisable energy (McDonald et al. 2002) and reduce CH_4 production. We hypothesised that a combination of commonly available feedstuffs (TMR) is suitable for rumen fermentation and CH_4 reduction *in vitro*.

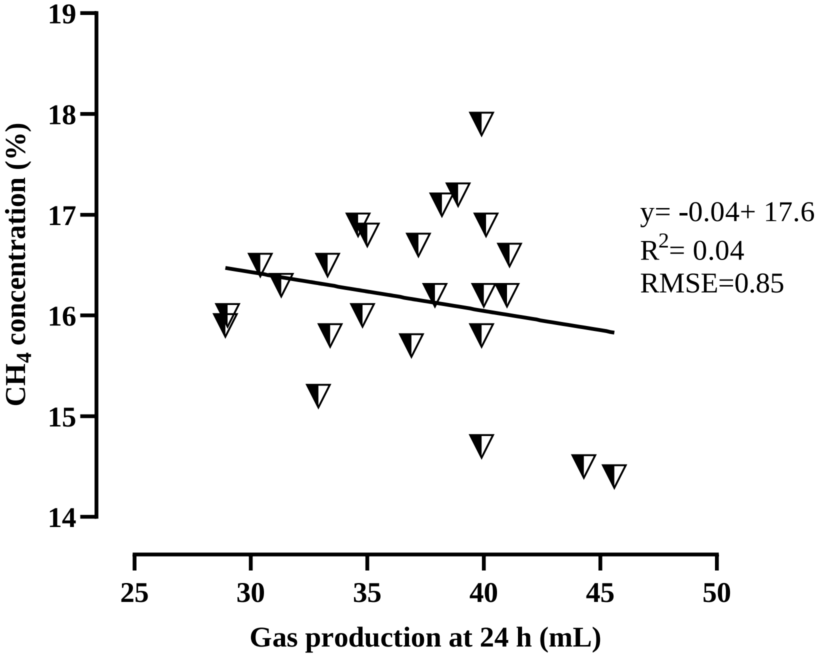

Fig.43: Comparison of gas production at 24 h and CH_4 concentration of different roughages based TMR using an *in vitro* ruminal fermentation technique.

In the current study, rice straw, German grass, Napier silage, and maize silage-based TMR-1, TMR-2, and TMR-5 resulted in lower gas production because of their lower non-structural carbohydrate and starch contents. The amount of gas produced from feedstuff relies primarily on its nutrient composition, rate, and extent of digestibility. It is important to note that a high concentration of NDF and ADF in feed impedes the breakdown of starch by rumen microbes, which in turn lowers the availability of carbohydrates that can be rapidly digested (Wilson and Hatfield 1997) and limits gas production (Njidda and Nasiru 2010). Moreover, the digestibility of these TMR was also low compared with that of the other treatments, likely owing to the high lignin content. Lignin is resistant to digestion by microbial enzymes and bound with cellulose and hemicellulose. Similar observations were reported by Hariadi and Santoso (2010), who indicated that higher lignin content in the ration reduced its digestibility. However, different roughage-based TMR-3 and TMR-6 produced a high amount of gas (mL), dOM (%), and ME (MJ/kg DM) compared to that of other possible treatments because of their excellent combination of feedstuffs. This combination created a better environment for engulfing readily fermentable CHO by rumen microbes, especially cellulolytic bacteria and fungi (Aregheore et al. 1999).

5.13. *In vitro* CH_4 production of different roughage-based TMR

CH_4 production usually tends to be higher (mL/200 mg DM) when different feedstuffs have higher gas production (Durmic et al. 2010, Njidda and Nasiru 2010, Jayanegara et al. 2011). This was consistent with our observation of gas production, dOM, and CH_4 production for all roughage-based TMR except RS_{TMR}-3 and GG_{TMR}-3, where CH_4 concentration (%) was significantly lower despite higher total GP (Fig. 44 and 45).

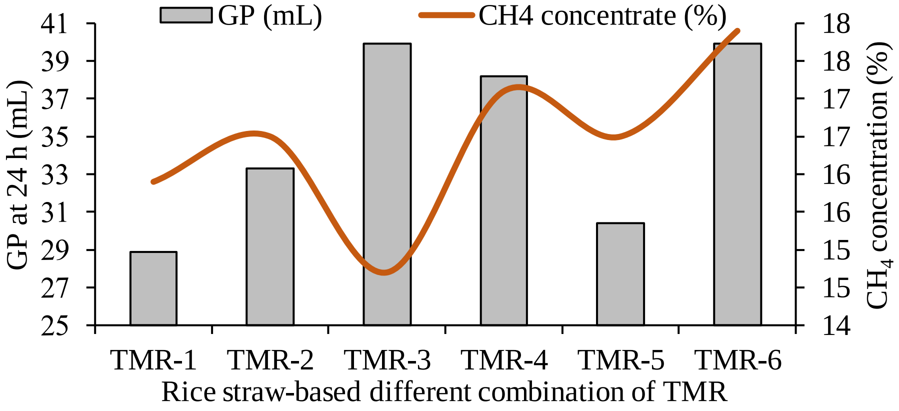

Fig. 44: Comparison between gas production at 24 h and CH_4 concentration of different combinations of rice straw-based TMR

Among the concentrates, RS_{TMR}-3 and GG_{TMR}-3 contained 60% soluble CHO-rich WB, which may have increased the population of ciliated protozoa, and stimulated their hydrogen transfer to methanogens, resulting in increased CH_4 production (Bonhomme 1990). Therefore, the high CH_4 production from grain by-products can be attributed to their higher NSC and dOM contents. In contrast, the lower concentrations of CH_4 produced by these TMR positively modulated rumen fermentation, resulting in the production of less CH_4 than would have been predicted given their greater total GP (Fig. 43, 44, and 45). Another factor may be the presence of secondary metabolites such condensed tannin and saponin found in Ipil-ipil may also have a role by inhibiting the growth of rumen microorganisms (Jayanegara et al. 2010). Some studies have also reported that condensed tannins are responsible for reducing the total protozoan population (Bhatta et al. 2009, Rira et al. 2019).

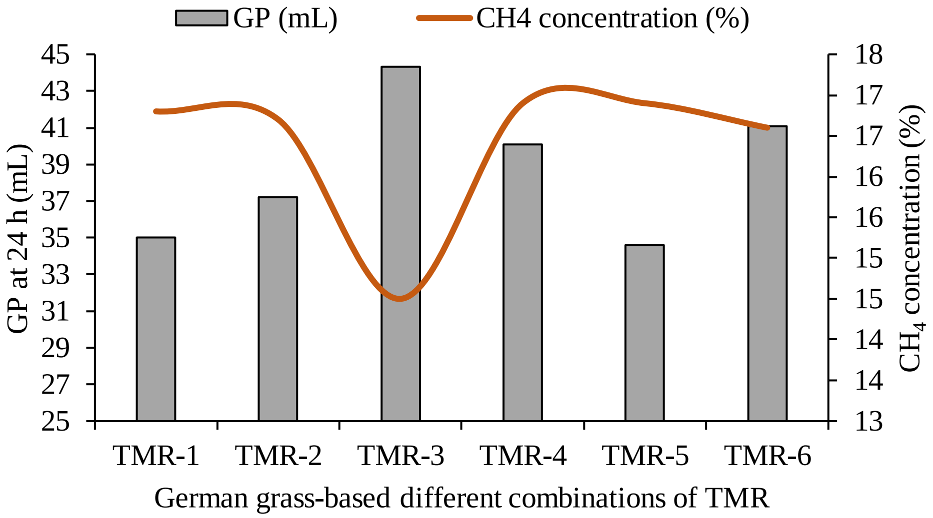

Fig. 45: Comparison between gas production at 24 h and CH_4 concentration of different combinations German grass-based TMR

Tannins have the ability to bind with sterols on the membranes of protozoan cells, which changes the permeability of the cell membranes and utterly destroys the protozoan cells (Saminathan et al. 2017). Saponin, a lipid molecule that alters the shape of the protozoan cell membrane, has the same function as tannin and has the ability to inhibit protozoan growth and alter the pattern of fermentation in the rumen. In the current study, RS_{TMR}-3 and GG_{TMR}-3, which contained tannin and saponin, retained 40% and 30% Ipil-ipil, respectively (2.6% DM in the leaves and stems; FAO 2009), which may reduce the protozoan population in the rumen, likely resulting in the lowest CH_4 concentration (14.7% and 14.5%, respectively) compared to that of other treatments. Similar results have been reported by Jayanegara et al. (2012). Additionally, decreasing the population of protozoa in the rumen is an efficient approach to increasing the bacterial population. This is due to the fact that even a partial reduction in the rumen's protozoan population can lead to an increase in the number of rumen bacteria (Gutierrez 2007).

One of the by-products of feed fermentation in the rumen is CH_4 gas, which is produced from CO_2 and H_2 by methanogenic bacteria. The amount of H_2 utilised in methanogenesis was 4 mol/mol CH_4, and the free energy transfer from this reaction was −134 kJ/mol CH_4. The conversion of pyruvate to acetate, when combined with $\Delta F = -55$ kJ/mol of the substrate, results in 1 mol of ATP. Therefore, the estimation of methanogenesis with $\Delta F = -134$ kJ/mol CH_4 produces equivalent energy of at least 3 moles of ATP/mol CH_4 (Czerkawski et al. 1986). This reflects the elevated production of CH_4 and increased energy expenditure. Collectively, higher CH_4 production feedstuffs are inefficient for livestock production (McDonald et al. 2002). The lowest CH_4 was produced by RS_{TMR}-3 and GG_{TMR}-3, which indicated that these TMRs are more effective for energy utilisation. According to McDonald (2002), feed efficiency in ruminants depends on the CH_4 production in the rumen. A higher CH_4 output indicates less efficient feeding practises.

5.14. *In vitro* VFA production of different roughage-based TMR

The formation of VFA in the rumen, which are the principal source of energy for ruminant animals, is influenced by the feed's quality, quantity, and ways of feeding (McCarthy et al. 1989, Casper et al. 1990). Acetate and butyrate may supply energy to animals; however, a major part of the metabolic glucose supply results from propionate, which is produced in the liver via gluconeogenesis (Cridland 1983). Dairy and animal fattening both benefit greatly from the use of VFA. VFA production can considerably deviate depending on the nature of the feed. Commonly, the molar ratios of acetate to propionate to butyrate vary from 75:15:10–40:40:20 (Bergman 1990).

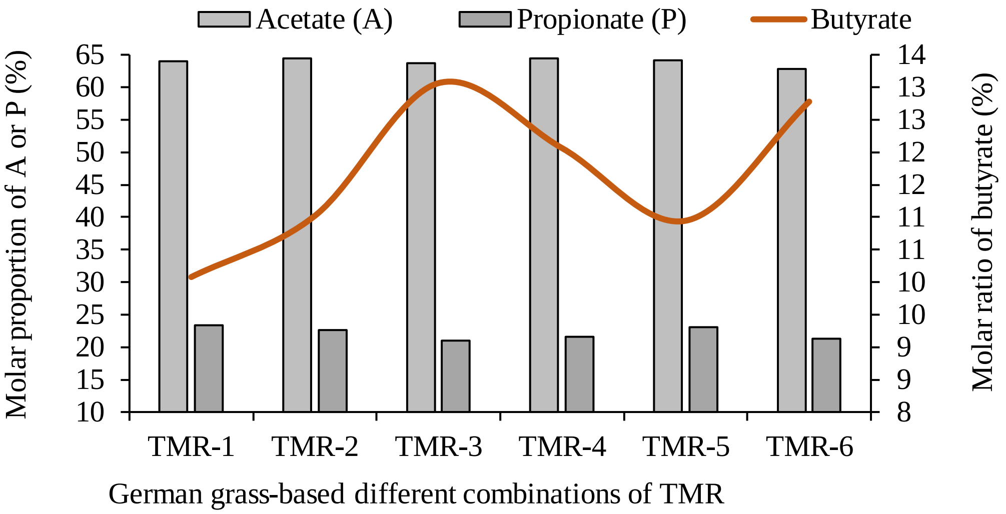

Fig. 46: Comparison between German grass-based different combinations of TMR with VFA production

In this study, the molar ratios of acetate, propionate, and butyrate in GG_{TMR}-3 and NS_{TMR}-3 were 64:21:13 and 63:21:13, respectively. Compared to the other treatments, significantly increased butyrate production was recorded in GG_{TMR}-3 and NS_{TMR}-3 (Fig. 46 and 47). The high molar proportion of propionate and butyrate production in the rumen is associated with a reduction in CH_4 production as an H_2 sink product (Nagaraja et al. 1997, Moss et al. 2000, Shibata and Terada 2010). Additionally, the total VFA concentration was higher in GG_{TMR}-3, NS_{TMR}-3, and MS_{TMR}-3, indicating that fermentation was relatively active with the addition of a high percentage of WB and KB.

The ratio of acetate (A) to propionate (P) indicates the efficiency of energy consumed by ruminants. Acetate is a non-glucogenic compound that can be oxidised by almost all body tissues. After absorption, it is used to synthesise milk fat, body fat, and muscle in growing animals. Conversely, propionate is a sugar precursor or primary glucogenic feed (Susanti et al. 2001) that increases milk yield. The A/P ratio ranged from 2.40–3.06. MS_{TMR}-1 and GG_{TMR}-3 treatments had the lowest (2.40) and highest (3.06) A/P ratio, respectively.

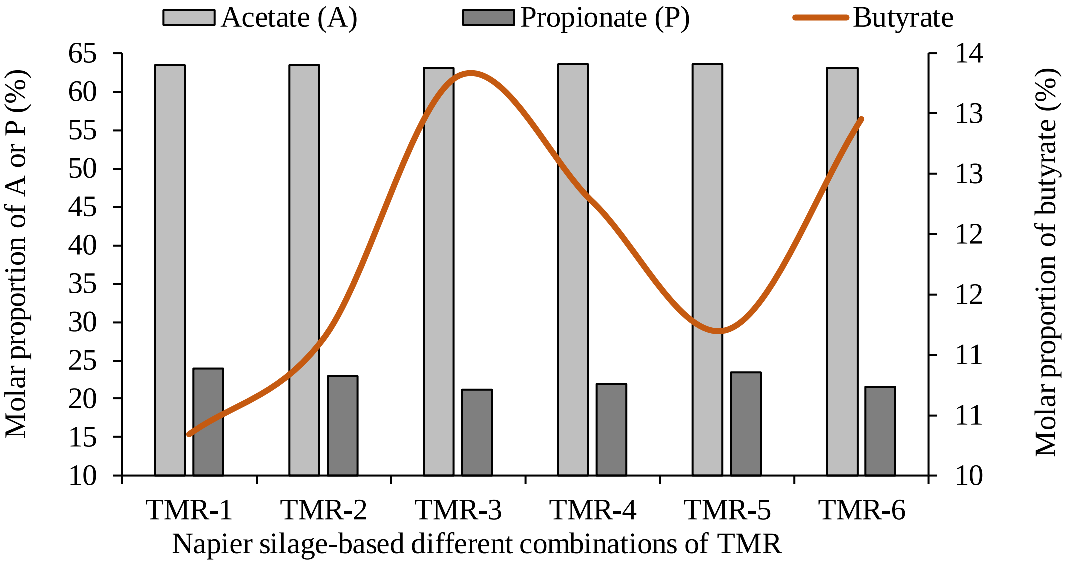

Fig. 47: Comparison between Napier silage-based different combinations of TMR with VFA production

5.15. Associative effects of different roughage-based TMR

Low-quality roughages are commonly used as cattle feed in Southeast Asia, and associations between feed ingredients for cattle have been described in the literature; however, information on the associative effects of available feed components for diet formulation remains limited. An associative effect may occur if there exists an interdependence between various single feeds. Rumen microbial fermentation of single and combined feedstuffs may deviate (Niderkorn and Baumont 2009), and the fermentation process may be different, which may lead to positive and negative associative effects in dairy animals.

The measured values of dOM, ME, and NEL in the different roughage-based TMR tended to be higher than their estimated values. A higher measured value indicated that the soluble fraction constitutes a substrate of rapid fermentation that facilitates the adhesion and colonisation of microorganisms, thereby increasing fermentation and reduce the incubation period (Shen et al.

2019). Other studies have come to the same conclusions; the use of tropical grasses and legumes together improved *in vitro* fibre degradation due to the favourable associative effects of both ingredients (Brown et al. 1991). The primary function of rumen microbes is to ferment and convert the substrates to CO_2, CH_4, H_2, and VFA (Liu et al. 2002). An increase in VFA may be responsible for the subsequent rise in GP, which may be linked to the interaction between fibre- and non-fibre-decomposing microbes or ruminal bacteria and protozoa (Liu et al. 2002, Robinson et al. 2009). When rice straw, German grass, Napier silage, and maize silage were fermented with Ipil-ipil and concentrates (RB, WB, and KB), interactions among the rumen microorganisms increased substrate fermentation and produced more gas and VFA, which resulted in a higher associative effect than those of individual feedstuffs (Sandoval-Castro et al. 2002). Therefore, the associated effects of legumes and concentrates may compensate for the lack of nutrients in rice straw, German grass, Napier silage, and maize silages. Low-quality energy and protein deficit roughages produced the lowest gas and digestibility. Supplementation with Ipil-ipil and different combinations of concentrates improved the GP and dOM of different roughage-based TMR. RS_{TMR}-6, NS_{TMR}-6, GG_{TMR}-3, and MS_{TMR}-3 exhibited more pronounced associative effects for efficient rumen fermentation and better feed efficiency. The differences in GP and their energy values between TMR and its components were likely caused by different microbial profiles, which can be related to the various substrate performances of the different protozoal, bacterial, and archaeal species in the rumen (Williams et at. 2010), and differences in the energy-protein ratio (Zebeli et al. 2008, Estrada-Lièvano et al. 2009, Berthiaume et al. 2010).

Poor quality roughage may be detrimental to the growth of rumen microorganisms or the ruminant itself (e.g. insufficient supply of nitrogen, sulphur, and phosphorus). However, positive associative effects indicate that other feedstuffs contain the required nutrients. Some studies have demonstrated that supplementing rapidly fermentable CHO sources (grain by-products; wheat bran) with roughage would improve microbial growth and feed degradability (Zicarelli et al. 2011, Belanche et al. 2012, Guadagnin et al. 2013). RS_{TMR}-6, NS_{TMR}-6, GG_{TMR}-3, and MS_{TMR}-3 were fermented with Ipil-ipil and concentrates; NFC from WB readily fermented cellulose and hemicellulose, and Ipil-ipil influenced rumen protein degradation between legume tannins and grass protein, which may have contributed to the highest positive associative effects (Niderkorn et al. 2012, Ling Sun et al. 2020).

In the current study, different roughage-based TMR-3 revealed that the measured values of CH_4-related traits were lower than the estimated values, indicating negative associative effects. However, the literature on this subject is scant. For instance, Jayanegara et al. (2013) observed that a combination of limited tannin-containing fodder and CT-rich plants resulted in an interactive effect on CH_4 emission, consequently reducing output more than the sum of the individual feeds.

5.16. Conclusions and perspectives for future research

Eighteen commonly available feedstuffs, including twelve roughages and six concentrates, were used as cattle feed for livestock production in Bangladesh. In this work, preliminary data on the chemical constituents, digestibility, and CH_4 production status of feedstuffs are provided that could be used to optimise diet formulation for ruminants in the future by selecting feedstuffs with low CH_4 output. It could also be demonstrated that the legume plants, Ipil-ipil (*Leucaena leucocephala*) and Gliricidia (*Gliricidia sepium*), exhibited wide nutritional diversity, and provided both protein and fibre for livestock. Therefore, supplementing legume plants to poor quality roughages in the study area would be a viable alternative to reduce CH_4 production and enhance animal production performance.

The chemical properties, rumen fermentation characteristics, and CH_4 production of some feeds are poor, while some single feeds are good quality. To improve roughage quality, supplementing graded levels of high-protein leguminous fodders, such as Ipil-ipil and Gliricidia, was combined with poor quality roughages. Based on these results, it was demonstrated that Ipil-ipil supplemented with low-quality roughages is more potent than Gliricidia. Adding 40% Ipil-ipil with rice straw and 30% Ipil-ipil with Napier silage effectively enhanced the dOM (18.1% and 15.7%, respectively) and reduced the CH_4 concentration (6.89% and 5.32%, respectively). Notably, no significant interaction was observed between Ipil-ipil and good quality roughages, such as German grass and maize silage. A better understanding of the associative effects between poor quality roughages and Ipil-ipil could help improve the feed utilisation efficiency and environmental hazards of the existing feeding systems, thereby contributing to the enhanced sustainable development of animal production. Finally, based on the data, the combination of roughages can be ranked as follows: RS + 40% > NS + 30% Ip > MS + 30% Ip > GG + 10% Ip.

Smallholder farmers in the tropics used available brans such as RB, WB, and KB as a single concentrate for rearing livestock. These brans must be categorised according to their performance efficiency as livestock feeds. The addition of RB as a single concentrate to the Ipil-ipil-based MF reduced the CP content and raised the structural carbohydrate, negatively impacting rumen fermentation and gas production. However, the reduced CH_4 production compared to that in WB and KB is a positive outcome.

Optimal combinations of single concentrates with roughages (TMR) have been identified to reduce CH_4 production, increase energy utilisation and dOM, and have optimal associative effects. These results imply that GG_{TMR}-3 and MS_{TMR}-3 are potent in maximising dOM (68.8% and 67.6%, respectively) and simultaneously mitigating CH_4 production, CH_4 concentration, and CH_4/dOM *in vitro*. Moreover, a combination of 40% WB and 30% KB with rice straw and Napier silage-based TMR-3 and TMR-6 is also a sustainable way to optimise fermentation capacity in the tropics.

Ipil-ipil of leguminous tree fodder is recommended as a good protein supplement to low-quality roughages for ruminants, especially in extensive production systems, with the potential to improve diet digestibility, protect dietary proteins from microbial activity, and mitigate enteric CH_4 production. However, for a better understanding, an *in vivo* study should be conducted to elucidate the rumen population dynamics and their inconsistent efficacy and optimise combinations to achieve endurable and feasible CH_4 mitigation in ruminants.

6. SUMMARY

Multiple analyses have shown that the rising human population, urbanization, and consumer preferences affect the demand for livestock products in developing countries. Concurrently, human population and urbanisation growth are reducing the likelihood of newly cultivated land producing feeds or restoring damaged pastures. Using low-quality roughage more efficiently is an option, but such roughages have drawbacks, including high structural carbohydrate and low nitrogen contents, which lead to poor palatability and nutrient utilisation including emission of methane (CH_4) that is harmful to the environment and a loss of energy to the animal. Using commonly accessible legumes and tree foliage could be a possibility to address the need for N and CH_4 mitigation due to their high crude protein and secondary compound contents. In order to evaluate the potential of specific combinations of roughages, concentrates, and other feeds, it is necessary to determine the feeding values, *in vitro* ruminal fermentation, and CH_4 production of single feeds, as well as the promising interaction (associative) effects between single feeds when combinded in a ration. This may allow for formulating a better balanced total mixed ration (TMR) for ruminants, which was the main goal of the present doctoral study.

Eighteen feedstuffs from Bangladesh were chosen, including roughages such as crop residues (rice straw, urea molasses treated straw and maize stover), silages (Napier silage and Maize silage), common grasses (German grass, Para grass and Napier grass) and leguminous fodder (Ipil-ipil, Glicidia, Alfalfa hay and Moringa tops) and concentrates (crushed wheat, crushed maize, Wheat bran, Kashari bran, and Rice bran). In Study 1, the single feeds were characterized by comprehensive chemical analysis and *in vitro* production of total gas and methane using the Hohenheim gas test. Based on the results of Study 1, rice straw, German grass, Napier silage, and maize silage were used in Study 2 and incubated *in vitro* alone or after substitution by Ipil-ipil and Gliricidia at levels of 10%, 20%, 30%, and 40%, respectively. In Study 3, mixtures of roughages and leguminous fodder (rice straw + 40% Ipil-ipil, German grass + 10% Ipil-ipil, Napier silage + 30% Ipil ipil, and maize silage + 30% Ipil-ipil) were combined with single concentrates in order to identify the most promising mixtures in regard to digestibility and methane production. Eventually, 24 TMR were formulated using six for each type of roughage in Study 4. These TMR were comprised of 60% of the same combination of roughage as used in Study 3 and 40% of various combinations of concentrates including TMR-1 (60% rice bran + 10% wheat bran + 30% kashari bran), TMR-2 (30% rice bran + 10% wheat bran + 60% kashari bran), TMR-3 (10% rice bran + 60% wheat bran + 30% kashari bran), TMR-4 (10% rice bran + 30% wheat bran + 60% kashari bran), TMR-5 (60% rice bran + 30% wheat bran + 10 kashari bran), and TMR-6 (30% rice bran + 60% wheat bran + 10% kashari bran) in order to identify suitable TMR for maximising ruminal fermentation and reducing the CH_4 production *in vitro*. In all studies, the total gas production (GP), digestibility of organic matter (dOM), metabolisable energy (ME), and net energy for lactation (NEL) were determined after incubating 200 mg of feed with a rumen fluid-buffer solution for 72 hours. In addition, the CH_4 concentration in the produced gas was measured after incubating 120 mg of feed for 24 hours. Measured values of the mixed feed were compared to estimated values, where the estimated values were weighted means of the values of the individual feeds, to determine the associative effects between the feed ingredients (Study 2 and Study 4).

In Study 1, the concentration of acid detergent fibre (ADF), neutral detergent fibre (NDF), and acid detergent lignin (ADL) was the lowest in leguminous fodder while the crude protein (CP) concentration was the hightest, followed by the common grasses and the silages. The crop residues showed the lowest CP and the highest cell wall fraction concentrations. The dOM, ME, and NEL of crushed wheat and crushed maize were significantly higher ($p<0.05$) than those of other feedstuffs. The same differences were found ($p<0.05$) for CH_4 concentration (% of GP) and CH_4 production (L CH_4/kg dOM). The dOM and ME of German grass and Ipil-ipil were higher ($p<0.05$), whereas the CH_4 concentration and CH_4 production were lower compared to that of crop residues and other common grasses. The CH_4 production of single feeds decreased with increasing concentrations of CP, ADF, and ADL, whereas it increased with NDF concentration. These findings enabled development of more balanced diets for ruminants with the aim of improved digestibility and reduced CH_4 emission while making use of widely available feed resources in Bangladesh.

Study 2 aimed to evaluate the *in vitro* ruminal fermentation, CH_4 production, and associative effects between low-quality roughages and CP supplements. The gas production after 24 hours (GP_{24}) and rumen fermentation rate increased ($p<0.05$) with increasing addition of Ipil-ipil to rice straw. It also resulted in a gradual increase of dOM and ME ($p<0.05$) of the mixture, reaching maximum values (59.1% and 7.60 MJ ME/kg DM) and maximum reductions in CH_4 concentration and CH_4/dOM (6.9% and 11.7%) compared to the control with Ipil-ipil addition at 40% of DM. When Ipil-ipil was added at 30% to Napier silage, the same trend was observed for dOM and ME

(56.5%, and 7.55 MJ ME/kg DM) and CH_4 concentration and CH_4/dOM (5.3% and 13.3%). Ipil-ipil did not significantly interact with high quality roughages such as German grass and maize silage. Increasing the addition of *Gliricidia* to the roughages in Study 2 led to a decrease in GP_{24}, dOM, ME, and CH_4 production. The highest levels of the associative effects ($p<0.05$) were seen when 40% Ipil-ipil was added to rice straw and 30% was added to Napier silage. In conclusion, adding Ipil-ipil to low-quality roughages is superior to *Gliricidia* and showed promising results, with the ranking as follows: rice straw + 40% Ipil-ipil > Napier silage + 30% Ipil-ipil > maize silage + 30% Ipil-ipil > German gras + 10% Ipil-ipil.

Study 3 and Study 4 had the overarching purpose to determine which combination of single concentrates and combinations of concentrates with roughage produced the best mixed feed in terms of rumen fermentation, CH_4 reduction potential, and associative effect. The CP and non-stach carbohydrate contents of the Ipil-ipil-based mixed feed was enhanced by adding Wheat bran and Kashari bran as single concentrates, which had a favourable effect on rumen fermentation and gas production but had the opposite effect on CH_4 production when compared to the addition of Rice bran. In Study 4, TMR were formulated using roughages and Ipil-ipil and addition of Wheat bran, Kashari bran, and Rice bran in various combinations. The TMR based on German grass and maize silage with specific bran combinations showed significant reductions in *in vitro* CH_4 production, CH_4 concentration, and CH_4/dOM while maximising GP, dOM, ME, and the associative effects.

To conclude, the results of chemical analyses and *in vitro* fermentation studies showed that specific combinations of roughages, protein feeds, and by-products available in Bangladesh have the potential to formulate rations for cattle that help making livestock production more sustainable. The results may be also relevant for other developing nations. It is suggested to verify the results of the present project by animal trials at local conditions.

7. ZUSAMMENFASSUNG

Zahlreiche Auswertungen haben gezeigt, dass die global wachsende Bevölkerung, der Wohlstand und die Verstädterung direkte Auswirkungen auf die Nachfrage nach tierischen Erzeugnissen in Entwicklungsländern haben. Sowohl die Bevölkerung als auch die zunehmende Verstädterung verringern die Wahrscheinlichkeit, dass neu bewirtschaftete Flächen Futtermittel liefern oder degradierte Weideflächen wiederhergestellt werden. Grobfutter minderer Qualität effizienter zu nutzen ist eine Option, den Futterbedarf zu decken. Allerdings haben viele Grobfutter Nachteile, wie z. B. einen hohen Gehalt an Strukturkohlenhydraten und wenig Stickstoff, was zu einer geringeren Schmackhaftigkeit und Nährstoffverwertung sowie zu höheren Emissionen von Methan (CH_4) führt. Dies schadet der Umwelt und verringert die Energieeffizienz der Tiere. Üblicherweise verfügbare Leguminosen und Baumlaub könnten aufgrund ihres hohen Gehalts an Protein und sekundären Inhaltsstoffen eine Möglichkeit sein, den Bedarf an Stickstoff zu decken und CH_4-Emissionen zu verringern. Um das Potential von Kombinationen von Grobfutter, Konzentraten und anderern Futtermitteln zu bewerten, müssen Futterwertkennzahlen wie in vitro-Pansenfermentation und die CH_4-Produktion von Einzelfuttermittel sowie die vielversprechenden Wechselwirkungen (assoziative Effekte) von Einzelfuttermittel in Mischungen bestimmt werden. Dies könnte es ermöglichen, eine ausgewogenere Totale Mischration (TMR) für Wiederkäuer zu formulieren, was das Hauptziel der vorliegenden Doktorarbeit war.

Achtzehn Futtermittel aus Bangladesch wurden ausgewählt. Dies schloss Grobfutter wie Ernterückstände (Reisstroh, Maisstroh und mit Harnstoffmelasse behandeltes Stroh), Silagen (Napier-Silage und Mais-Silage), Gräser (German-Gras, Para-Gras und Napier-Gras) und Leguminosen (Ipil-ipil, Glicidia, Luzerne-Heu und Moringa-Spitzen) sowie Konzentrate und Koppelprodukte (Weizenschrot, Maisschrot, Weizenkleie, Kashari-Kleie und Reiskleie) ein. In Studie 1 wurden die Einzelfuttermittel durch umfassende chemische Analysen und die in vitro-Gasbildung mit dem Hohenheimer Futterwerttest charakterisiert. Basierend auf den Ergebnissen von Studie 1 wurden in Studie 2 Reisstroh, German-Gras, Napier-Silage und Mais-Silage ausgewählt und einzeln oder mit im Austausch zu 10 %, 20 %, 30 % bzw. 40 % gegen Ipil-ipil und Gliricidia in vitro inkubiert. In Studie 3 wurden Mischungen aus Grobfutter und Leguminosen (Reisstroh + 40 % Ipil-ipil, German-Gras + 10 % Ipil-ipil, Napier-Silage + 30 % Ipil-ipil und Mais-Silage + 30 % Ipil-ipil) jeweils mit einzelnen Konzentraten kombiniert, um die vielversprechendsten Mischungen hinsichtlich Verdaulichkeit und CH_4-Produktion zu identifizieren. Schließlich wurden in Studie 4 24 TMR – jeweils 6 für jedes Grobfutter – formuliert. Diese TMR bestanden zu 60 % aus der gleichen Kombination von Grobfutter wie in Studie 3 und zu 40 % aus verschiedenen Kombinationen von Konzentratfutter, einschließlich TMR-1 (60 % Reiskleie + 10 % Weizenkleie + 30 % Kashari-Kleie), TMR-2 (30 % Reiskleie + 10 % Weizenkleie + 60 % Kashari-Kleie), TMR-3 (10 % Reiskleie + 60 % Weizenkleie + 30 % Kashari-Kleie), TMR-4 (10 % Reiskleie + 30 % Weizenkleie + 60 % Kashari-Kleie), TMR-5 (60 % Reiskleie + 30 % Weizenkleie + 10 % Kashari-Kleie) und TMR-6 (30 % Reiskleie + 60 % Weizenkleie + 10 % Kashari-Kleie). Dies hatte das Ziel, geeignete TMR für die Maximierung der Pansenfermentation und die Reduktion der CH_4-Produktion in vitro zu ermitteln. In allen Studien wurde die Gesamtgasproduktion (GP), die Verdaulichkeit der organischen Substanz (dOM), die Umsetzbare Energie (ME) und die Netto-Energie-Laktation (NEL) nach 72-stündiger Inkubation von 200 mg mit Pansenflüssigkeit und Pufferlösung bestimmt. Außerdem wurde die CH_4-Konzentration im produzierten Gas gemessen, nachdem 120 mg Futter 24 Stunden inkubiert wurden. Die gemessenen Werte der Mischrationen wurden mit geschätzten Werten verglichen, wobei die geschätzten Werte gewichtete Mittelwerte der Daten der Einzelfuttermittel waren, um die Wechselwirkungen zwischen den Futtermitteln zu bestimmen (Studie 2 und Studie 4).

Den Ergebnissen von Studie 1 zufolge war die Konzentration an Säure-Detergentien-Faser (ADF), Neutral-Detergentien-Faser (NDF) und Säure-Detergentien-Lignin (ADL) bei Leguminosen am niedrigsten, wohingegen die Leguminosen die höchste Konzentration an Rohprotein (XP) aufwiesen, gefolgt von den Gräsern und den Silagen. Die Ernterückstände wiesen den niedrigsten und die höchsten Konzentrationen an XP bzw. Zellwandfraktionen auf. Von geschrotetem Weizen und Mais waren dOM, ME und NEL signifikant ($p<0,05$) höher als bei den anderen Futtermitteln. Die gleichen Unterschiede wurden für die CH_4-Konzentration (% des GP) und die CH_4-Produktion je kg dOM festgestellt ($p<0,05$). Die dOM und ME von German-Gras und Ipil-ipil waren höher ($p<0,05$), wohingegen die CH_4-Konzentration und die CH_4-Bildung im Vergleich zu den Ernterückständen und anderen Gräsern niedriger waren. Die CH_4-Bildung von Einzelfuttermitteln nahm mit steigender Konzentration an XP, ADF und ADL ab, während sie mit der Konzentration an NDF und vermehrter GP zunahm.

In Studie 2 wurde die in vitro-Pansenfermentation, CH_4-Reduktion und die Wechselwirkungen zwischen minderqualitativen Grobfuttermitteln und Proteinsupplementen untersucht. Die Gasbildung nach 24 Stunden (GP24) und die Pansenfermentationsrate stiegen ($p<0,05$) mit zunehmender Zugabe von Ipil-ipil zu Reisstroh. Dies führte zudem zu einem Anstieg von dOM und ME ($p<0,05$) in der Mischung und erreichte Werte von bis zu 59,1% bzw. 7,60 MJ ME/kg TM und eine maximale Reduzierungen der CH_4-Konzentration und dem CH_4/dOM-Verhältnis (6,89% bzw. 11,7%) im Vergleich zur Kontrolle, wenn Ipil-ipil bei 40% TM zugesetzt wurde.

Wenn Ipil-ipil zu 30 % zu Napier-Silage zugegeben wurde, wurde derselbe Trend für dOM und ME (56,5 % bzw. 7,55 MJ ME/kg TM) und die CH_4-Konzentration und das CH_4/dOM-Verhältnis (5,32 % bzw. 13,3 %) beobachtet. Ipil-ipil zeigte keine signifikanten Interaktionen mit hochwertigen Grobfuttermitteln wie Deutschem Weidelgras und Mais-Silage. Eine Erhöhung des Anteils von Gliricidia bei den Grobfuttermitteln führte zu einem Rückgang von GP_{24}, dOM und ME sowie der CH_4-Produktion. Die deutlichste Wechselwirkung ($p<0,05$) wurde festgestellt, wenn 40% Ipil-ipil zu Reisstroh und 30% zu Napier-Silage gegeben wurden. Zusammenfassend wurde festgestellt, dass die Zugabe von Ipil-ipil zu minderqualitativen Grobfuttermitteln im Vergleich zu Gliricidia vielversprechende Ergebnisse zeigte, wobei die Rangfolge wie folgt war: Reisstroh + 40% > Napier-Silage + 30% Ipil-ipil > Mais-Silage + 30% Ipil-ipil > German-Gras + 10% Ipil-ipil.

Sowohl Studie 3 als auch Studie 4 hatten das übergeordnete Ziel, zu bestimmen, welche Kombination von einzelnen Konzentraten mit Grobfutter das beste Mischfutter hinsichtlich Pansenfermentation, CH_4-Reduktionspotenzial und Wechselwirkungen ergab. Die Konzentrationen an XP und Nicht-Stärke-Polysacchariden von auf Ipil-ipil basierendem Mischfutter wurde durch die Zugabe von Weizenkleie und Kashari-Kleie erhöht, was vorteilhafte Auswirkungen auf die Pansenfermentation und die Gasproduktion hatte, aber abträgliche Konsequenzen für die CH_4-Produktion im Vergleich zur Zugabe von Reiskleie. In Studie 4 wurden TMR mit Ipil-ipil und der Zugabe von Weizenkleie, Kashari-Kleie und Reis-Kleie in verschiedenen Kombinationen verwendet. Die TMR, die aus German-Gras oder Mais-Silage mit bestimmten Kombinationen aus Kleien bestanden, zeigten eine signifikante Verringerung der in vitro-CH_4-Bildung, der CH_4-Konzentration und des CH_4/dOM-Verhältnisses bei gleichzeitiger Maximierung von GP, dOM, ME sowie der Wechselwirkung.

Die Ergebnisse der Studien zu chemischen Analysen der Inhaltsstoffe und der in vitro-Fermentation zeigten, dass bestimmte Kombinationen aus in Bangladesch verfügbaren Grobfutter- und Proteinfuttermitteln sowie Nebenprodukten des Lebensmittelsektors die Möglichkeit bieten, Rationen für Wiederkäuer zu formulieren, die zu einer nachhaltigeren Tierhaltung beitragen. Diese Erkenntnisse könnten auch für andere Entwicklungländer von Bedeutung sein. Es wird empfohlen, die Ergebnisse des vorliegenden Projekts unter den jeweiligen lokalen Bedingungen in Tierversuchen zu verifizieren.

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DECLARATION IN LIEU OF AN OATH ON INDEPENDENT WORK

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic:

"Approaches to increase digestibility of Bangladesh ruminant feed resources in order to mitigate enteric methane production".

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. 3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath. I confirm that the declaration above is correct.

I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

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