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Comparing Rumen Fluid to Buffer Ratios to Estimate *in vitro* Degradability, Fermentation, and Methane Profiles of Seven Forages at Two Incubation Times

Research Article

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ABSTRACT

Using different rumen fluid (RF) or RF to buffer (B) ratios (RFB) could be a limiting factor in evaluating numerous feeds for obtaining reproducible estimates of *in vitro* rumen degradability. Thus, two duplicated factorial experiments (exp) compared the effect of changing the RFB ratios on degradability, fermentation, and methane profiles of seven forages during *in vitro* incubations with 40 mL of buffered RF (inoculum, RI) for 48 and 96 hours. Exp 1 (7×2×2) involved 7 forages (F) and 2 RFB ratios (1:2 and 1:4), for two incubation times (T, 48 and 96 hours). The main effects of F and T were significant ($P<0.05$) but the effect of RFB ratio was not always significant ($P>0.05$) for the mean *in vitro* dry matter degradability (IVD) and organic matter degradability (IVOMD). The mean IVD and IVOMD of each forage and mean of all forages were significantly ($P<0.05$) higher after 96 h than 48 h of incubation. The F × RFB interaction was significant ($P<0.05$) for IVD and IVOMD depending on different RFB ratios. In exp 2 (7×2) involving the same 7 forages and 2 RFB ratios, total gas volume and methane were significantly ($P<0.001$) higher for the 1:2 than the 1:4 RFB ratio. Methane concentration was higher for the high fibrous than the low fibrous forages. Ammonia concentration, total gas volume, and methane production correlated positively with IVD. It appeared that the degradability, fermentation, and methane profiles of different forages were influenced by the RFB ratios, and *in vitro* incubation times. However, the changes in IVD and fermentation profiles were not proportionate to the change in RFB from 1:4 to 1:2. IVD for 1:2 and 1:4 ratios are very highly correlated at both 48 and 96 h. Thus, the use of diluted rumen fluid with a 1:4 RFB ratio is proposed suitable for subsequent studies to rank a wide range of feed ingredients for both degradability and methane measurements. However, in places where it is easy to collect RF can continue *in vitro* trial with a 1:2 RFB ratio.

KEY WORDS fermentation, forages, inoculums, *in vitro,* methane.

INTRODUCTION

For production and digestibility studies researchers need ruminant animals. They are important to understand ruminant nutrition and feeding practices, but animal trials require considerable amounts of labour, feed, animals, and time. Then researcher tried *in vitro* techniques. By using *in vitro* techniques, researchers can minimize the use of those resources. The research article published by Tilley and Terry (1963) is one of the pioneers to introduce *in vitro* technique. Widespread use of *in vitro* techniques to evaluate ruminant feeds has largely been due to high analytical

capacity and low cost (Yáñez-Ruiz *et al.* 2016). The objective of *in vitro* experimentation is to create an environment that is similar to the environment present in the animals. Although these techniques are more rapid and precise and require less substrate than *in vivo* procedures, they still require an inoculum to create the fermentative environment.

For *in vitro* experiment researcher need rumen fluid. To collect RF from animal, it requires surgically modified animals that raise ethical and moral issues. Rumen fluid contains different types of microorganism like bacteria, archea, protozoa, and fungi. These microorganisms are essential for degradability, fermentation and gas production trial. In ruminant animals, saliva is added through the mouth to the rumen. Saliva content phosphate and bicarbonate those act as a buffer. Consequently for *in vitro* trial buffer is added with RF and buffer is produced by using phosphate and bicarbonate salt. Buffer maintains the pH, provides mineral supplements to the microbes and creates environments so that microbes can work on feed. Hence, for degradation and fermentation experiments, researchers have used different concentrations of inoculum. For example, some researchers (Chaudhry, 2008; Chaudhry and Khan, 2012; Navarro-Villa *et al.* 2011) have used a 1:4 ratio of rumen fluid to buffer (RFB) and others (Homem Junior *et al.* 2015) have used either 1:2 or (Mahfuz *et al.* 2014; Chowdhury *et al.* 2018) 1:3 or (Budiyono *et al.* 2014) 1:1 RFB ratios. It was found that gas production was varied in different published values.

Hidayat *et al.* (1993) observed that the total VFA production was increased with the increasing bacterial density in the inoculum. Navarro-Villa *et al.* (2011) found that the ratio of rumen fluid to buffer had an effect on types of VFA production but did not affect total VFA production. The effect of the ratio of rumen fluid to buffer on acetic, propionic and butyric acids was dependent on feed.

Rymer *et al.* (1999) reportedhigher rumen fluid concentration decreased final culture pH ($P<0.01$) and increased total gas production ($P<0.05$). They suggested that when the concentration of rumen fluid was low, more degraded OM was diverted from gas production, presumably to microbial growth. Microbes might use some gas as an energy source to increase their biomass. Navarro-Villa *et al.* (2011) reported increasing the proportion of rumen fluid in the mixture increased methane production and dry matter disappearance and reduced pH ($P<0.01$).

Results were consistent among feeds tested. In their report methane production was dependent on types of feed, and this ranking was maintained among all combinations of quantities of feed incubated and the ratio of rumen fluid to buffer. They concluded that rumen fluid and buffer mixture at a 1:2 ratio was a satisfactory combination with their buffer system.

Budiyono *et al.* (2014) observed that higher RF inoculums caused biogas production rate and efficiency to increase more than two times. Homem Junior *et al.* (2015) reported that incubation without buffer solution increases the methane production of corn silage. Patra and Yu (2013) evaluated *in vitro* fermentation technique containing different bicarbonate concentrations (80, 100, and 120 mM) in the buffer. The reported that bicarbonate concentrations above 80 mM should be avoided to minimize non-microbial CO_2 production associated with changes in pH. Yáñez-Ruiz *et al.* (2016) suggested that available data do not allow for recommendations on an ideal RF:B. A ratio of 1:2 appears to generate the most reliable results for 24 h incubations. However, this ratio and the amount of substrate incubated needs to be considered based on the frequency of gas sampling and the duration of the incubation depending on the research objectives.

To research with a large number of samples need more RF, means need more animal, especially for small ruminant animals, need a large number of fistulated animal. In some countries, it is difficult to maintain large number of fistulated animals for a long time. Using less RF and more buffer could resolve this problem. In some other places like in developing countries researchers can easily get RF from the abattoir, for them preparing buffer will be costly. Pell and Schofield (1993) recommended minimum concentration of 20 mL inoculum/100 mL incubation medium. As many researchers suggested for 1:2 RFB, therefore, it was important to conduct a comparative study to observe the effect on degradability, fermentation profile, and gas production of different concentrations (1:4 RFB) of inoculum to determine the extent to which experimental technique may have influenced the interpretation of *in vitro* results. This was achieved by carrying out two experiments each with a specific different objective related to the overall aim. In our previous paper, it was observed that low-quality forages are degraded up to 96 h, this is why in this experiment two different incubation times (48 and 96 h) were chosen.

MATERIALS AND METHODS

Experiment one

Comparing the effect of different RFB ratios on the *in vitro* degradability and fermentation profiles of forages

Seven forages (rice straw, wheat straw, rye grass hay, rye grass silage, sugarcane bagasse, rapeseed plant, and ryegrass) were used in two different concentrations of inoculum (RFB ratios) for two different incubation times to observe the effect of inoculum on forage degradability and fermentation (pH, ammonia production, total gas, methane and VFA) profiles. A 7 × 2 × 2 factorial arrangement in duplicate was used to assess the degradability and fermen-

tation profiles of seven forages with two RFB ratios (1:2 and 1:4) at two incubation times (48 h and 96 h). In exp 2 total gas volumes were recorded for 0 to 96 h and methane production for a fixed period of 96 h only. A 7 × 2 factorial arrangement in duplicate was used to assess the gas and methane production in the presence of seven forages with two RFB ratios (1:2 and 1:4) at two incubation times (48 h and 96 h).

Collection of forage samples

Representative samples of dried rice straw (Variety, IR50) and sugarcane bagasse were collected from Bangladesh, whereas those of wheat straw (Variety, Einstein), ryegrass hay, dried ryegrass, ryegrass silage and rapeseed plant at its green and pre- flower stage were collected from the Newcastle University's Farm. The samples of dried ryegrass were prepared after cutting random samples of fresh grass with a hand cutter directly from a field in mid-May before their oven drying as described later. Representative samples of forages were re-dried at 60 ˚C in an oven and ground through a 1 mm sieve by using a grinder (Christy mill, Christy and Norris Ltd, Suffolk, United Kingdom).

Collection of rumen fluid sample

The procedures used for the collection of rumen fluid and preparation of buffer solutions were the same as described by Khan and Chaudhry (2010). Representative samples of were obtained from two fistulated sheep (Lleyn breed) with mean a live weight of 81 kg just before their morning feeding. These sheep were managed under a project license authorized by the UK home office under the Animal and Scientific Procedures Act 1986 of the UK. The detailed composition of the feed given to sheep was the same as previously reported by Khan and Chaudhry (2010). The RF was transported in two insulated pre-warmed flasks under anaerobic conditions to the laboratory. The fluid was pooled in equal proportions before its use as a source of inoculums. The RF was strained through four layers of cheesecloth into pre-warmed flasks under CO_2 before its mixing with the pre-warmed phosphate-bicarbonate (McDougall, 1948), which was then edscrew-capped and kept at 38-39 ˚C in a water bath. The RF was mixed with the pre-warmed buffer at either a 1:4 RFB ratio by mixing one part of RF and four parts of the buffer solution or a 1:2 ratio by mixing one part of RF and two parts of the buffer solution.

In exp 1, the incubations of forages were conducted in 50 mL polypropylene tubes each containing 0.4 g of the ground (1 mm) forages with 40 mL of two different RFB ratios (1:2 and 1:4 ratio) according to the above described experimental designs. The tubes were purged with CO_2, sealed with rubber stoppers fitted with pressure release valves and incubated at 39 ˚C in a temperature-controlled water bath. After 48 and 96 h, the tubes were submerged in crushed ice to stop further fermentation. The liquids and residues were separated by centrifuging the tubes at 3000 rpm for 10 min. Then the pH of each RF supernatant was measured immediately with a pH meter (Jenway Ltd, model 3340). Each RF supernatant was also collected to determine VFA and ammonia concentration. For the determination of VFA, 2 mL supernatants were collected to which 0.25 Ml of deproteinizing solution (200 g/Lmetaphosphoric acids in distilled water) (Chaudhry and Khan, 2012) were added and kept at 4 ˚C in a refrigerator until analysis. For ammonia concentration, 2 mL of supernatant were acidified with 2 mL of 1 N HCl and kept in a fridge. The residues were washed with distilled water, dried, and weighed to determine DM and OM to estimate their respective degradability values. The procedures used for the analysis and determination of *in vitro* DM (IVD), OM degradability (IVOMD), ammonia and VFA were the same as described by Chaudhry (2008) and Chaudhry and Khan (2012). Washed residues were dried in an oven at 60 ˚C for 48 h. The calculation of the IVD degradability of each forages was done by deducting the weight of the dried residue from the pre-incubated weight of the same forages. Each dried residue was then transferred to a muffle furnace at 550 ˚C for 5 h. After cooling, the ashed residues were weighed to estimate the OM of the residue. IVOMD of the forage was then calculated by deducting the OM of the residue from the OM of the forage sample. To determine ammonia the supernatants from each tube for each incubation time were collected after centrifuging the relevant samples in tubes at 3000 rpm for 10 min, acidified with 1 (N) HCl and kept in a fridge. The acidified samples were analyzed for ammonia by using ammonium test kit (containing sodium nitroprusside and sodium hydroxide solution, Merk, Germany) and a spectroquent colorimeter (Merk, Germany) at 660 nm. Ammonium sulfate solution was used as a standard.

Experiment 2

Measuring the gas and methane production of forage samples

The procedures used for the measurement of gas production were as described by Chaudhry and Khan (2012). After 6, 24, 30, 48, 54, 72, 80 and 96 h of incubation, gas production was recorded. Methane was determined according to the method described by Upstill-Goddard *et al.* (1996) with some modifications (Chaudhry and Khan, 2012).

Statistical analysis

The data from exp 1 were analyzed by using the General Linear Model of Minitab 19 in a 7 × 2 × 2 factorial arrangement. The main effects of forages, RFB ratio, time and their 2 and 3-way interactions were considered for for-

age degradability, pH, ammonia production, total and individual VFA production for each incubation time. The data from exp2 were analyzed by using general linear model (GLM) of Minitab 19 according to a 7 × 2 factorial arrangement. The main effects of forages and RFB ratio and their interactions were considered for total gas and methane production. Significant differences between means for each main effect and their interactions were compared by using the Tukey's test for their significance at $P < 0.05$. The data were further analyzed by using the Pearson's Correlation in Minitab to study possible relationships as determined by 'r' between ammonia concentration, gas, and methane production and *in vitro* degradability of various forages of this study. Fitted line plot regression was carried out between ammonia concentration, gas and methane production and *in vitro* degradability of various forages at two RFB ratios (1:2 and 1:4) and combined at 96 h. The data were also analyzed by using the Pearson's Correlation in Minitab 19 to study possible relationships as determined by 'r' between two RFB ratios (1:2 and 1:4) *in vitro* degradability of various forages of this study at 48 and 96 h. Fitted line plot regression was carried out between *in vitro* degradability of various forages at two RFB ratios (1:2 and 1:4) at 48 and 96 h.

In experiments 1 and 2, orthogonal comparisons were used to determine the effects of different treatments. In experiment 1, the null hypotheses that were tested were:

(a) The concentration of rumen fluid did not affect degradability of forages.

(b) The concentration of rumen fluid had no effect on fermentation (pH, ammonia production, total gas, methane and VFA) profiles.

In experiment 2, the null hypotheses that were tested were:

(a) The concentration of rumen fluid did not affect gas production from 0 h to 96 h.

(b) The concentration of rumen fluid did not affect methane production from 0 h to 96 h.

RESULTS AND DISCUSSION

Experiment one

Comparing the effect of different RFB ratios on the *in vitro* degradability and fermentation profiles of forages

As shown in Tables 1-3, the main effects of forage, inoculum and T were always significant ($P<0.05$) for IVD, IVOMD, and most fermentation profiles. However, their two and three-way interactions showed significance or non-significance with variable P-values. There were predictable, statistically significant differences between the IVD and IVOMD of the forages, where less fibrous materials based on immature plants, (ryegrass, rapeseed plant and silage) had higher values than those of the mature materials, (rice and wheat straw, hay and sugarcane bagasse). The IVD of ryegrass was highest and IVD of sugarcane bagasse was lowest at both incubation times. The IVD and IVOMD values of forages were higher at 1:2 than the 1:4 RFB ratio at both incubation periods except rapeseed plant and ryegrass showed higher IVD at 1:4 ratio. Again, the IVD and IVOMD of all forages were higher after 96 h than 48 h of incubation (Table 1) which was expected. Hence the interaction between forage and ratio was significant ($P<0.003$). IVD at 1:2 and 1:4 RFB ratio had positive correlation at 48 ($r=0.868$) and 96 h ($r=0.992$) incubation periods (Figures 14 and 15).

The value of pH largely varied with the type of forages, and RF:B ratio. The pH of the rumen fluid incubating sugarcane bagasse was highest followed by wheat straw and pH was lowest in the ryegrass at 48 h but in form of silage at 96 h. The pH was significantly ($P<0.001$) higher in the 1:4 RFB ratio at both incubation times (Table 1). The pH value was generally larger at a shorter incubation time (48 h). However, the pH value was higher for rapeseed plants and rye grass for longer incubation time that caused the F × T interaction significant ($P<0.001$).

The ammonia value also changed significantly ($P<0.001$) in the forages. Ammonia levels were higher in fluid containing the more degradable forages. In all instances, ammonia increased with incubation time (96 versus 48 h) g and usually, but not always ammonia levels were higher ($P<0.001$) when the incubation ratio of 1:2 was used. Ammonia was higher for ryegrass and rapeseed plant than the other forages and it was lowest in sugarcane bagasse (Table 5). Ammonia concentration had a positive correlation with IVD ($r=0.554$; $P<0.003$) at 96 h (Figure 5). Although the correlation value($r=0.997$; $P<0.001$) was excellent for the 1:2 RFB ratio, it ($r=0.718$; $P<0.004$) was very good for the 1:4 ratio (Figures 6 and 7).

Total VFA concentration (mmol/L) varied significantly ($P<0.001$) among the different forages. VFA concentration was highest for silage and lowest for sugarcane bagasse for both the incubation times (Table 2). The VFA concentration also changed significantly ($P<0.001$) in different RFB ratios. The VFA concentration was higher for the 1:2 ratio than the 1:4 ratio. At 48 h, VFA concentration for high-quality forages was more than 100 mmol/L but in low quality forages it was less than 100, but at 96 h VFA concentration was more than 100 mmol/L for all forages.

The molar proportion of acetic and propionic acid varied significantly ($P<0.001$) with the type of forages. The molar proportion of acetic acid was highest for sugarcane bagasse and lowest for ryegrass at 48 h and for silage at 96 h (Table 3), on the other hand, molar proportion of propionic acid was highest in silage and lowest in sugarcane bagasse for both the incubation times.

Table 1 IVD and IVOMD (g/kg) of different forages and pH of rumen fluid inoculum (RI) following incubation with at different rumen fluid to buffer (RFB) ratios for two time periods (T)

Item	T	RI	Hay	RP	RG	RS	SB	Si	WS	Mean
		1:2	567	857	871	451	164	780	400	584
	48	1:4	557	891	886	423	172	793	300	575
IVD		Mean	562	874	879	437	168	787	350	**579**
		1:2	733	901	934	570	271	882	535	689
	96	1:4	739	906	941	568	256	835	479	675
		Mean	736	904	938	569	264	859	507	**682**
			Pooled SEM= 33.8; P for F < 0.001; P for RI < 0.05; P for T < 0.001							
			P for F × RI < 0.003; P for F × T × 0.001; P for T × RI < 0.8; P for F × T × RI < 0.3							
		1:2	544	877	865	423	127	744	352	562
	48	1:4	518	885	880	402	107	763	265	546
		Mean	531	881	873	413	117	754	309	**554**
IVOMD		1:2	725	929	961	582	247	875	500	684
		1:4	714	903	920	565	231	823	452	663
	96	Mean	720	916	941	574	239	849	476	**673**
			Pooled SEM= 35.6; P for F < 0.001; P for RI < 0.001; P for T < 0.001							
			P for F × RI < 0.3; P for F × T < 0.001; P for T × RI < 0.3; P for F × T × RI < 0.09							
		1:2	6.97	7.04	6.82	7.04	7.14	6.93	7.08	7.00
	48	1:4	7.04	7.10	7.05	7.24	7.32	6.98	7.22	7.13
		Mean	7.01	7.07	6.93	7.14	7.23	6.96	7.15	**7.07**
pH		1:2	6.88	7.03	7.05	7.02	7.12	6.84	7.01	6.99
		1:4	6.98	7.12	7.01	7.03	7.19	6.96	7.10	7.06
	96	Mean	6.93	7.08	7.03	7.03	7.16	6.90	7.06	**7.02**
			Pooled SEM= 0.016; P for F < 0.001; P for RI < 0.001; P for T < 0.001							
			P for F × R < 0.09; P for F × T < 0.001; P for T × RI < 0.003; P for F × T × RI < 0.001							

IVD: *in vitro* dry matter degradability and IVOMD: *in vitro* organic matter degradability.
T: incubation time; RI: rumen inoculum; Hay: ryegrass hay; RP: rapeseed plant; RG: dried ryegrass; RS: rice straw; SB: sugarcane bagasse; Si: ryegrass silage and WS: wheat straw.
SEM: standard error of the means.

Table 2 Rumen ammonia and VFA concentration (mg/L) of rumen fluid incubated with different forages with buffered rumen inoculum (RI) at two RFB ratios for two time periods (T)

Item	T	RI	Hay	RP	RG	RS	SB	Si	WS	Mean
		1:2	137	166	167	132	112	146	98	137
	48	1:4	95	158	163	69	56	149	68	108
		Mean	116	162	165	101	84	148	83	**123**
Rumen Ammonia		1:2	272	284	292	212	180	273	144	237
		1:4	151	161	182	138	129	166	89	145
	96	Mean	212	223	237	175	155	220	117	**191**
			Pooled SEM= 8.48; P for F < 0.001; P for RI < 0.001; P for T < 0.001							
			P for F × RI < 0.2; P for F × T < 0.02; P for T × RI < 0.001; P for F × T × RI < 0.001							
		1:2	145	150	149	94	72	160	95	124
	48	1:4	116	111	120	73	61	145	75	100
		Mean	131	131	135	84	67	153	85	**112**
VFA		1:2	159	145	158	156	131	166	146	152
		1:4	112	134	137	125	123	156	130	131
	96	Mean	136	140	148	141	127	161	138	**141**
			Pooled SEM= 4.05 P for F < 0.001; P for RI < 0.001; P for T < 0.001							
			P for F × RI < 0.001; P for F × T < 0.001; P for T × RI < 0.5; P for F × T × RI < 0.06							

VFA: Volatile fatty acids.
T: incubation time; RI: rumen inoculum; Hay: ryegrass hay; RP: rapeseed plant; RG: dried ryegrass; RS: rice straw; SB: sugarcane bagasse; Si: ryegrass silage and WS: wheat straw.
SEM: standard error of the means.

Table 3 Molar proportions of various VFA (mol/100 mol) in the rumen fluid inoculum (RI) incubated with different forages at different RFB ratios for two-time periods (T)

Item	T	RI	Hay	RP	RG	RS	SB	Si	WS	Mean
Molar proportions of acetic acid	48	1:2	61.2	61.4	55.6	61.0	62.6	54.6	59.9	59.5
		1:4	63.0	63.3	55.8	60.1	64.3	59.6	62.3	61.2
		Mean	62.1	62.4	55.7	60.6	63.5	57.1	61.1	**60.3**
	96	1:2	63.5	62.0	57.6	62.0	64.6	55.5	61.7	61.0
		1:4	60.8	60.5	58.0	63.2	62.2	55.9	60.7	60.2
		Mean	62.2	61.3	57.8	62.6	63.4	55.7	61.2	**60.6**
		Pooled SEM= 0.425; P for F < 0.001; P for RI < 0.6; P for T < 0.2								
		P for F × R < 0.2; P for F × T < 0.001; P for T × RI < 0.001; P for F × T × RI < 0.06								
Molar proportions of propionic acid	48	1:2	30.4	27.3	33.0	26.7	26.8	36.0	29.2	29.9
		1:4	29.5	26.5	32.9	27.1	26.1	33.0	28.1	29.0
		Mean	30.0	26.9	33.0	26.9	26.5	34.5	28.7	**29.5**
	96	1:2	26.0	30.0	30.4	26.0	25.7	34.3	25.5	28.3
		1:4	27.7	27.6	33.0	27.8	27.6	35.1	29.6	29.8
		Mean	26.9	28.8	31.7	26.9	26.7	34.7	27.6	**29.0**
		Pooled SEM= 0.472; P for F < 0.001; P for RI < 0.93; P for T < 0.6								
		P for F × RI < 0.5; P for F × T < 0.6; P for T × RI < 0.2; P for F × T × RI < 0.9								
Molar proportions of butyric acid	48	1:2	6.40	5.73	6.00	6.72	7.74	6.54	7.06	6.60
		1:4	6.22	5.56	5.99	6.82	7.54	6.00	6.79	6.42
		Mean	6.31	5.65	6.00	6.77	7.64	6.27	6.93	**6.51**
	96	1:2	7.65	4.55	7.66	7.14	7.34	5.86	7.74	6.85
		1:4	7.69	6.59	4.32	6.42	7.45	6.77	7.05	6.61
		Mean	7.67	5.57	5.99	6.78	7.40	6.32	7.40	**6.73**
		Pooled SEM= 0.149; P for F < 0.001; P for RI < 0.6; P for T < 0.2								
		P for F × RI <0.2; P for F × T < 0.001; P for T × RI < 0.001; P for F × T × RI < 0.06								
Molar proportions of minor VFA	48	1:2	3.00	3.55	4.45	2.70	2.61	3.93	3.83	3.44
		1:4	2.25	3.70	4.41	3.03	2.05	2.40	2.81	2.95
		Mean	2.63	3.63	4.43	2.87	2.33	3.17	3.32	**3.19**
	96	1:2	2.88	3.34	4.31	2.87	2.39	3.20	2.42	3.06
		1:4	3.88	5.35	4.78	2.58	2.85	3.40	2.70	3.65
		Mean	3.38	4.35	4.55	2.73	2.62	3.30	2.56	**3.35**
		Pooled SEM= 0.118; P for F < 0.001; P for RI < 0.6; P for T < 0.2								
		P for F × RI < 0.2; P for F × T < 0.001; P for T × RI < 0.001; P for F × T × RI < 0.06								

VFA: Volatile fatty acids.
T: incubation time; RI: rumen inoculum; Hay: ryegrass hay; RP: rapeseed plant; RG: dried ryegrass; RS: rice straw; SB: sugarcane bagasse; Si: ryegrass silage and WS: wheat straw.
SEM: standard error of the means.

The molar proportion of butyric acid was highest for sugarcane bagasse at 48 h and for hay at 96 h and it was lowest for the rapeseed plant (Table 3). Molar proportions of minor VFA were highest for ryegrass and lowest for sugarcane bagasse at 48 hour. The molar proportion of acetic acid was higher (P<0.001) for 1:4 RFB ratio at 48 h but not at 96 h. There were no significant changes in the molar proportion of propionic, butyric, and minor volatile fatty acids in different RFB ratios.

Experiment two

Measuring the gas and methane production of forage samples

Total gas volume was significantly (P<0.001) higher for the RFB ratio of 1:2 than 1:4 and it was highest when ryegrass forage was used. Total gas volume was highest for rapeseed plant and lowest for sugarcane bagasse at 1:2 ratio (Figure 1), but at 1:4 ratio total gas volume was second highest in silage and lowest in wheat straw (Figure 2). Gas volume had a positive correlation with IVD (r= 0.647, P<0.001**)** at 96 h (Figure 8). In the 1:2 RFB ratio the correlation value (r=0.919; P<0.001) was higher than the correlation value in the 1:4 RFB ratio (r=0.702; P<0.006) (Figures 9 and 10).

In the present study, the concentration of methane in total gas was higher for the high than the low fibrous forages except for sugarcane bagasse. Among the high fibrous forages, methane concentration was highest in the presence of wheat straw and lowest in the presence of sugarcane bagasse. On the other hand, among the low fibrous forages methane concentration was the lowest in the presence of

ryegrass (Figure 3). The RFB ratio also had a significant effect on methane concentration. The methane concentration was higher at 1:2than the 1:4 RFB ratio.

Hour	0	06	24	30	48	54	72	80	96
SEM	0	0.48	0.48	1.50*	1.73**	2.08**	2.38***	2.53***	2.55***

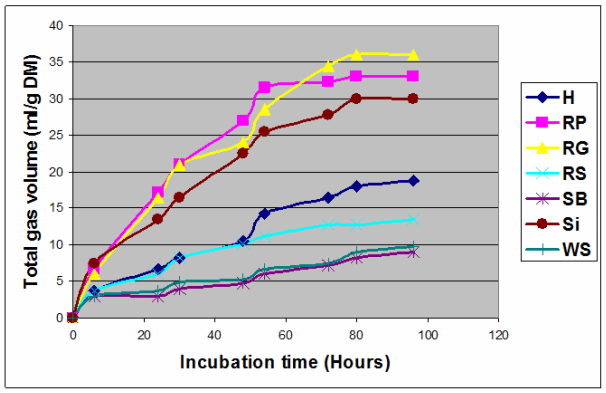

Figure 1 Total gas volume for different forages at 1:2 rumen fluid to buffer (RFB) ratio

Hour	0	06	24	30	48	54	72	80	96
SEM	0	0.13	0.35*	0.50**	0.60***	0.68***	0.83***	0.88***	0.88***

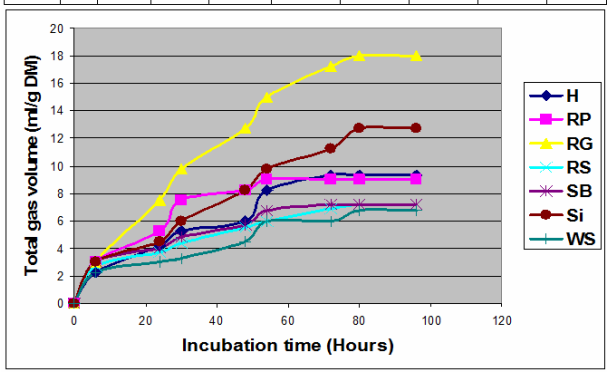

Figure 2 Total gas volume for different forages at 1:4 rumen fluid to buffer (RFB) ratio
* (P<0.05); ** (P<0.01) and *** (P<0.001)

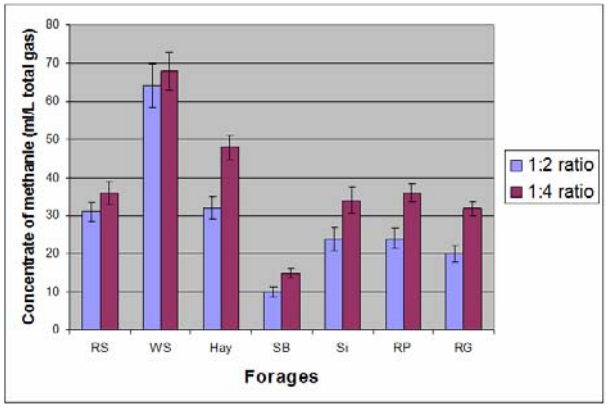

Figure 3 Concentration of methane (mL/L total gas) for different forages after incubations with rumen fluid (RF) inoculum at two rumen fluid to buffer (RFB) ratios

As total gas production was higher for low fibrous forages, total methane production was also higher for low fibrous forages at 1:2 RFB ratio; however, at 1:4 RFB ratio, methane productions in wheat straw and hay were higher than rapeseed plant and silage. Methane production had a positive correlation with IVD (r=0.707, P<0.001**)** at 96 h (Figure 11).

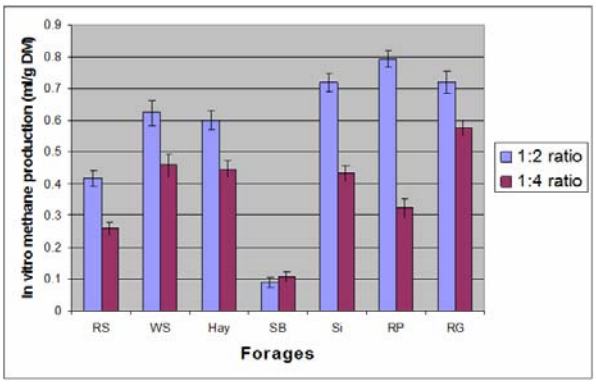

Figure 4 Methane production (mL/g DM) of different forages after incubation with rumen fluid (RF) inoculum at two rumen fluid to buffer (RFB) ratios

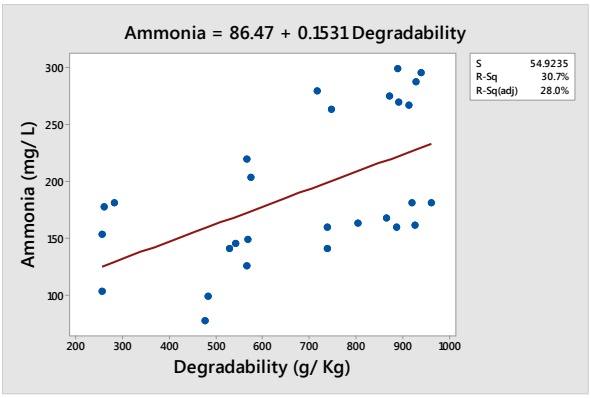

Figure 5 Relationship between degradability (g/kg) and ammonia (mg/L) in rumen fluid (RF) at 96 h (r=0.554; P<0.003)

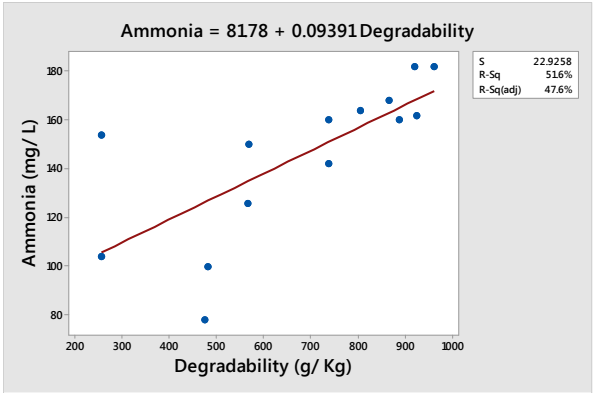

Figure 6 Relationship between degradability (g/kg) and ammonia (mg/L) in rumen fluid (RF) at 96 h in 1:4 rumen fluid to buffer (RFB) (r=0.718; P<0.004)

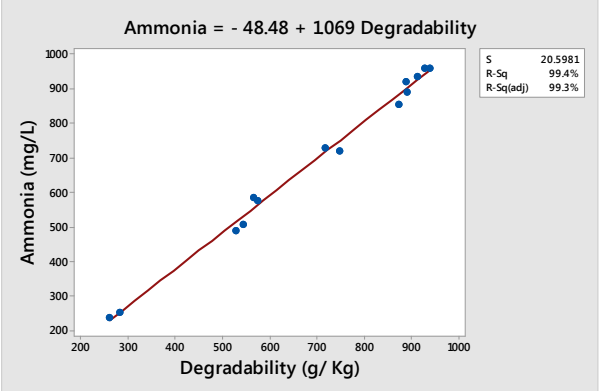

Figure 7 Relationship between degradability (g/kg) and ammonia (mg/L) in rumen fluid (RF) at 96 h in 1:2 rumen fluid to buffer (RFB) (r=0.997; P<0.001)

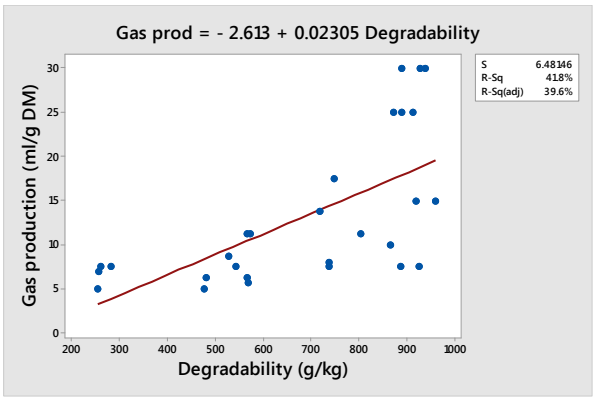

Figure 8 Relationship between degradability (g/kg) and gas production (mL/g DM) at 96 h (r=0.647; P<0.001)

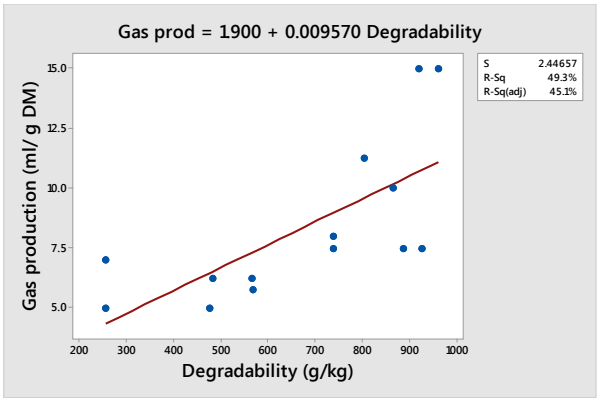

Figure 9 Relationship between degradability (g/kg) and gas production at 96 h in 1:4 rumen fluid to buffer (RFB) (r=0.702; P<0.006)

In the 1:2 RFB ratio the correlation value (r=0.868; P<0.001) was higher than correlation value in the 1:4 RFB ratio (r=0.669; P<0.01) (Figures 12 and 13). For the 1:2 RFB ratio, methane production was highest in rapeseed plant followed by silage and ryegrass, on the other hand for 1:4 RFB ratio methane production was highest in ryegrass followed by wheat straw and hay. Methane production fluctuated largely in the presence of rapeseed plant for different RFB ratios (Figure 4).

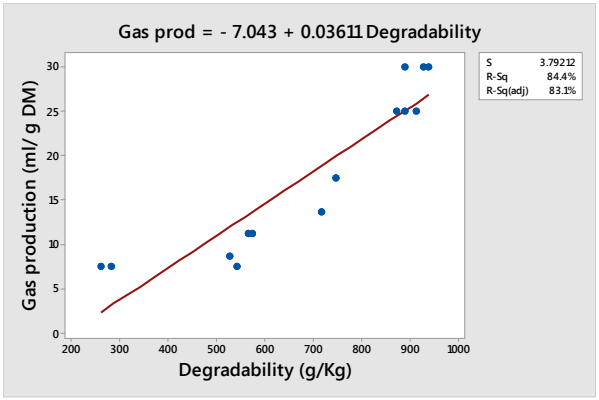

Figure 10 Relationship between degradability (g/kg) and gas production at 96 h in 1:2 rumen fluid to buffer (RFB) (r=0.919; P<0.001)

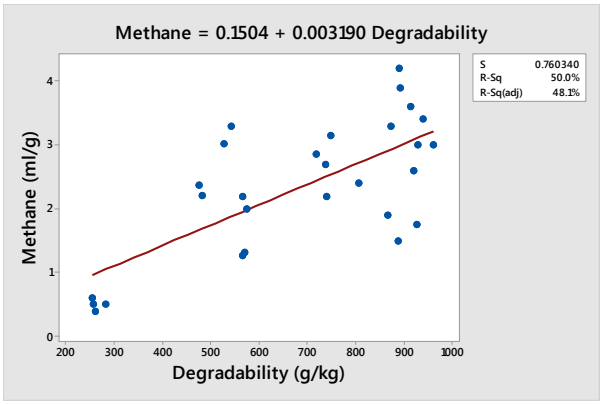

Figure 11 Relationship between degradability (g/kg) and methane production (mL/g) at 96 h (r=0.707; P<0.001)

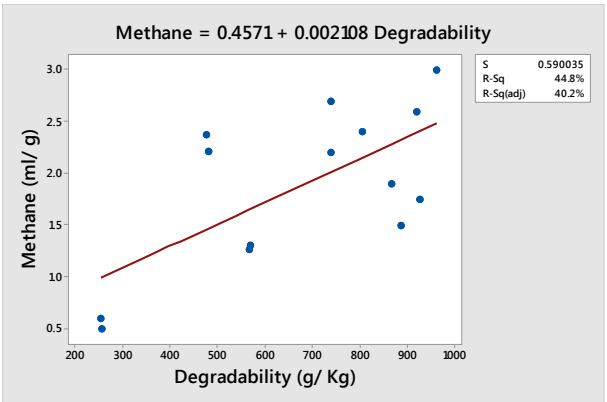

Figure 12 Relationship between degradability (g/kg) and methane production (mL/g) at 96 h in 1:4 rumen fluid to buffer (RFB) (r=0.669; P<0.01)

The main objective of the research was to compare the effect of two RFB ratios on degradability, fermentation profiles and gas and methane production. The degradability of forages, pH, ammonia and VFA concentration was affected by the RFB ratios.

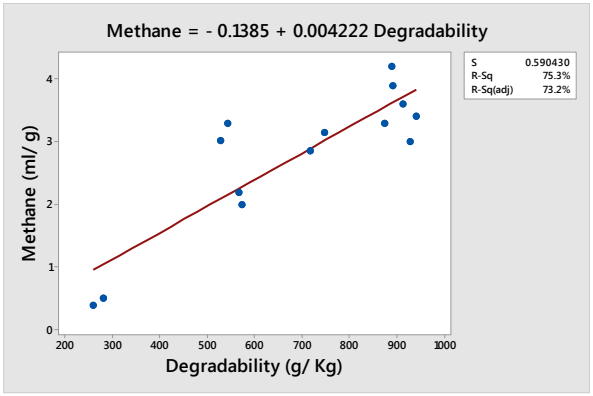

Figure 13 Relationship between degradability (g/kg) and gas production at 96 h in 1:2 rumen fluid to buffer (RFB) (r=0.868; P<0.001)

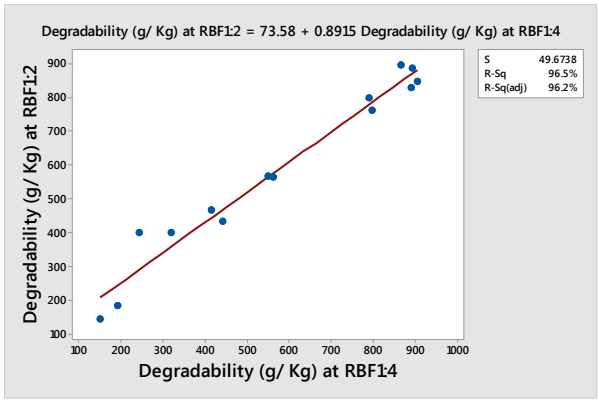

Figure 14 Relationship between degradability (g/kg) at 1:2 rumen fluid to buffer (RFB) and 1:2 RBF ratio at 48 h (r=0.868; P<0.001)

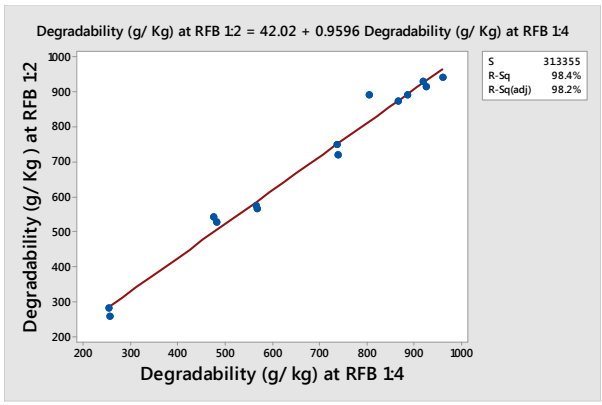

Figure 15 Relationship between degradability (g/kg) at 1:2 rumen fluid to buffer (RFB) and 1:2 RBF ratio at 96 h (r=0.992; P<0.001)

However, ranking of degradability of forages did not affect by RFB ratio. Those forages are more degraded in 1:2 ratio are also more degraded in the 1:4 ration, again those forages that are less degraded in 1:2 ratio are also less degraded in 1:4 ration Likewise, Homem Junior *et al.* (2015) did not find any significant difference in degradability of corn silage for non-buffered RF and 1:2 RFB. As predicted the IVD and IVOMD were higher for the immature and relatively less fibrous materials. It was noted in Khan and Chaudhry (2011) that the nutritive value of ryegrass was more acceptable and sugarcane bagasse was least acceptable; that also reflects their variable IVD and IVOMD. The IVD and IVOMD of forages can increase even after 48 h of incubation. As a result, both IVD and IVOMD of forages were higher at 96 h than 48 h of incubations. This agreed well with the report of Khan and Chaudhry (2011) who also found higher IVD and IVOMD of forages at longer incubation times.

The 1:2 RFB ratio decreased the pH value as compared to the 1:4 ratio. Rymer *et al.* (1999) also observed similar results for change in pH. During ensiling and haymaking some of the carbohydrates were converted to acids (Li *et al.* 2015; Ren *et al.* 2019) and thus might have caused lower pH when silage or hay was used as a substrate.

One approach is to estimate ruminal CP degradation by determining ammonia-N production (Hristov *et al.* 2019). The forages that contained more CP also had more degradable CP and greater ammonia. Likewise, Chaudhry and Khan (2012) observed higher ammonia in higher CP-containing mixed feed. The 1:2 RFB ratio increased the ammonia concentration in RF of respective samples. In higher concentration of rumen fluid, there was more ammonia than the lower concentration of inoculum from the beginning. In addition, there might be some soluble nutrients already available in the higher inoculum that might have helped to produce higher ammonia in those samples. These observations might have caused a higher correlation between ammonia and IVD for the 1:2 RFB ratio. During longer incubation time, it is possible that the more rumen microbes got more time to degrade more forages that might have caused higher ammonia concentration at longer incubation time.

The 1:2 RFB ratio increased the VFA concentrations in the corresponding samples. Like ammonia, with higher buffer concentration there were lessVFA than the lower buffer concentration from the beginning. Hidayat *et al.* (1993) observed that the total VFA production was increased with the increasing bacterial density in the inoculum. Again some soluble nutrients available in the higher RF concentration might have helped to produce higher VFA in those samples. Higher VFA concentration might have caused lower pH in higher RF concentration. However, it was interesting to report that the ranking of forages for pH, ammonia, and VFA aswas not affected by changing the RFB ratio.

High fibrous feeds normally produce more acetate (Suarez *et al.* 2007), which might have caused higher acetate proportion in large fiber-containing forage such as sugarcane bagasse. The difference in forage amount also did

not change the ranking of forages for different fatty acid proportions. Navarro-Villa *et al.* (2011) also reported that acetic, propionic, and butyric acids were dependent on feed.

Total gas volume and methane production were influenced by the RFB ratio and were higher in the 1:2 RFB ratio. Other researchers (Budiyono *et al.* 2014; Homem Junior *et al.* 2015; Rymer *et al*. 1999) also observed higher gas and methane production in a higher concentration of rumen fluid. Rymer *et al.* (1999) suggested that when the concentration of rumen fluid was low, more degraded OM was diverted from gas production, presumably to microbial growth. Microbes might use some gas as an energy source to increase their biomass. That might have also influenced the correlation values between IVD and gas and methane production. Total gas and methane production also depended on forage quality. Indeed less fibrous but high degradable forages produced more gas during their *in vitro* incubations.

Navarro-Villa *et al.* (2011) mentioned that methane production was depended on types of feed, and this ranking was maintained among all combinations of quantities of feed incubated and ratio of rumen fluid to buffer. This was re-confirmed by the positive correlation that was observed between total gas and methane production with IVD. Concentration of methane in total gas largely depended on the type of forage. It is often assumed that less fibrous and highly degradable forages produced comparatively larger amounts of methane than high fibrous and low degradable forages.

Higher amount of ether extract (EE) in sugarcane bagasse might have reduced methane concentration in the presence of sugarcane bagasse as large amount of dietary EE can reduce methane production in ruminants (Giger-Reverdin *et al.* 2003). On the other hand, the lowest amount of total phenolics, saponins and tannins in wheat straw caused the highest concentrate of methane in wheat straw as these components are known to inhibit methane (Puchala *et al.* 2005; Patra *et al.* 2006). Indeed higher amount of total phenolics, saponins and tannins in ryegrass caused lowest concentration of methane among the high-quality forages.

The degradability, ammonia and pH for rapeseed plant at 1:4 RFB ratio either did not change or very little change between 48 and 96 h. That means that the degradation and gas production of rapeseed plant peaked at 48 h at a 1:4 RFB ratio.

As the ranking of forages for degradability, pH, ammonia and VFA were not affected by the change in RFB ratios, even the correlation value of degradability between the two was higher in both the incubation times, it is safe to continue the use of diluted inoculum with 1:4 RFB ratio without affecting the ranking of a wide range of feed ingredients for their use to formulate cost-effective ruminant diets. This will also reduce the need to collect high volumes of RF, which is not easy to obtain due to increasingly strict health and safety regulations, scheduling, and cost implications regarding the collection of RF from surgically modified animals or even abattoirs in some countries. However, in places where it is easy to collect RF can continue *in vitro* trial with a 1:2 RFB ratio to reduce the cost for preparing buffer.

CONCLUSION

It appeared that although the change in RFB ratio was able to influence the degradability of forages; and fermentation profiles of 7 forages, these changes were not proportionate to the change in the RFB ratio. Both RFB ratios were able to differentiate these forages for degradability and methane emission in a similar manner for their ranking. Therefore, the use of diluted rumen fluid with a 1:4 RFB ratio is proposed for subsequent studies to rank a wide range of feed ingredients for their possible inclusion in formulating ruminant diets. However in places where it is easy to collect RF can continue *in vitro* trial with 1:2 RFB ratio.

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