Influence of Temperature and pH on Fermentation Pattern and Methane Production in the Rumen Simulating Fermenter (RUSITEC)

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ABSTRACT : An experiment was conducted to study the effect of temperature and pH on *in vitro* nutrient degradability, volatile fatty acid profile and methane production. The fermenter used was the semi-continuous system, known as the rumen simulation technique (RUSITEC). Sixteen cylinders were used at one time with a volume of 800 ml, the dilution rate was set at 3.5%/hour, the infused buffer being McDougall's artificial saliva. Basal diet (9.6 g DM) used in RUSITEC consisted of (DM) 6.40 g Timothy hay, 1.86 g crushed corn and 1.34 g soybean meal. The food for the fermentation vessel was provided in nylon bags, which were gently agitated in the liquid phase. The experiment lasted for 17 d with all the samples taken during the last 5 d. Treatments were allocated at random to four vessels each and were (1) two temperature levels of 39°C and 41°C (2) two pH levels of 6.0 and 7.0. The total diet contained (g kg^{-1} DM) 957 OM, 115 CP and 167 MJ kg^{-1} (DM) GE. Although increase in temperature from 39°C to 41°C reduced degradation of major nutrients *in vitro*, it was non-significant. Interaction effect of temperature with pH also reflected a similar trend. However, pH showed a significant ($p<0.05$) negative effect on the degradability of all the nutrients *in vitro*. Altering the *in vitro* pH from 7 to 6 caused marked reduction in DMD from 60.2 to 41.8, CPD from 76.3 to 55.3 and GED from 55.3 to 35.1, respectively. Low pH (6) depressed total VFA production (61.9 vs. 34.9 mM) as well as acetate to propionate ratio *in vitro* (from 2.0 to 1.5) when compared to pH 7. Compared to pH 7, total gas production decreased from 1,841 ml to 1,148 ml at pH 6, CO_2 and CH_4 production also reduced from 639 to 260 ml and 138 to 45 ml, respectively. This study supported the premise that pH is one of the principal factors affecting the microbial production of volatile fatty acids and gas. Regulating the ruminal pH to increase bacterial activity may be one of the methods to optimize VFA production, reduce methane and, possibly, improve animal performance. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 3 : 376-380)***

Key Words : Temperature, pH, Rumen Fermentation, Volatile Fatty Acids, Methane, RUSITEC

INTRODUCTION

The awareness of greenhouse gases and global warming are relatively recent issues and there is still much uncertainty, debate and misunderstanding as to their overall importance. The issues are extremely complex and currently there is lack of knowledge as to all factors involved and their relative importance. Domestic livestock have been implemented as significant components from several points ranging from land clearance, intensive land-use practices, enteric fermentation, to waste disposal. Clearly, we need more research and understanding of factor involved and documentation of their relative importance (Young, 2002). Ruminal methanogenesis is a nutritionally wasteful process that has been implicated in global warming (Duxbury et al., 1993). Reducing methane production from enteric fermentation by nutritional management, animal selection and rumen modifications are possible, but require a long-term research and development commitment (Hegarty, 2002). The impact of the environment on diet digestibility, rumen parameters and methane production is dependent on many factors. The ambient temperature at which methane measurements are made might have a significant impact on the estimate for the emission of methane from ruminants (Moss, 2002). In an earlier experiment in our lab, it was recorded that the mean rumen temperature could increase to 41°C at 33°C of air temperature with 70% RH (Higuchi, K., personnel communication). The combined effect of temperature and pH on the methane production has not been studied extensively since it is difficult to segregate the effects in *in vivo* experiments. The present study was therefore conducted to examine the effect of temperature as well as pH on the volatile fatty acid profile and methane production *in vitro*.

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MATERIALS AND METHODS

Apparatus and method

The fermenter used was the semi-continuous system similar to the one developed by Czerkawski and Breckenridge (1977), known as the rumen simulation technique (RUSITEC) to monitor the effects of treatments (temperature and pH) on rumen fermentation and gas production. The system (Kajikawa et al., 2003) was equipped with sixteen cylinders to study the treatment effect in four replicates. Treatments were allocated at random to four vessels each and were (1) two temperature levels of 39°C and 41°C (2) two pH levels of 6.0 and 7.0. The pH of eight fermenters were maintained at 6.0 by altering the

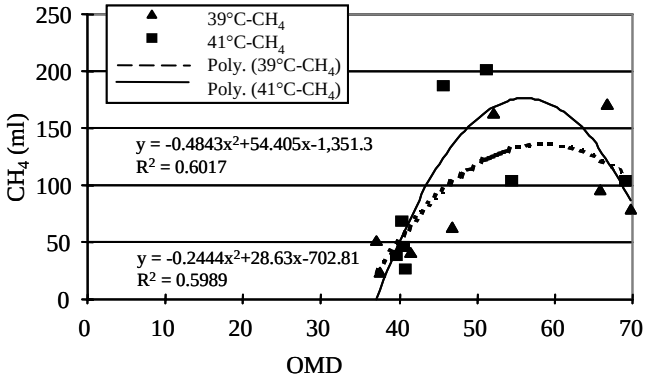

Figure 1. Relationship between OMD and methane production.

composition of the infused buffer and were kept at 39°C and whereas in another 8 fermenters the pH was maintained 7.0 and were kept at 41°C by thermostatically controlled water baths. All the fermenters were filled with 400 ml strained rumen fluid and 400 ml of artificial saliva (Mc Dougall, 1948). Rumen inoculum for the fermentation vessels were obtained from a pooled sample of strained rumen contents and rumen solids removed before the morning feed from a cannulated cow receiving Timothy hay: corn: soybean meal (7:2:1) fed for maintenance. A precision pump guaranteed a continuous buffer infusion rate set at 3.5% per hour. Basal diet used in RUSITEC consisted of (DM) 6.40 g Timothy hay, 1.86 g crushed corn and 1.34 g soybean meal. The food for the fermentation vessel was provided in nylon bags (10×20 cm, mean pore size 50 μm, ANKOM Technology, NY, USA). At the beginning of the experimental period one of the two nylon bags was filled with 70 g solid rumen content for easier establishment of favorable fermentation conditions and the other one with the respective diet, which were gently agitated in the liquid phase. Subsequently, each day, one bag was replaced starting with the bag containing solid rumen content thus achieving a general feed incubation period of 48 h. While the bag was being changed, the vessels were flushed with CO_2 to help maintain anerobiosis. After removal from the fermenters, feed bags were gently squeezed and washed with cold water until the outflow was clear and allowed to dry to a constant weight at 60-70°C for 48 h. The experiment lasted for 17 d with all the samples taken during the last 5 d.

Experimental measurements

During the last 5 days of the experiment, fermentation products were determined on the samples taken from the liquid overflow. Formalin (1% v/v) solution was added to the over flow to prevent fermentation. Culture pH was measured using a pH electrode in samples of fermentation fluid withdrawn at the time of feeding. Volatile fatty acids were determined by gas chromatography (6890 series with flame ionization detector, Hewlett-Packerd, Wilmington, DE, USA) on a glass column with 5% Thermon 1,000 and 0.5% H_3PO_4 on 80/100 mech Chromosorb W (Wako Pure Chemical Ltd., Osaka). The total gas produced in each fermenter was collected in gas proof bags. Gas production was quantified in a dry gas meter. In the gas samples, contents of CH_4 and CO_2 were analyzed by gas chromatograph GC-8A (Shimadzu Corp., Kyoto, Japan) on Polapack Q (Waters Corp., MA, USA) column. The degradability of the nutrients was estimated from the DM remaining in the bags after 48 h incubation in the RUSITEC vessels. Apparent nutrient degradation was computed from the nutrient contents present in the nylon bags before and after incubation. The diet and dried fermentation residue samples were analyzed for total ash and nitrogen by the Kjeldahl method (AOAC, 1995). Neutral detergent fibre (NDF), Acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined by the method of van Soest et al. (1991). Gross energy content of the samples was analyzed in an adiabatic bomb calorimeter (CA-4 PJ, Shimadzu, Japan).

Table 1. Nutrient composition of different ingredients (% DM) used in rumen simulating fermenter (RUSITEC)

	Corn	Soybean meal	Timothy hay
Dry matter	86.9	88.2	88.8
Organic matter	98.8	94.1	95.1
Crude protein	5.95	49.6	4.99
Neutral detergent fibre	12.1	16.5	64.5
Acid detergent fibre	3.69	10.7	44.0
Acid detergent lignin	0.351	0.721	6.85
Ash	1.16	5.94	4.91
GE (MJ/kg)	16.4	17.6	16.6

Statistical analyses

Statistical evaluation was carried out by analysis of variance (ANOVA) using SAS/STAT Version 9.1 package with temperature and pH as variables. The interaction effect of temperature vs. pH was also calculated. The probability values of temperature, pH and the interaction effect were presented in the tables. A multiple regression analysis (SAS program (REG Procedure); model $Y = X_1X_1X_1$) was done to depict the quadratic relationship between OMD and methane production.

RESULTS AND DISCUSSION

Nutrient composition of the ingredients used in the study is given in Table 1. The total diet (6.40 g Timothy hay +1.86 g corn+1.34 g soybean meal) contained (g kg^{-1} DM) 957. 3 OM, 114.5 CP and 16.68 MJ kg^{-1} DM of GE.

The effect of *in vitro* temperature and pH on the nutrient degradability and VFA profile is presented in Table 2. Although increase in temperature from 39°C to 41°C

Table 2. Effect of temperature and pH on nutrient degradability, pH and volatile fatty acids in the rumen simulating fermenter (RUSITEC)

	Temperature		pH		Probability		
	39°C	41°C	pH 7	pH 6	Temperature	pH	Temp.×pH
Degradability of nutrients (%)							
DMD	53.2	48.8	60.2	41.8	0.209	0.000	0.257
CPD	73.2	58.5	76.3	55.3	0.064	0.001	0.059
OMD	52.2	47.7	59.4	40.5	0.213	0.000	0.255
GED	47.6	42.8	55.3	35.1	0.211	0.000	0.253
VFA profile							
Total VFA	48.5	48.3	61.9	34.9	0.981	0.001	0.141
Acetic acid	26.6	26.3	35.4	17.5	0.939	0.000	0.162
Propionic acid	14.6	14.2	17.4	11.4	0.790	0.002	0.065
Butyric acid	5.90	6.20	7.00	5.10	0.477	0.004	0.506
Iso-valeric acid	1.10	1.40	1.60	1.00	0.232	0.023	0.255
A/P	1.7	1.8	2.0	1.5	0.425	0.000	0.851
pH	6.50	6.50	6.81	6.19	0.942	0.000	0.184

reduced degradation of major nutrients *in vitro*, however it was non-significant ($p>0.05$). Interaction effect of temperature with pH also reflected a trend similar to that of sole temperature. Moss (2002) recorded non-significant effect of ambient temperature on apparent digestibility in sheep fed with dehydrated *Medicago sativa*. Bhatta et al. (2004, 2005) in a recent study conducted in sheep housed under two management systems (asbestos roof shed vs. open corral) in a semi-arid region of India, also did not observe any significant ($p<0.05$) effect of environmental temperature on nutrient digestion *in vivo*. However, pH showed significant ($p<0.05$) negative effect on the degradability of all the nutrients *in vitro*. Altering the *in vitro* pH from 7 to 6 caused significant reduction in DMD from 60.2 to 41.8, CPD from 76.3 to 55.3 and GED from 55.3 to 35.1, respectively. Erfle et al. (1982) reported that protease activity measured in bacteria from the artificial rumen maintained at pH 5 was only 22 per cent of that at pH 7. Inhibition of hay fermentation was recorded when the final *in vitro* pH was less than 5.7 (Russell, 1998) and it was indicated that pH values of less than 6.0 was detrimental. Mould and Orskov (1983) through *in vitro* experiments observed that even modest decline in pH could have a negative impact on *in vitro* ruminal cellulose digestion since, ruminal cellulolytic bacteria couldn't easily adapt to low pH. It was reported that rumen is well buffered around pH 6.8; the buffering capacity is poor below pH 6.0. A major consequence when ruminal pH falls below 6 is that fibre digestion declines dramatically (Russell and Wilson, 1996). This was attributed to two reasons, firstly, enzymes necessary for fibre breakdown do not function effectively at pH<6.0 and secondly, growth rate of fibrolytic activity declines markedly at low pH. Not only are these bacteria not able to obtain the sugars necessary for growth, low pH impedes growth itself. Fibrolytic bacteria were unable to maintain the pH inside their cells when ruminal pH was low. This incapacitates the cell machinery making growth impossible (Russell and Wilson, 1996). Observed responses to lowered pH was summarized by other workers (a) as reduced numbers of proteolytic microbes and their respective proteases (Erfle et al., 1982), (b) reduced bacterial attachments to fiber (Hoover, 1986), (c) as well as reduced ATP yield per unit of carbohydrate fermented (Strobel and Russell, 1986) as mediating mechanisms. The results of the present study on nutrient degradability support the earlier *in vitro* findings.

However, Beauchemin et al. (2003) recorded non-significant relationships ($R^2<0.03$) between rumen pH and NDF digestibility. This contrast with other *in vitro* studies with pure cultures and contribution of other minor fibrolytic organisms in fiber digestion was attributed as the possible reason for these findings.

Total VFA production reflects the net effect of the individual VFA. Low pH (6) depressed the total VFA production (61.9 vs. 34.9 mM) as well as acetate to propionate ratio *in vitro* (Table 2). Decreased production of acetic acid with the proportional increase in propionic acid production resulted in lower acetic to propionic acid ratio (from 2.0 to 1.5), when pH was reduced from 7 to 6. Erfle et al. (1982) in a concentrate-corn silage diet observed A/P ratio of 4.3 decreasing to 0.9 as the pH was decreased from 7 to 5. Lana et al. (1998) reported that ruminal pH was positively correlated with acetate to propionate ratio *in vitro* and *in vivo*. Our study corroborates the concept that pH was having major impact on acetate: propionate ratio.

The effect of temperature and pH on the total gas production and the composition of gas are presented in Table 3. Unlike *in vitro* degradability, an increase in temperature from 39°C to 41°C increased the total gas and methane production; however, the values were not significant. Kurihara et al. (1995) in a study with ruminants fed high concentrate diets under heat exposure also did not observe any increase in methane production. Moss (2002) recorded significant reduction in methane production when

Table 3. Effect of temperature and pH on the gas production in the rumen simulation technique (RUSITEC)

	Temperature		pH		Probability		
	39°C	41°C	pH 7	pH 6	Temperature	pH	Temp.×pH
Total gas production (ml)	1,418	1,570	1,841	1,148	0.397	0.002	0.759
Carbon dioxide production (ml)	415	484	639	260	0.313	0.000	0.349
Methane production (ml)	86.1	97.1	138	45.0	0.565	0.000	0.565
Methane (ml/g DM)	6.44	7.27	10.4	3.37	0.462	0.001	0.486
Methane (ml/g OM)	6.74	7.60	10.8	3.52	0.356	0.011	0.012
Methane energy (Kj)	3.40	3.84	5.46	1.78	0.565	0.000	0.565
Methane energy (%GE)	2.13	2.40	3.41	1.11	0.565	0.000	0.565
Methane energy (%DE)	4.29	5.49	6.63	3.15	0.344	0.014	0.374

the ambient temperature was raised and this was associated with a reduced rumen DMD. Rumen temperature and pH were raised in the cold environment which was associated with enhanced microbial and cellulolytic respectively in the rumen and corresponds to an increased level of methane produced per kg DM intake. Here the environmental temperature has affected both the rumen temperature and rumen pH (Moss, 2002). However, effect of rumen temperature and pH on methane production couldn't be separated because of *in vivo* conditions. In our study gas production was greatly influenced by *in vitro* pH. Total gas production was decreased from 1,841 ml to 1,148 ml at pH 6, CO_2 and CH_4 productions also reduced from 639 to 260 ml and 138 to 45 ml, respectively. Decreased CH_4 production at lower pH agrees with the results of Erfle et al. (1982). Van Kessel and Russell (1996) reported that, when the ruminal bacteria were incubated in a medium containing 100 mM acetate, the addition of HCl caused a dramatic decrease in methane production, and no methane was detected at pH values less than 6.0. When the acetate concentration of the medium was decreased, the pH-dependent inhibition of methane production could be completely reversed. At low pH, CO_2 no longer acts as an acceptor for protons produced during fermentation, when the H_2 concentration increases lactate dehydrogenase reaction shifts towards lactate production and stimulate propionate synthesis either via acrylate (direct pathway) or via succinate (transcarboxylate pathway) (Hungate, 1966). The pH sensitivity of ruminal bacteria was dictated by intracellular pH and the pH gradient across the cell membrane (Russell, 1992). Some starch-digesting ruminal bacteria can metabolize sugar when the intracellular pH is low, and the decline in intracellular pH prevents toxic accumulation of intracellular VFA anions (Russell, 1991). Low pH caused marked decrease in the acetate to propionate ratio, which was mirrored by a reduction in methane production. This result was consistent with the idea that propionate production and methanogenesis are competing and alternative mechanisms of reducing equivalent disposal.

Van Kessel and Russell, (1996) observed *in vitro*, using rumen fluid sampled from animals fed on roughage-based diets, that ruminal methanogens lose the ability to use H_2 at low pH, giving rise to free H_2 in the gas phase when the pH was less than 5.5. Thus on roughage diets, a low pH leads to a decrease in methanogenesis independent from propionate formation. Based on these results, Russell (1998) concluded that many starch-fermenting bacteria prefer to produce propionate, and H_2 would not be available for methanogenesis. However, when the final pH was less than 5.3, acetate to propionate ratio increased dramatically, and large amounts of H_2 were detected. These results hypothesized that ruminal bacteria that produce propionate could be even more sensitive to pH than some bacteria that produce acetate and H_2 (Russell, 1998). In another study, mixed ruminal bacteria from forage-fed cow converted CO_2 and H_2 to methane, but no methane was detected with ruminal fluid from the concentrate fed cow. When the pH was increased to 7.0, the ruminal fluid from the concentrate-fed cow produced methane, but the lag time was 4 h longer. Based on the zero-time intercept, concentrate-fed cow had approximately 10-fold fewer methanogens than forage-fed cow (Van Kessel and Russell, 1996). Results of the present experiment further prove the conclusion that ruminal methanogens are very sensitive to low pH and pH-dependent decreases in the ratio of acetate to propionate are probably caused by an inhibition of methanogenesis.

Graph 1 depicts the relationship between OMD and methane production *in vitro*. At lower and at higher OMD, there is non-significant relationship between the two parameters, however at in-between levels there was a quadratic relationship and methane production tends to increase under high temperature. Kurihara et al. (1995) in a study with ruminants fed low concentrate diets under heat exposure also recorded an increase in methane production. From the results, it was apparent that reducing the pH although reduced the methane production, but simultaneously reduced the digestibility as well. So, the ideal feeding strategy should be one which can reduce the methane production without compromising the digestibility. This could be achieved by improving the quality of the diet, if the diet digestibility is above 65 percent; then the methane production could also be minimized to a great extent. Developments of management strategies to mitigate methane emissions from cattle are possible and desirable.

Not only will enhanced utilization of dietary carbon improve feed efficiency and animal productivity, but a decrease in methane emissions will reduce the contribution of ruminant livestock to the global methane inventory.

CONCLUSIONS

From the results of the present study it could be concluded that temperature (41°C) didn't have significant effect on the methane production *in vitro* and pH was one of the principal factors affecting the microbial production of volatile fatty acids and methane. Regulating the ruminal pH to increase bacterial activity might be one of the methods to optimize VFA production, increase feed digestibility, reduce methane and, possibly, animal performance.

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