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Full Length Research Paper

Effects of different levels of phosphate in comparison with citrate buffer in culture media on *in vitro* ruminal fermentation and methanogenesis of a starch-rich feed mixture

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Compared to a phosphate free medium containing citrate buffer, effects of phosphate concentrations (10, 20, 50 ,and 100 mmol/L) in the modified Menke and Steingass's medium on methanogenesis of a maize-rich substrate (*Leymus chinensis* hay:maize meal = 1:4) were determined in comparison with using an *in vitro* cumulative gas production technique. After the 48 h batch cultures, phosphate additions decreased total gas production (GP_{48}, ml/g dry matter (DM)) ($P < 0.05$) as well as the gas production rate to reach maximum digestion (RmaxG, mL/h) ($P < 0.01$). Total volatile fatty acids (VFA) concentrations (mmol/L) were decreased by 28%, while the phosphate concentration increased up to 50 to 100 mmol/L ($P < 0.05$). Volatile fatty acid production shifted from an initially acetate dominated production towards a propionate production as demonstrated by the ratio of non-glucogenic to glucogenic acids (NGR) ($P < 0.01$). Although, the molar proportions of CO_2, CH_4, and H_2 in the fermentation gases were not altered, the net fractional CH_4 production (ml/g DM) in the phosphate levels of 10, 20, 50, and 100 mmol/L were reduced by 6.2, 11.8, 18.57, and 26.2%. The results obtained in the present study showed that a phosphate-mediated reduction in methane emissions occurred without the expense of a reduction in the digestion of the substrate, and the mechanism that phosphate inhibited acetoclastic methanogenesis on rice roots may exist in the rumen.

Key words: Rumen, methanogenesis, phosphate buffer, citrate buffer, *in vitro* batch culture.

INTRODUCTION

The anaerobic conversion of organic matter to CH_4 in the rumen involves a consortium of rumen microorganism with the final step affected by methanogens (McAllister et al., 1996). Primary digestive microorganism (e.g. bacteria, protozoa, and fungi) hydrolyze protein, starch, and plant-cell-wall polymers to produce amino acids and sugars. These products are immediately fermented to volatile fatty acids (VFA), H_2, CO_2, and CH_4. Methanogenesis in ruminant represented a loss of 2 to 12% of the gross energy consumed by the animal; besides, its contribution to climatic change and global warming caused more attention in recent years (Johnson and Johnson, 1995). A study reported that addition of >20 mmol/L of phosphate in the media specifically inhibited acetotrophic methanogenesis and acetate contributed about 50 to 60% of the total methanogenesis on rice roots (Conrad et al., 2000). Hydrogen and carbon dioxide are widely believed to be the principal precursors used by

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Abbreviations: ADFom, Acid detergent fibre corrected for residual ash; **AGRS,** automated gas recording system; **CB,** citrate buffer; **DM,** dry matter; **GP_{48},** gas production after 48 h incubation; **IVDMD,** *in vitro* dry matter disappearance; **NDFom,** neutral detergent fibre corrected for residual ash; **NGR,** ratio of non-glucogenic to glucogenic acids; **PB,** phosphate buffer; **NPM,** net CH_4 production; **$R_{max}G$,** maximum gas production rate; **$TR_{max}G$,** time which the maximum gas production rate is reached; **VFA,** volatile fatty acids.

Table 1. Composition of citrate buffer (CB) and phosphate buffers (PB) with different concentrations of phosphate used as the macro-mineral solutions for the basal medium preparations.

Ingredients[b]	CB	Phosphate levels (mmol/L)[a]			
		10	20	50	100
$Na_2HPO_4 \cdot 12H_2O$ (g/L)	0	0.90	1.79	4.46	8.95
KH_2PO_4, anhydrous (g/L)	0	1.07	2.14	5.35	10.71
$C_6H_8O_7 \cdot H_2O$ (g/L)	0.35	0	0	0	0
$Na_3C_6H_5O_7 \cdot 2H_2O$ (g/L)	6.51	0	0	0	0

[a]The phosphate levels obtained by modifying basal medium of Menke and Steingass (1988), and same in other tables. [b]Ingredients with different levels expressed in the macro-mineral solutions.

rumen methanogens to produce methane (Hungate et al., 1970). But the *Methanosarcina* species, which has been confirmed to be a group of acetotrophic methanogens present in the rice root (Großkopf et al., 1998; Chaban et al., 2006), also exist in the rumen, though not the dominant methanogenic population (Janssen and Kirs, 2008).

Rumen fermentation *in vitro* was used to obtain data of methane production from various feeds (Rossi et al., 2001), and it has been extensively applied to determine effects of feed additives on methane production in the last decades. The aim of this work was to assess if *in vitro* rumen methanogenesis could also be affected by the phosphate addition.

MATERIALS AND METHODS

Substrate and its chemical analysis

Samples of Chinese wild ryegrass (*Leymus chinensis*) at late-bloom stage were chopped with a paper cutter and oven-dried at 65°C over night. Oven dried ryegrass hay and maize meal were ground in a Wiley mill to pass through 2-mm screen. Samples of hay and maize meal were analyzed following AOAC (1999) for dry matter (DM, ID 930.5), crude protein (ID 984.13), and ash (ID 942.05). Both neutral detergent fibre (NDFom) and acid detergent fibre (ADFom) were analyzed (Van Soest et al., 1991) and corrected for residual ash content. Starch-rich substrate used in the latter batch culture was prepared by mixing 20% of ryegrass hay and 80% of maize meal. The chemical compositions of the substrate (per kg DM) were 91 g crude protein, 268 g NDFom, 82 g ADFom, 642.7 g starch, and 26 g ash.

Media preparation

Basal medium without Na_2HPO_4 and KH_2PO_4 were prepared by modifying the medium of Menke and Steingass (1988) in which phosphate buffer was replaced by citrate buffer (CB, 1.67 mmol/L $C_6H_8O_7 \cdot H_2O$ and 22.80 mmol/L $Na_3C_6H_5O_7 \cdot 2H_2O$) to maintain the buffering capacity. In the phosphate buffer treatments (PB), 10, 20, 50, and 100 mmol/L phosphate concentrations were prepared by modifying the concentration levels of $Na_2HPO_4 \cdot 12H_2O$ and KH_2PO_4 (Table 1) in the modified medium of Menke and Steingass (1988). All media were bubbled with CO_2 until the pH reached 6.7.

In vitro batch culture

Amount of 500 mg of the substrate were weighed into 100 ml Hungate's screw-capped bottles. Rumen fluids were obtained from three rumen-cannulated lactating Holstein dairy cows at 2 h prior to the morning feeding. The cows were daily fed 8.0 kg Chinese wildrye grass hay and 6.0 kg commercial concentrate and cared under the Guidelines of the Beijing Municipal Council on Animal Care. A total of 25 ml strained rumen fluids (pH 6.4) were mixed into each bottle with 50 ml pre-warmed media, purged with N_2 for 5 s to remove air and were sealed. 25 ml of inoculum plus 50 ml medium without substrate were served as a blank control. Triplicate bottles for each buffer treatment were immediately connected to gas channel inlets of an Automated Gas Production Recording System (AGRS) as modified from Theodorou et al. (1994) and Cone et al. (1996). The cumulative gas production (2.5 to 3.0 ml) calibrated for each vent was automatically recorded in the AGRS driven by a differential pressure switch (pressure range: 20 to 300 pa, Huba Control Inc. USA). Extra triplicate bottles were connected to air sampling bags to collect whole fermentation gas of the incubation for gas analysis. All bottles were incubated at 39°C for 48 h.

After the incubation, bottles were open and pH was determined in culture fluids. Samples of 1.0 ml culture fluid were mixed with 0.3 ml of 25% (w/v) meta-phosphoric acid solution for 30 min and centrifuged at 15,000 ×g for 10 min at 4°C. Supernatants were analyzed for ammonia N and VFA (Yang et al., 2005). Proportions of CH_4, CO_2, and H_2 were estimated by injecting 1.0 ml gas sample in gas chromatograph (GC522, Wufeng Instruments, Shanghai, China) equipped with flame ionization detector and a stainless steel column (2 m, ∅3 mm) packed with TDX-01. Injector oven, column oven, and detector temperatures were 150, 80, and 200°C, respectively. The cultures of each bottle was filtered with a Nylon bag (8 × 12 cm, 42 μm pore size) and dried at 65°C for 48 h to determine the *in vitro* dry matter disappearance (IVDMD).

Biometric analysis

Data of the cumulative gas production were fitted to a mono-phasic model (Groot et al., 1996) as: $GP_t = A/(1 + (C/t)^B)$, where GP_t is the cumulative gas production (ml/g DM) at incubation time t (h), A is a asymptotic gas production (ml/g DM), C is the time (h) at which half of A is reached, and B is a sharpness parameter determining the shape of the curve. A, B, and C were calculated by the NLIN procedure of SAS (1999). Maximum gas production rate ($R_{max}G$, ml/h) and the time at which $R_{max}G$ is reached ($TR_{max}G$, h) were calculated with A, B, and C (Yang et al., 2005).

The sum of the analyzed CH_4, CO_2, and H_2 was calculated as total gas proportion, which excluded nitrogen, oxygen, and water

Table 2. Effects of citrate buffer (CB) and phosphate buffers (PB) with different phosphate concentrations in the basal medium on *in vitro* kinetic gas production characteristics of the maize rich substrate.

Parameter	CB	Phosphate concentration (mmol/L)				SEM	*P* values		
		10	20	50	100		CB vs. PB	L[a]	Q[b]
IVDMD[c]	0.72	0.74	0.73	0.74	0.78	0.058	NS	NS	NS
GP_{48}[d]	267.2	232.1	235.2	212.2	207.8	10.85	*	NS	NS
A[e]	276.9	245.0	248.4	223.0	220.5	12.21	*	NS	NS
B[e]	1.71	1.45	1.4	1.51	1.50	0.02	**	NS	NS
C[e]	5.2	5.3	5.0	4.9	5.0	0.30	NS	NS	NS
$R_{max}G$[f]	32.1	27.9	27.7	27.4	26.5	1.78	*	NS	NS
$TR_{max}G$[f]	2.4	1.7	1.9	1.7	1.8	0.13	**	NS	NS

*,**P < 0.05 and P < 0.01, respectively; NS, P > 0.05. SEM = standard error of the mean in all tables. [a]Linear effect of phosphate buffer solutions in all tables. [b]Quadratic effect of phosphate buffer solutions in all tables. [c]*In vitro* dry matter disappearance. [d]Cumulative gas production (ml/g DM) after 48 h fermentation. [e]A is an asymptotic gas production (ml/g DM), B is a sharpness parameter determining the shape of the curve, and C is the time (h) at which half of A is reached (Groot et al., 1996). [f]$R_{max}G$ is maximum rate of in vitro gas production (ml/h), and $TR_{max}G$ is the time (h) of $R_{max}G$ occurred (Yang et al., 2005).

vapor. Net CH_4 productions (NMP, ml/g DM) were calculated as the total gas production multiplied by the molar proportion of CH_4. Ratio of non-glucogenic to glucogenic acids (NGR) was calculated according to Ørskov (1975) as: NGR = (Acetate + 2 × Butyrate + Valerate)/(Propionate + Valerate), where VFAs were expressed in molar proportion.

Data were analyzed by the general linear model procedure of SAS (1999) with the model including treatments of CB and PB using a multiple comparison test (Tukey/Kramer). The contrasts were used to assess the effects of phosphate levels (CB versus PB), and both linear and quadratic orthogonal contrasts were tested only within the PB treatments using the regression procedure of SAS (1999). Least square means and standard errors of means (SEM) were calculated with the least-squares means (LSMEANS) statement of the general linear model procedure. The partial correlation coefficients and P values between IVDMD and GP_{48} or total VFA were tested by the correlation procedure of SAS (1999). A level of P < 0.05 was chosen as the minimum for acceptable statistical significance.

RESULTS

In Table 2, no significant difference occurred in IVDMD and C due to whatever use of buffers. GP_{48}, A, $R_{max}G$, and $TR_{max}G$ in PB were lower than those in CB (P < 0.05). Neither linear nor quadratic effect of the phosphate addition occurred on IVDMD and all kinetic gas production parameters. Compare to citrate buffer, the use of phosphate buffers in media decreased total potential gas production by 10.2 to 20.3% and gas production rate by 13.0 to 17.4% (Figure 1).

The value of pH in PB was lower than those in CB (P < 0.05), but all pH values were in normal range. The ammonia N did not differ due to the use of PB (Table 3). Total VFA in CB was greater than those in PB, and a decreasing linear effect of phosphate on total VFA occurred within PB (P < 0.01). Compared to CB, total VFA decreased by a maximum 28%, while the phosphate concentration increased up to 50 to 100 mmol/L (P < 0.01).

In molar proportions of VFA, propionate and butyrate in CB were lower than those in PB (P < 0.01), and a linear effect of phosphate occurred in the PB treatments (P < 0.05). The phosphate concentrations in the PB treatments did not alter valerate and branch chained VFA proportions. NGR in CB was significantly greater than those in PB (P < 0.01). Both linear (P < 0.01) and quadratic effect (P < 0.05) occurred for NGR within the PB treatments, which implicated that VFA production changed from an initially dominated acetate towards the production of propionate.

The phosphate concentrations in the PB treatments did not alter molar proportions of CO_2, CH_4, and H_2. NMP in PB was significantly lower than that in CB (P < 0.05), and a quadratic effect of phosphate occurred in the PB treatments (P < 0.01). Compared to CB, NMP in PB were reduced by 6.2, 11.8, 18.57, and 26.2% for 10, 20, 50, and 100 mM phosphate concentration, respectively (P < 0.05).

DISCUSSION

The original phosphate concentration in the basal medium (Menke and Steingass, 1988) is about 20 mmol/L. Assuming a daily fluid flow through the rumen is 240 L for dairy cattle with 20 kg of daily DM intake, the estimated concentrations of phosphorus in the diets are indeed too high to be economically or environmentally sound. A reduction in methane production at the expense of an increase in phosphorus excretion will not be necessarily a good thing from an environmental perspective. Therefore, the high level of phosphate in medium is of interest in terms of research but not for practical purpose until now.

Microbial fermentation of organic matters produces gas as one of the end-products providing the foundation of the strong correlation between OM digestibility and

Table 3. Effect of citrate buffer (CB) and phosphate buffers (PB) with different phosphate concentrations in the basal medium on fermentation characteristics of the maize rich substrate.

Parameter	CB	Phosphate concentration (mmol/L)				SEM	*P* values		
		10	20	50	100		CB vs. PB	L	Q
pH	6.57	6.43	6.40	6.41	6.37	0.013	**	NS	NS
Ammonia N (mmol/L)	37.6	32.7	35.4	37.3	35.7	2.17	NS	NS	NS
Total VFA (mmol/L)	155.5	129.1	127.8	111.3	115.0	0.52	**	*	NS
VFA (molar, %)									
Acetate	68.3	64.3	64.5	63.7	63.2	0.52	**	NS	NS
Propionate	17.6	19.5	19.7	20.0	21.3	0.46	**	*	NS
Butyrate	10.4	12.5	11.9	12.1	11.6	0.22	**	NS	NS
Iso-butyrate	0.24	ND [a]	0.16	0.47	0.20	0.18	NS	NS	NS
Valerate	1.49	1.48	1.60	1.72	1.49	0.09	NS	NS	NS
Iso-valerate	1.84	2.04	1.93	1.86	2.07	0.09	NS	NS	NS
NGR [b]	4.75	4.31	4.21	4.14	3.85	0.11	**	**	*
Fermentation gas (molar, %)									
CO_2	78.2	78.7	77.8	78.2	78.9	0.39	NS	NS	NS
CH_4	21.7	21.2	22.1	21.7	20.9	0.40	NS	NS	NS
H_2	0.05	0.06	0.07	0.07	0.10	0.01	NS	NS	NS
NMP [c] (ml/g DM)	57.9	54.3	51.0	47.1	42.7	3.01	*	NS	**

*,**P < 0.05 and P < 0.01, respectively; NS, P > 0.05. [a]Not detected. [b]Ratio of non-glucogenic to glucogenic acids (Ørskov, 1975). [c]Net methane production.

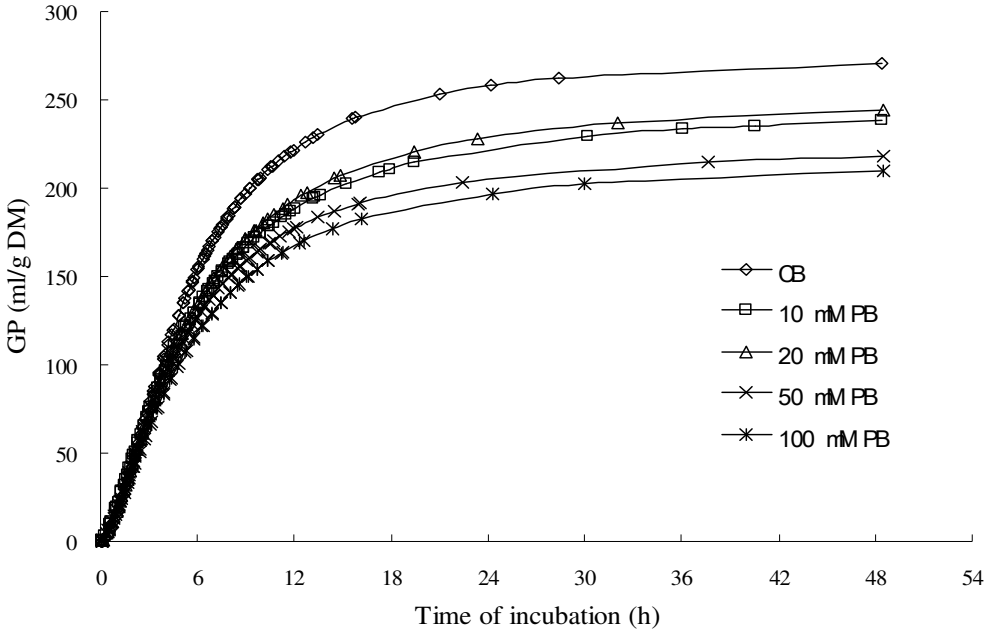

Figure 1. Kinetic gas production profiles of the substrate consisted of *L. chinensis* hay and maize meal in 1 to 4 portions in the presence of citrate buffer (CB) and different phosphate levels in phosphate buffers (PB).

volume of gas produced (Blummel and Ørskov, 1993), and results should be found to provide comparable estimates for digestibility of feeds. A poor correlation occurred between IVDMD and GP_{48} (r = -0.18; P = 0.50)

or total VFA production (r = -0.43; P = 0.11), and a comparative high IVDMD occurred in PB, suggesting that the use of IVDMD, estimated by the nylon bag washing procedure, may be not sensitive enough to phosphate concentration treatments in medium buffers instead of nutrient differences in chemical composition of substrate.

Volatile fatty acids are the end products of rumen microbial fermentation and represent the main supply of metabolizable energy for ruminants (Van Soest, 1982). Therefore, a reduction in their production would be nutritionally unfavorable for the animal. Although, a fair good correlation (r = 0.96, P = 0.02) was observed between GP_{48} and total VFA, total VFA concentrations significantly declined with the increase of phosphate levels in media. These results suggest that rumen microorganism was indeed sensitive to higher phosphate.

For the purpose of maintaining the buffer capacity in the phosphate-free medium, appropriate buffer pair should be selected. Normally, marble (0.5 to 2.0 mm size, consisting of $CaCO_3$) was chosen to be the alternative (Lu et al., 2005). However, CO_2 would be released in such a buffer as marble reacted to the VFA, and consequently affected the fermentation gas component. Citrate buffer provided a pH of 6.6 as those in PB. A literature indicated that citrate could be decomposed by methanogens to acetate, CO_2, and hydrogen (Gamez et al., 2009). Therefore, there should be a ruminal degradation of citrate in our study. This degradation might provoke an increase of the gas production (GP_{48} and A) and the gas production rate (RmaxG), an increase of the ruminal pH, and changes in the VFA production (Tables 2 and 3). There is a significant effect of the buffer (CB versus PB) on *in vitro* ruminal gas production, and on pH and VFA concentrations/molar proportions. As observed by Gamez et al. (2009), similarly higher molar acetate proportion in CB might also be caused by citrate buffer. Higher proportion of acetate results in more production of H_2, a substrate that methanogenic archaea used to reduce CO_2 and produce CH_4 (Ellis et al., 2008), this may be one of the reason that CH_4 production in CB was higher than that in PB treatments.

The VFA production changed from an initially dominated acetate towards the production of propionate, and these results implicate that higher levels of phosphate in medium buffers could provide more glucogenic energy equivalent in the rumen. As much as 25% of the decrease in acetate to propionate ratio could be explained by the effect of pH declined from 6.5 to 5.8 (Russell, 1998). Low rumen pH results in lower acetate and butyrate concentrations and greater propionate molar proportions (Calsamiglia et al., 2008). The ruminal pH with phosphate buffer was significantly lower than with CB, and this effect could induce changes in the ruminal microbiota communities. The shift of VFA proportions may also be caused by lower pH that occurred in PB treatments whereas the digestion of organic matter is likely hampered due to lower microbial population activity.

CH_4 should be viewed as an every sink where hydrogen from rumen microorganisms drains, allowing a greater total yield of ATPs. H_2 and CO_2 are believed to be the main precursors for CH_4 synthesis in the rumen (Hungate et al., 1970). However, the fact that the stable CH_4 proportion and less net fractional production of methane suggest that CO_2 and H_2 may not be limiting factors for hydrogenotrophic methanogens to produce CH_4. With the presence of three interacting metabolic groups of strictly anaerobic microbes to produce CH_4 (Ferry, 1997), CH_4 depressed effects of phosphate may be caused by its inhibition on acetate-producing bacteria that use pyruvate-ferredoxin oxidoreductase to metabolize pyruvate to acetyl-SCoA. The mechanism that phosphate inhibited acetoclastic methanogenesis on rice roots (Conrad et al., 2000) might also exist in the rumen, and some ruminal methanogens might also metabolize acetate to produce CH_4. Further confirmation is necessarily made by analyzing the methanogenic community affected by phosphate addition to classify such mode of methane production.

Conclusions

Rumen microorganism was indeed sensitive to increased levels of phosphate in the culture medium. The batch culture results implicated that a phosphate-mediated reduction in methane emissions occurred without the expense of a reduction in the digestion of the substrate. The mechanism that phosphate inhibited acetoclastic methanogenesis on rice roots would also exist in the rumen environment.

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