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Low Ruminal pH Reduces Dietary Fiber Digestion via Reduced Microbial Attachment

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ABSTRACT : *In vitro* rumen incubation studies were conducted to determine effects of initial pH on bacterial attachment and fiber digestion. Ruminal fluid pH was adjusted to 5.7, 6.2 and 6.7, and three major fibrolytic bacteria attached to rice straw in the mixed culture were quantified with real-time PCR. The numbers of attached and unattached *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcoccus albus* were lower ($p<0.05$) at initial pH of 5.7 without significant difference between those at higher initial pH. Lowering incubation media pH to 5.7 also increased bacterial numbers detached from substrate regardless of bacterial species. Dry matter digestibility, gas accumulation and total VFA production were pH-dependent. Unlike bacterial attachment, maintaining an initial pH of 6.7 increased digestion over initial pH of 6.2. After 48 h *in vitro* rumen fermentation, average increases in DM digestion, gas accumulation, and total VFA production at initial pH of 6.2 and 6.7 were 2.8 and 4.4, 2.0 and 3.0, and 1.2 and 1.6 times those at initial pH of 5.7, respectively. The lag time to reach above 2% DM digestibility at low initial pH was taken more times (8 h) than at high and middle initial pH (4 h). Current data clearly indicate that ruminal pH is one of the important determinants of fiber digestion, which is modulated via the effect on bacterial attachment to fiber substrates. (**Key Words** : Bacterial Attachment, Fiber Digestion, pH, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcoccus albus*).

INTRODUCTION

The main source of energy for ruminants is fiber, which is digested by a consortium of anaerobic microorganisms residing in the rumen. Understanding mechanisms involved in fiber digestion by rumen microorganisms, therefore, has been a major interest of ruminant nutritionists (Kobayashi et al., 2004).

It has been well documented that bacterial populations in the rumen at a given time largely determine the extent and rate of fiber degradation (Akin and Barton, 1983; Miron et al., 2001; Pan et al., 2003; Khampa et al., 2006). Also, the attachment of fibrolytic bacteria is an obligatory step in fiber degradation, which was proven in previous studies with major fibrolytic bacteria such as *Fibrobacter succinogenes* S85 (Gong and Forsberg, 1989), *Ruminococcus albus* SY3 (Miron et al., 1998) and *Ruminococcus flavefaciens* 007 (Stewart et al., 1990).

Rumen pH, together with microbial population, nature of substrates, environmental factors such as temperature and existence of cations and soluble carbohydrates have been suggested as factors governing bacterial attachment (Miron et al., 2001). Ruminal pH is one of the most important of these factors, because fibrolytic bacteria are very sensitive dependent on pH change. In pure culture studies, the number of adhesion cells of *F. succinogenes* to cellulose decreased when pH was reduced from 6.0 to 4.5 with the numbers being maintained between pH 6.0 and 7.0, and falling abruptly above pH 7.5 (Roger et al., 1990). In another study in which the adhesion of *R. albus* was investigated, change in pH between 5.5 and 8.0 had little effect but below pH 5.0 there was a marked decrease (Morris, 1988).

Fiber digestion decreases at low rumen pH, especially below pH 6.0, as observed previously in studies using continuous culture of mixed ruminal microorganisms (Slyter, 1986), *in vitro* rumen culture (Grant and Weidner, 1992; Hu et al., 2005), *in sacco* disappearance (Mould and Ørskov, 1983) and *in vivo* tests (Mould et al., 1984). It is

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apparent that low ruminal pH changes the rumen microbial population from fibrolytic to amylolytic (Tajima et al., 2001). Mourino et al. (2001) reported that bacterial adhesion was severely inhibited by initial low pH (5.5) compared to values above pH 6.0, and low adhesion of rumen microorganisms was considered a causative factor of reduced fiber digestion at low ruminal pH, although the results were obtained from the measurement N and P content of microbial mass attached to pure cellulose substrate.

From the literature survey, no direct evidence appears to have been reported on the relationship among rumen pH, bacterial attachment and fiber digestion in complex rumen microbial ecosystem. Therefore, *in vitro* studies with mixed rumen microorganisms were conducted to determine effects of initial pH on fiber digestion and fibrolytic bacterial attachment by real-time PCR.

MATERIALS AND METHODS

Ruminal inocula and culture media

Rumen contents were obtained from three rumen fistulated Holstein steers (average 550 kg) which was fed twice daily at 09:00 and 18:00 with a mixture of 40% concentrate and 60% timothy hay for more than 2 wk. Rumen contents which had a range of pH 6.5-6.7 were collected before morning feeding, homogenized under anaerobic conditions, and strained through four layers of cheesecloth. The strained rumen fluids from three steers were mixed in equal amount and were used as a source of microorganisms. Rice straw ground to pass a 2 mm screen was used as a carbon and energy source. The basal medium used in this experiment was on buffer solution of McDougall (1948).

In vitro fermentation

Bacterial attachment to rice straw under different pH conditions was tested using an *in vitro* culture system with some modification by Sung et al. (2006). The rumen fluids-basal medium mixture was prepared by mixing one volume of rumen fluid and two volumes of the basal medium described above. The prepared medium was adjusted to 6.7 (high pH), 6.2 (middle pH) and 5.7 (low pH) using 5 N HCl, and made up to the same volume using distilled water. Five hundred mg of ground rice straw was added to 30 ml of pH-adjusted incubation medium in 60 ml serum bottle and incubated at 39°C with continuous shaking at 120 rpm. The triplicate cultures were respectively scarified to analysis bacterial attachment and fermentation parameters at 0, 2, 4, 8, 12, 24 and 48 h of incubation. The whole procedure was carried out under anaerobic conditions (oxygen-free CO_2). Bacterial detachment tests were carried out by washing filter paper (0.5×2.0 cm) which was pre-incubated in rumen fluid at pH 6.7 for 12 h. The filter papers were washed in buffer solutions (100 ml) having a pH of 6.7, 6.2 and 5.7 for 30 min in a shaker at 70 rpm.

Quantification of cellulolytic bacteria

Sample preparation : The culture was centrifuged at 160×g for 10 min to separate rice straw and culture medium. Collected rice straw was suspended in 50 ml of 0.9% saline solution and centrifuged three times at 160×g for 10 min to remove easily detachable bacteria. After centrifugation, rice straw was dried using a lyophilizer (Ilshin, Korea) and kept at -80°C until bacterial measurement. The collected culture medium and saline solution which was used for washing rice straw were used for analysis of suspended (unattached) bacterial population. The mixture of collected culture medium and saline solution was centrifuged at 16,000×g for 10 min. After centrifugation, the bacterial pellet was resuspended with 0.9% saline solution and centrifuged at 16,000×g for 10 min to obtain a bacterial pellet.

DNA extraction : Total DNA was extracted according to the method described by Purdy et al. (1996). In detail, 0.5 g of dried rice straw or centrifuged culture pellet was mixed with 0.35 ml of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0), 0.5 ml of Tris-buffered phenol and 0.25 g of sterilized glass bead (0.5 mm, BioSpec., Product Inc., USA.). The tubes were shaken for 2 min, stood on ice for 2 min and this step was repeated three times. After adding 40 μl of 10% sodium lauryl sulfate solution, tubes were centrifuged at 13,000×g for 2 min and supernatant was collected. The remaining pellet was resuspended in 20 μl of TE buffer, then centrifuged at 13,000×g for 2 min and the supernatant was collected. Total DNA was collected from pooled supernatant using a hydroxyapatite chromatography column (Hydroxyapatite Bio-Gel HTP Gel, Bio-Rad Laboratories, Inc., USA). The RNA was removed by DNAse-free pancreatic RNAse A treatment and subsequent gel filtration (MicroSpin S-200 HR Columns, Amersham Biosciences, UK). The Purity and concentration of total DNA were checked using a Biomate 5 spectrophotometer (Thermo Spectronic, USA).

PCR primer : Species-specific PCR primers for *F. succinogenes*, *R. flavefaciens* and *R. albus* were selected from the literature (Koike and Kobayashi, 2001). Primers for *F. succinogenes*, *R. flavefaciens* and *R. albus* were: Fs219f (5'-GGT ATG GGA TGA GCT TGC-3') and Fs654r (5'-GCC TGC CCC TGA ACT ATC -3'); Rf154f (5'- TCT GGA AAC GGA TGG TA-3') and Rf425r (5'-CCT TTA AGA CAG GAG TTT ACA A-3'); Ra1281f (5'-CCC TAA AAG CAG TCT TAG TTC G-3') and Ra1439r (5'-CCT CCT TGC GGT TAG AAC A-3'), respectively. Amplification sizes from PCR reactions for the three bacterial species were 446, 259 and 175 bp and annealing

temperatures were 62, 55 and 55°C, respectively.

Real-time PCR : Bacterial DNA was amplified and quantified with an iCycler iQ real-time PCR system (Bio-Rad Inc. USA). The iQ Syber Green Supermix (Bio-Rad INC. USA) was used for PCR amplification according to the manufacturer's protocol. PCR conditions were: one cycle of initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 30 s, followed by annealing at each temperature of strains for 30 s and then an extension at 72°C for 30 s. Thereafter, the melting point of PCR product was analyzed to detect specificity of application. The melting curve was obtained by a 0.1°C/s increase of heating temperature from 65 to 95°C with fluorescence detection at 0.1°C intervals.

Bacterial population was defined as log copy number of 16S rDNA which was calculated from a standard curve of control plasmid. The control plasmid had an insert of specific fragment of 16S rDNA amplified with primers specific to each species (*F. succinogenes*, *R. flavefaciens* and *R. albus*). The control plasmid was constructed by using the pGEM-T and pGEM-T Easy Vector System (Promega, USA) according to the manual procedure. The standard curves were respectively made by plotting C_t values for serial dilutions of the each control plasmid for each species.

Determination of DM digestibility and fermentation parameters

The DM digestibility was calculated by the difference between dry matter before and after incubation. The change of pH was measured using a Mettler Delta 340 pH meter (Mettler Electronics, UK). The accumulated head gas pressure was measured using a pressure transducer and recorded using a digital readout voltmeter (Laurel Electronics, USA). For VFA determination, 1 ml of culture supernatant was treated with 200 μl of meta-phosphoric acid for 30 min at room temperature and stored at -20°C until individual VFA were analyzed using a HP 6890 gas chromatograph (Hewlett Packard, USA) equipped with a flame inonization detector. The VFA were separated with a 30 m×0.32 mm×0.25 μm sized HP-FFAP capillary column using nitrogen as a carrier gas.

Statistical analysis

All experimental cultures were done in triplicate. The data were analyzed according to a complete randomized design with a one-way analysis of variance of pH treatment. The statistical model was: $Y_{ij} = \mu_i + T_{ij}$, where i was the number of treatments and j was the number of replication tubes. When the overall treatment effect was significant ($p<0.05$), the differences between treatment means were tested with the LSD test using the SAS program (SAS, 1985).

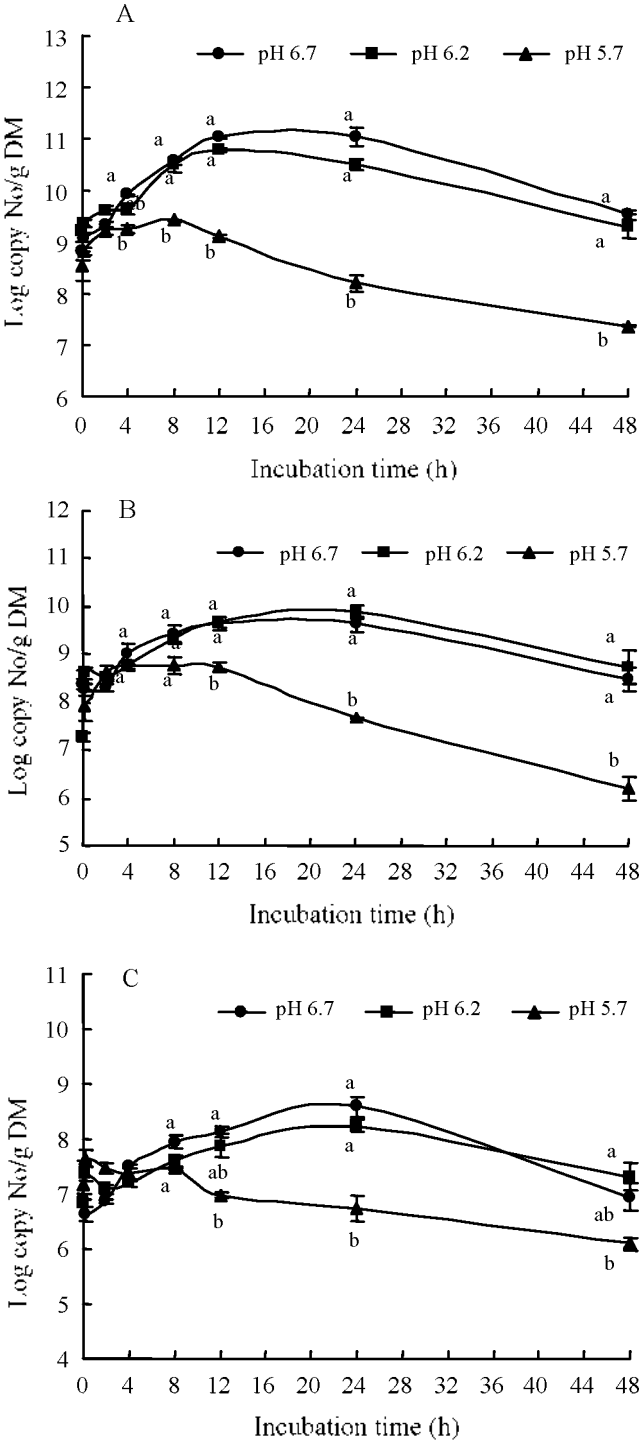

Figure 1. The attachments of *F. succinogenes* (A), *R. flavefaciens* (B) and *R. albus* (C) on rice straw as influenced by different pH during *in vitro* rumen fermentation ($^{a, b, c}$ Means with different letters differ at $p<0.05$).

RESULTS

Bacterial attachment

Active attachment of three major fibrolytic bacteria to rice straw took place during early incubation (Figure 1).

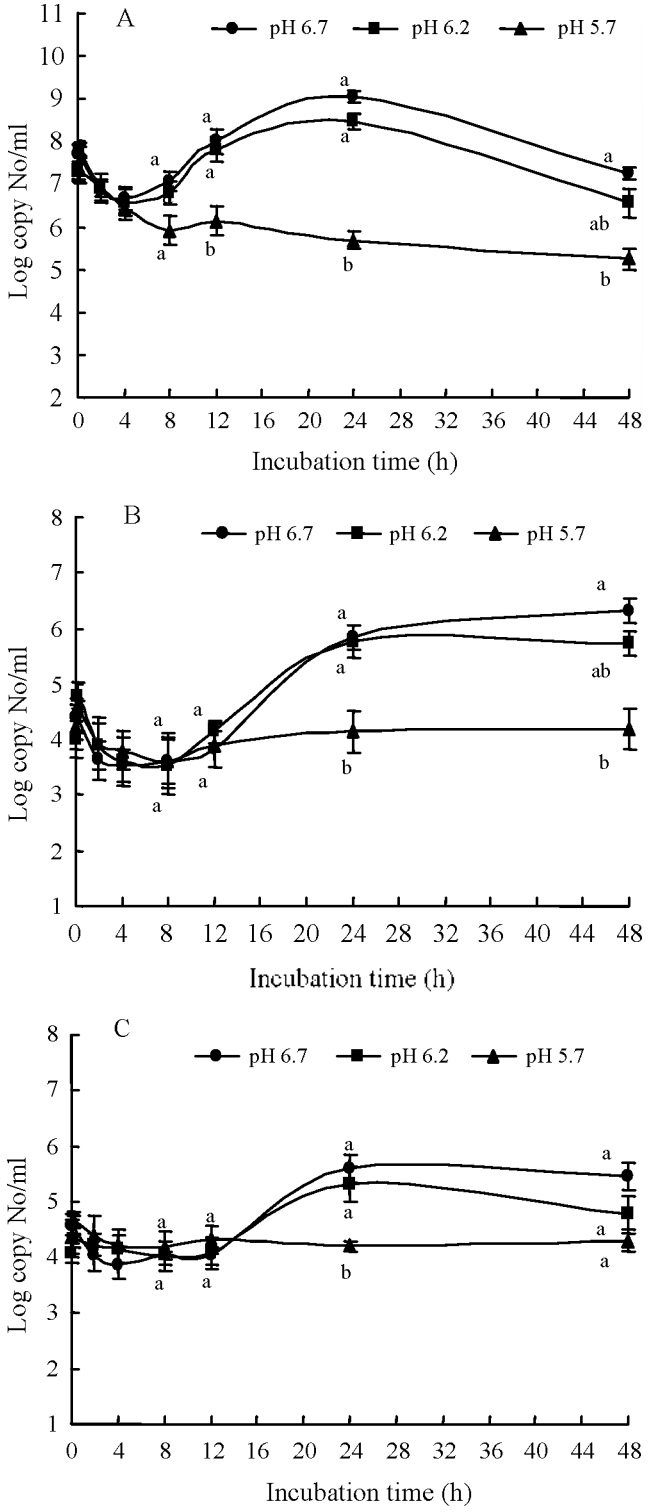

Figure 2. The unattached population of *F. succinogenes* (A), *R. flavefaciens* (B) and *R. albus* (C) as influenced by different pH during the *in vitro* rumen fermentation ([a, b, c] Means with different letters differ at p<0.05).

When it was computed from attached (Figure 1) and unattached bacterial population (Figure 2), more 70% of total bacteria were attached to rice straw within 2 hr in all three species regardless of initial pH.

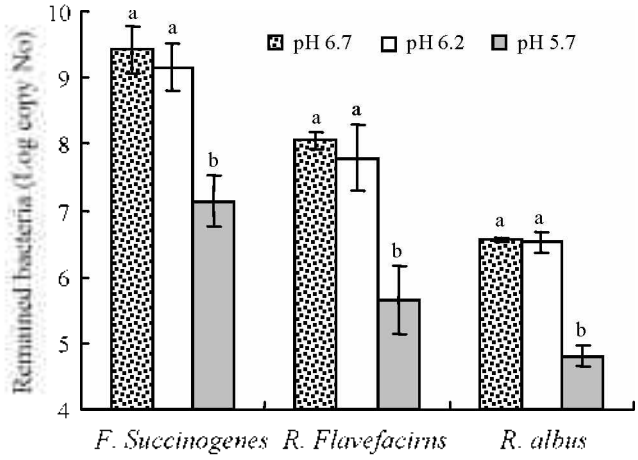

Figure 3. Effects of different pH in washing solution on the detachment of bacteria colonized on filter paper ([a, b, c] Means with different letters differ at p<0.05).

The attachment of *F. succinogenes* to rice straw at low (5.7) initial pH was significantly (p<0.05) less than at middle (6.2) and high (6.7) initial pH (Figure 1A). However, no significant differences in bacterial attachment were detected between high and middle initial pH. The attached population at pH 5.7 was highest at 8 hr and then decreased, while that at higher pH remained increased until 12 to 24 h. Low initial pH reduced (p<0.05) attachment of *R. flavefaciens* compared to middle and high initial pH (Figure 1B); peak attachment was reached at 12, 24 and 4 h incubation in high, middle and low pH conditions, respectively. In common with the other two bacterial species, the attachment of *R. albus* was also pH dependent with significantly (p<0.05) lower attachment being achieved at low initial pH than with either the middle or high initial pH; there was no significant difference between the two higher pH conditions (Figure 1C).

Unattached bacteria

There was a tendency for a reduced number of unattached *F. succinogenes* (those in incubation medium) during the early stage of incubation (4 h) which then reached a peak at 24 h incubation in cultures at pH 6.7 and 6.2 (Figure 2A). However, the number of *F. succinogenes* in the pH 5.7 of culture decreased up to 8 hrs of incubation, and thereafter the numbers remained similar during the incubation. The number of unattached *F. succinogenes* was higher at high and middle initial pH compared to low pH, as was the case with attached *F. succinogenes*. A similar trend was observed in unattached *R. flavefaciens* (Figure 2B) and *R. albus* (Figure 2C) with numbers at the two higher initial pH (6.2 and 6.7) being much larger compared to low (5.7) initial pH, although the time when effects of pH became significant was different among the three bacterial species.

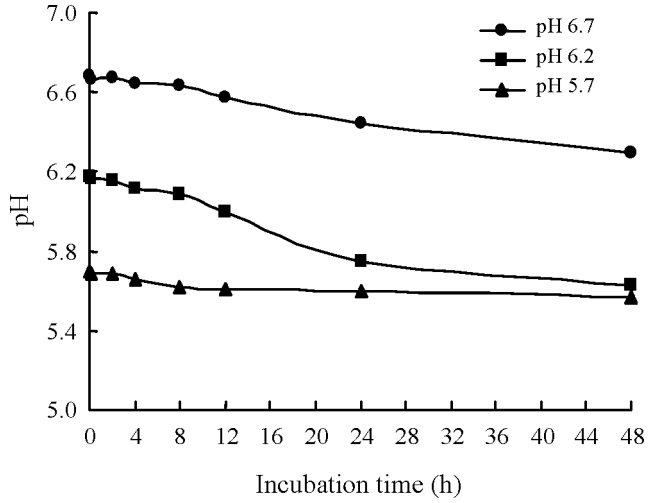

Figure 4. The pH of incubation medium as influenced by initial pH.

Bacterial detachment

Filter paper was incubated for 12 h and washed with solutions having three different pH (6.7, 6.2 and 5.7) to see if pH of washing solution could influence bacterial detachment. The number of remained *F. succinogenes* on filter paper after washing with pH 5.7 buffer solution was significantly ($p<0.05$) lower than with pH 6.7 and 6.2 (Figure 3). A similar result was obtained in the degree of detachment of *R. falvefacines* and *R. albus*.

Fermentation parameters and dry matter digestibility

The pattern of media pH change during incubation at three different initial pHs is presented in Figure 4. The pH remained above 6.0 during 48 h incubation when initial pH was 6.7. However, the pH decreased below 6.0 after 24 h incubation when initial pH was 6.2. Gas production (Figure 5A) was dependent on initial pH, and was significantly higher ($p<0.05$) at higher initial pH. Total gas production after 48 h incubation at middle and high initial pH was 2 and 3 times, respectively, higher than that at low initial pH. Incubation of rice straw at higher initial pH also resulted in higher total VFA production (Figure 5B), with a pronounced ($p<0.05$) effect apparent after 8 hr incubation. The average increases in total VFA production at the two higher initial pHs were, respectively, 1.2 and 1.6 times that at low initial pH. Figure 6 shows the digestibility of rice straw DM as influenced by different initial pH: a distinctive trend for higher DM digestibility was observed at higher initial pH. Raising initial pH from 5.7 to 6.2 and 6.7 improved DM digestibility by 2.8 and 4.4 times, respectively. The lag time (to reach above 2% DM digestibility) for rice straw DM digestion at high and middle initial pH was 4 h and that at low initial pH was 8 h.

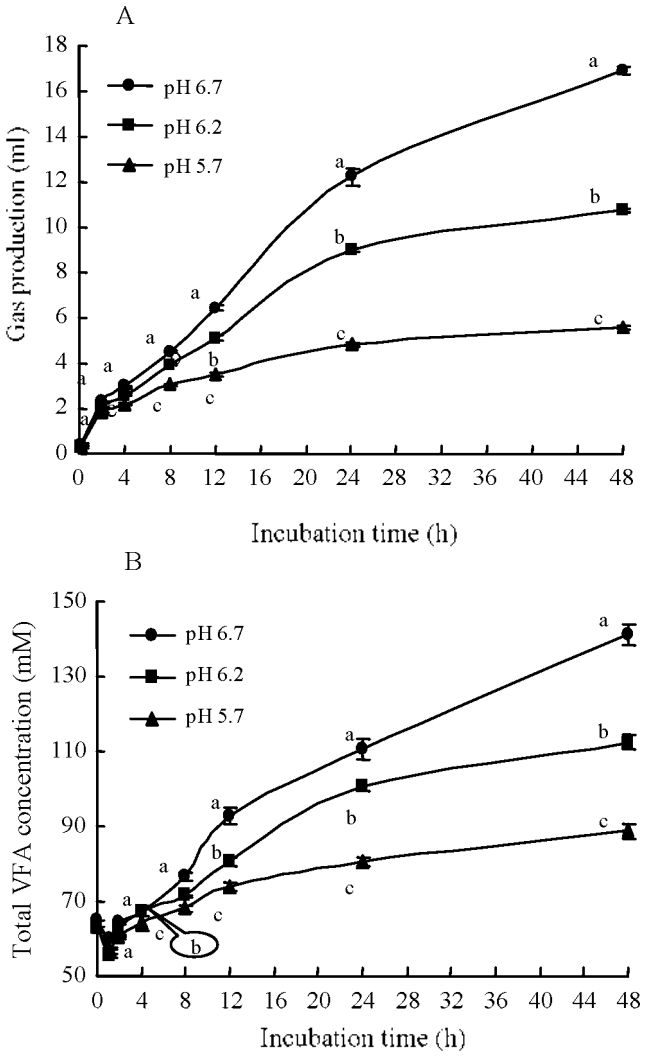

Figure 5. Gas production (A) and total VFA concentration (B) as affected by different initial pH ($^{a, b, c}$ Means with different letters differ at $p<0.05$).

DISCUSSION

Results obtained in the present study clearly indicate that initial ruminal pH is one of the important factors influencing attachment of major fibrolytic bacteria to fiber substrates thereby affecting their digestion. The influence of initial pH was especially pronounced when initial pH was lower than 6.0. The importance of ruminal pH on microbial attachment has been reported in previous studies, the results of which, however, seem equivocal. For instance some studies reported that bacterial attachment was not changed in the pH range 5.3 to 6.0 in cultures of *F. succinogens* (Gong and Forsberg, 1989), was stable in the pH range 3.3 to 7.5 in cultures of *R. flavefaciens* (Roger et al., 1990), or bacterial attachment to cellulose was increased when pH was increased from 5.5 to 6.0 (Mourino et al., 2001). In addition, the attachment of *F. succinogenes* to cellulose was

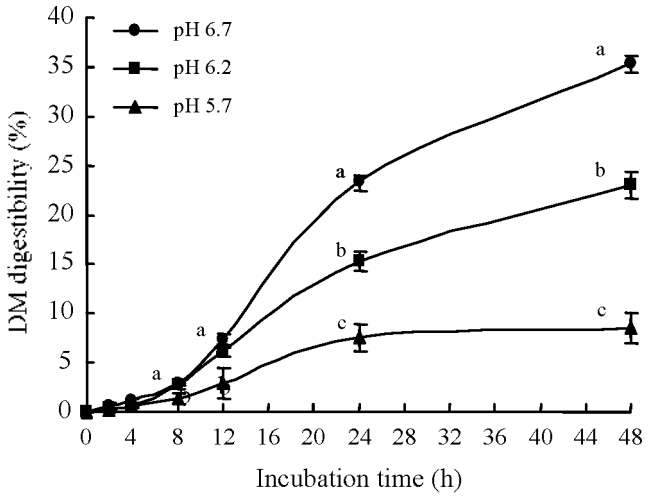

Figure 6. Rice straw digestibility as influenced by different initial pH ($^{a, b, c}$ Means with different letters differ at $p<0.05$).

increased when pH was increased from 4.5 to 6.0 (Roger et al., 1990) and the attachment of *R. albus* was remarkably decreased in the pH range from 5.0 to 5.5. The discrepancy among studies may arise from differences in culture condition, type of substrates and bacterial quantification method.

The exact mechanism involved in reduced attachment at low initial pH is not evident. However, based on the present study, reduced bacterial growth and accelerated detachment at low initial pH may have contributed to lower attachment. As shown in Figure 2, incubating rice straw at pH 5.7 resulted in the lowest concentration of three fibrolytic bacteria in the medium, indicating inhibited bacterial growth at low initial pH. A similar result has been reported by Russell and Wilson (1996), who observed that predominant species of ruminal cellulolytic bacteria did not grow at low pH. In the study of Slyter (1986) the cellulolytic capability of the continuous culture system was low when the pH condition was maintained below 6.0 due to diminished number of cellulolytic bacteria. Bhat et al. (1990) also reported that maximum attachment of both *F. succinogenes* and *R. flavefaciens* to barley straw was achieved at pH 6.0. The decline of cellulose degradation efficiency at lower pH was attributed to the inhibition of cellulolytic bacteria, since most ruminal cellulolytic bacteria are pH-sensitive (Russell and Wilson, 1996). The pH sensitivity can be explained by intracellular pH regulation of cellulolytic bacteria. When the extracellular pH of acid-sensitive bacteria declines, the intracellular pH is relatively stable, but the increase in the transmembrane pH gradient cause a logarithmic accumulation of intracellular fermentation acid anions and hence leads to anion toxicity and product inhibition (Russell and Wilson, 1996; Weimer, 1996).

Another possible mechanism for reduced attachment at low initial pH is detachment of bacteria from the surface of substrate. As indicated in Figure 3, when colonized cells on filter paper were washed with different pH solutions, the cells remaining on filter paper were decreased with lower pH of the washing solution, which is indicative of increased bacterial detachment at low environmental pH. There was almost a 100 fold difference in the remaining bacteria on filter paper between the low and two higher initial pH conditions, regardless of bacterial species. Binding to ligand using cellulose binding protein is one of the bacterial attachment mechanisms (Pell and Schofield, 1993), and the ligand binding might have been hampered by low pH and caused bacterial detachment in this experiment.

High initial pH led to an increase in DM digestibility, gas production and total VFA production in this experiment (Figures 5 and 6), which was probably the result of an increase in attached bacterial population caused by higher initial pH. This study clearly shows that attachment of fibrolytic bacteria is a pivotal process in the substrata surface phenomenon for fiber digestion and that mechanisms of both attachment and digestion are apparently influenced by pH as an environmental factor in the ruminal microbial ecosystem. The role of bacterial populations attached to fibrous substrata has been addressed previously in microscopic observations (Cheng et al., 1980; Cheng et al., 1984; Bae et al., 1997). Koike et al. (2003) quantified cell numbers of *F. succinogenes*, *R. flavefaciens* and *R. albus* attached to stem by competitive PCR and showed that numbers of all three species increased gradually with increased NDF disappearance. Additional support for the relationship between attachment and subsequent fiber degradation was given by studies in which wild isolates of *R. albus* strains degraded better than mutant strains lacking attachment ability (Morris and Cole, 1987) and a normal type of *F. instinalis* DR7 had higher total activities of cellulose-degrading enzymes than the mutant (Miron and Forsberg, 1998).

Bacteria associated with substrata have been mentioned in previous reports (McAllister et al., 1994; Miron et al., 2001). The populations associated with feed particles are numerically predominant and occupy from 70 to 80% of the total microbial population in the rumen (Craig et al., 1987; Forsberg and Lam, 1977). These bacteria rapidly associated with and attached to recently ingested feed particles within 5 min (Bonhomn, 1990), and then primary colonizers formed a rich biofilm on the substrata surface together with additional colonizers. They were responsible for 88 to 91% of ruminal endoglucanase and xylanase activity (Minato et al., 1993). Similarly, in our study the attached population of *F. succinogenes*, *R. flavefaciens* and *R. albus* reached >70% of total number in each strains shortly after inoculation (in 2 h) at all three initial pH and the highest digestion rate of rice straw was achieved thereafter. Roger

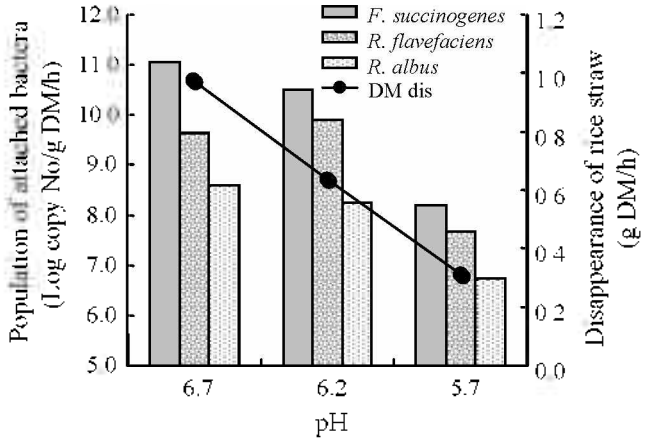

Figure 7. The relationship between initial pH, DM digestibility and bacterial attachment to rice straw after 24 h incubation.

et al. (1990) found that *R. flavefacines* attached to cellulose within 1 min of first contact and active attachment of *F. succinogenes* took place within 30 min in the *in vitro* study. Attachment of *ruminococci* species to damaged plant occurred within 1 to 5 min after their inoculation (Latham et al., 1978).

It is commonly observed that low pH<6.0 severely inhibits fiber digestibility in the rumen (Weimer, 1996; Russell and Rychlik, 2001), and this inhibition is strong when low pH is maintained for extended time of periods. The present study also showed that digestion of rice straw was strongly inhibited by low pH with a longer lag time for active digestion (Figure 6). Only very weak fermentation occurred at pH 5.7 and subsequently little gas or VFA was produced (Figures 3 and 4). In similar studies, Grant and Weidner (1992) reported that the lag in NDF digestion increased as pH fell from 6.8 to 5.5 and decreasing pH of buffer to <6.0 dramatically decreased NDF digestion rate for alfalfa hay and corn silage. Hu et al. (2005) showed that cellulose degradation did not occur at pH<5.5, whereas at pH>6.0 there was increased cellulose degradation. Also, in the same study, no VFA or reducing sugar were produced at pH<5.5 and only a small amount of gas was produced at pH 4.5-5.5.

The present study clearly showed that a strong relationship exists between the initial pH, the rate of major ruminal bacterial attachment and the rate of fiber digestion, as shown in Figure 7. It is apparent that the rate of fiber digestion was pH dependent, possibly modulated via modified bacterial attachment to fiber substrates. Maintaining pH>6.0 is critical for efficiency of microbial attachment and fiber digestion in the rumen.

ACKNOWLEDGEMENT

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REFERENCES

Akin, D. E. and F. E. Barton. 1983. Rumen microbial attachment and degradation of plant cell walls. Fed. Proc. 42:114-121.

Bae, H. D., T. A. McAllister, E. G. Kokko, F. L. Leggett, L. J. Yamke, K. D. Jakober, J. K. Ha, H. T. Shin and K. J. Cheng. 1997. Effect of silica on the colonization of rice straw by ruminal bacteria. Anim. Feed Sci. Technol. 65:165-181.

Bhat, S., R. J. Wallace and E. R. Orskov. 1990. Adhesion of cellulolytic ruminal bacteria to barley straw. Appl. Environ. Microbiol. 56:2698-2703.

Bonhomme, A. 1990. Rumen ciliates: Their metabolism and relationships with bacteria and their hosts. Anim. Feed Sci. Technol. 30:203-266.

Cheng, K. -J., J. P. Fay, R. E. Howarth and J. W. Costerton. 1980. Sequence of events in the digestion of fresh legume leaves by rumen bacteria. Appl. Environ. Microbiol. 40:613-625.

Cheng, K. -J., C. S. Stewart, D. Dinsdale and J. W. Costerton. 1984. Electron microscopy of bacteria involved in the digestion of plant cell walls. Anim. Feed Sci. Technol. 10:93-120.

Craig, W. M., G. A. Broderick and D. B. Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. J. Nutr. 117:56-64.

Forsberg, C. W. and R. Lam. 1977. Use of adenosine-5-triphosphate as an indicator of the microbial biomass in rumen contents. Appl. Environ. Microbiol. 33:528-534.

Gong, J. and C. W. Forsberg. 1989. Factors affecting adhesion of *Fibrobacter succinogenes* S85 and adherence defective mutants to cellulose. Appl. Environ. Microbiol. 55:3039-3044.

Grant, R. J. and S. J. Weidner. 1992. Digestion kinetics of fiber: Influence of *in vitro* buffer pH varied within observed physiological range. J. Dairy Sci. 75:1060-1068.

Hu, Z. -H., H. -Q. Tu and R. -F. Zhu. 2005. Influence of particle size and pH on anaerobic degradation of cellulose by rumen microbes. Int. Biodeter. Biodegr. 55:233-238.

Khampa, S., M. Wanapat, C. Wachirapakorn, N. Nontasol, M. A. Wattiaux and P. Rowlison. 2006. Effect of levels of sodium DL-malate supplementation on ruminal fermentation efficiency of concentrates containing high levels of cassava chip in dairy steers. Asian-Aust. J. Anim. Sci. 19:368-375.

Kobayashi, Y., S. Koike, H. Taguchi, H. Itabashi, Dong K. Kam and J. K. Ha. 2004. Recent advances in gut microbiology and their possible contribution to animal health and production-Review. Asian-Aust. J. Anim. Sci. 17:877-884.

Koike, S. and Y. Kobayshi. 2001. Development and use of competitive PCR asays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. FEM Microbiol. Letters. 204:361-366.

Koike, S., J. Pan, Y. Kobayashi and K. Tanaka. 2003. Kinetics of *in sacco* fiber-attachment of representative ruminal cellulolytic

bacteria monitored by competitive PCR. J. Dairy Sci. 86:1429-1435.

Latham, M. J., B. E. Brooker, G. L. Petipher and P. J. Harris. 1978. *Ruminoccocus flavefaciens* cell coat and adhesion to cotton cellulose and cell leaves of perennial ryegrass. Appl. Environ. Microbiol. 35:156-165.

McAllister, T. A., H. D. Bae, G. A. Jines and K. -J. Cheng. 1994. Microbial attachment and feed digestion in the rumen. J. Anim. Sci. 72:3004-30018.

McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J. 43:99-109.

Minato, H., M. Mitsumori and K. J. Cheng. 1993. Attachment of microorganisms to solid substrates in the rumen. pp. 139-145 in Proc. Mie bioforum on Genetics, Biochemistry and Ecology of Lignocellulose Degradation. Uni Publisher, Tokyo..

Miron, J. and C. W. Forsberg. 1998. Features of *Fibrobacter intestinalis* DR7 mutant which is impaired with its ability to adhere to cellulose. Anaerobe 4:35-43.

Miron, J., D. Ben-Ghedalia and M. Morrison. 2001. Invited Review: Adhesion mechanisms of rumen cellulolytic bacteria. J. Dairy Sci. 84:1294-1309.

Morris, E. J. 1988. Charateristics of the adhesion of *Ruminococcus albus* to cellulose. FEMS Microbiol. Letters. 51:113-117.

Morris, E. J. and O. J. Cole. 1987. Relationships between cellulolytic activity and adhesion to cellulose in *Ruminococcus albus*. J. Gen. Microbiol. 133:1023-1032.

Mould, F. L. and E. R. Orskov. 1983. Manipulation of rumen fluid pH and its influence on cellulolysis *in sacco*, dry matter degradation and the rumen microflora of sheep offered either hay or concentrate. Anim. Feed Sci. Technol. 10:1-14.

Mould, F. L., E. R. Orskov and S. O. Mann 1984. Associative effects of mixed feeds. I Effects of type and level of supplementation and the influence of the rumen pH on cellulolysis *in vivo* and dry matter degradation of various roughages. Anim. Feed Sci. Technol. 10:15-20.

Mourino, F., R. Akkarawongsa and P. J. Weimer. 2001. Initial pH as a determinant of cellulose digestion rate by mixed ruminal microorganisms *in vitro*. J. Dairy Sci. 84:848-859.

Pan, J., S. Koike, T. Suzuki, K. Ueda, Y. Kobayashi, K. Tanaka and Okubo. 2003. Effect of mastication on degradation of orchardgrass hay stem by rumen microbes: fibrolytic enzyme activities and microbial attachment. Anim. Feed Sci. Technol. 106:69-79.

Pell, A. N. and P. Schofield. 1993. Microbial adhesion and degradation of plant cell walls. pp. 397-423 in Forage Cell Wall Structure and Digestibility. ASA-CSSA-SSSA, Madison, WI.

Purdy, K. J., T. M. Embley, S. Takii and D. B. Nedwell. 1996. Rapid extraction of DNA and RNA from sediments by novel hydroxyapatite spin-colum method. Appl. Environ. Microbial. 62:3905-3970.

Roger, V. R., G. Fonty, S. Komisarczuk-Bondy and P. Gouet. 1990. Effects of physicochemical factors on the adhesion to cellulose avicel of the rumen bacteria *Ruminococcus flavefaciens* and *Fibrobactor succinogenes* subsp. *succinogenes*. Appl. Environ. Microbiol. 56:3081-3087.

Russell, J. B. and D. B. Wilson. 1996. Why are cellulolytic bacteria unable to digest at low pH? J. Dairy Sci. 79:1503-1509.

Russell, J. B. and J. L. Rychlik. 2001. Factors that alter rumen microbial ecology. Sci. 292:1119-1122.

SAS (Statistical Analysis System Institute). 1989. SAS/STATTM User's Guide: Statistics, Version 6, 4th Edition. Vol. 2, Cary, NC.

Slyter, L. L. 1986. The ability of pH-selected mixed ruminal microbial population to digest fiber at various pHs. Appl. Environ. Microbiol. 52:390-391.

Srinivas, B. and U. Krishnamoorthy. 2005. Influence of diet induced changes in rumen microbial characteristics on gas production kinetics of straw substrates *in vitro*. Asian-Aust. J. Anim. Sci. 18:990-996.

Stewart, C. S., S. H. Duncan and H. J. Flint. 1990. The properties of forms of *Ruminococcus flavefaciens* which differ in their ability to degrade cotton cellulose. FEMS Microbiol. Lett. 72:47-50.

Sung, H. G., D. M. Min, D. K. Kim, D. Y. Li, H. J. Kim, S. D. Upadhaya and J. K. Ha. 2006. Influence of transgenic corn on the in vitro rumen microbial fermentation. Asian-Aust. J. Anim. Sci. 19:1761-1768.

Tajima, K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Environ. Microbiol. 67:2766-2774.

Weimer, P. J. 1996. Why don't ruminal bacterial digest cellulose faster? J. Dairy Sci. 79:1496-1502.