# Effects of Methylcellulose on Fibrolytic Bacterial Detachment and *In vitro* Degradation of Rice Straw

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**ABSTRACT:** Two *in vitro* experiments were conducted to evaluate the effect of methylcellulose (MC) on i) bacterial detachment from rice straw as well as ii) inhibition of bacterial attachment and fiber digestibility. To evaluate the effect of MC on fibrolytic bacterial detachment (Exp 1), *in vitro* bacterial cultures with 0.1% (w/v) MC solution were compared with cultures without MC after 8 h incubation. The effect of MC on inhibition of bacterial attachment was determined by comparing with real-time PCR the populations of *F. succinogenes*, *R. flavefaciens* and *R. albus* established on rice straw pre-treated with 0.1% MC with those on untreated straw after incubation for 0, 6 and 12 h (Exp 2). The major fibrolytic bacterial attachment on rice straw showed significantly lower populations with either the addition of MC to the culture or pre-treated rice straw compared to controls ($p<0.05$). Also, the digestibility of rice straw with MC was significantly lower compared with control ($p<0.05$). The *F. succinogenes* population did not show detachment from rice straw, but showed an inhibition of attachment and proliferation on rice straw in accordance with a decrease of fiber digestion. The detachments of *Ruminococcus* species co-existed preventing the proliferations with subsequent reduction of fiber degradation by MC during the incubation. Their detachments were induced from stable colonization as well as the initial adhesion on rice straw by MC in *in vitro* ruminal fermentation. Furthermore, the detachment of *R. albus* was more sensitive to MC than was *R. flavefaciens*. These results showed the certain evidence that attachment of major fibrolytic bacteria had an effect on fiber digestion in the rumen, and each of fibrolytic bacteria, *F. succinogenes*, *R. flavefaciens* and *R. albus* had a specific mechanism of attachment and detachment to fiber. (**Key Words:** Methylcellulose, Bacterial Detachment, Fiber Digestion, *F. succinogenes*, *R. flavefaciens*, *R. albus*)

## INTRODUCTION

Based on their inhabiting environments, rumen bacteria are categorized as i) free-living bacteria in rumen liquid, ii) bacteria loosely attached to feed particles, iii) bacteria firmly adhered to feed particles, iv) bacteria associated with rumen epithelium, and v) bacteria attached to protozoa and fungal sporangia (Cheng and Costerton, 1980; Czerkawski and Cheng, 1988; McAllister et al., 1994). Microbes in rumen liquid (20 to 30% of total microbes) including free-living bacteria and bacteria detached from solid substrate, have little direct involvement in insoluble feed particles

digestion (Miron et al., 2001). Microbes lived by attaching to feed particles (compartment 2 and 3) are numerically predominant (70 to 80% of microbial matter and microbial ATP production) and play a key role in feed particle digestion in the rumen (Craig et al., 1987; Miron et al., 2001; Trabalz Marinucci et al., 2006). Fibrolytic microbes such as *F. succinogenes*, *R. flavefaciens*, and *R. albus* are responsible for fibrin degradation as the primary degraders of forage cell walls (Windham and Akin, 1984; McAllister et al., 1994; Sung et al., 2007; Wang et al., 2012). Their attachments on plant tissues for fiber digestion were closely related with enzyme activity (Brock et al., 1982; Williams and Strachan, 1984; Michalet-Doreau et al., 2001; Hwang et al., 2008) and mutant cells lacking the adhesion ability were not effective in digesting fiber (Miron et al., 1998; Reddy and Morrison, 1998; Miron and Forsberg, 1999). Sodium-carboxymethylcellulose (CMC) and methylcellulose (MC) did strongly inhibit both the adhesion of the major rumen fibrolytic species to fibrous feed particle (Kudo et al., 1987; Rasmussen et al., 1989; Roger et al., 1990), and cellulose

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degradation (McAllister et al., 1994; Miron et al., 2001). In the previous studies, MC was used for bacterial detachment in the rumen (Kudo et al., 1987; Ranila and Carro, 2003; Martinez et al., 2009), the objective of this study was to evaluate the effect of MC on fibrolytic bacterial detachment from particles and/or attachment and rice straw digestion in the rumen.

## MATERIALS AND METHODS

### *In vitro* experiments and substrates

Three cannulated Holstein steers (740±10 kg body weight) were used as donors of rumen fluid for *in vitro* experiments. Timothy and concentrate in the ratio of 60 to 40 were fed at 2% of body weight twice a day (09:00 and 17:00). The rumen contents were collected from the fistulated steers after 1 h of morning feeding. Rumen fluid containing the ingesta was homogenized with a mixer (Mini mixer, Hanil, Korea) under $O_2$-free $CO_2$ gas, and then filtered through 8 layers of cheesecloth. Rice straw was ground through a 2 mm screen and dried after washing with hot distilled water to removing dust particles.

### Incubation

Sixty 60 mL of rumen fluid-buffer mixture, comprising McDougall buffer and rumen liquor in the ratio of 2 to 1, was dispensed anaerobically into 120 mL of serum bottles, filled with $O_2$-free $CO_2$ gas, containing 0.5 g of substrate, and then capped with a rubber stopper. The serum bottles were held in a shaking incubator at 39°C.

### Experimental design

*Exp. 1*: Experiment 1 was conducted to evaluate the effect of Methylcellulose (MC) addition on bacterial detachment and fiber digestibility during rumen fermentation. The rumen culture was incubated with rice straw as the substrate for 8 h. MC (0.1% w/v) was added to one half of the rumen cultures after 8 h incubation and all cultures were incubated for a total of 48 h. Methylcellulose solution was prepared by dissolving methylcellulose powder (Sigma Mo262) in boiling water.

*Exp. 2*: Experiment 2 was conducted to evaluate the effect of methylcellulose pre-treatment on bacterial attachment and fiber degradation. The rumen culture was prepared without pre-treated substrate for control and with MC pretreatment for the test cultures. Pre-treated substrates were prepared by soaking the ground rice straw in 0.1% of methylcellulose solution for 16 h and dried at 65°C for 72 h. The triplicate cultures were sampled to analyze bacterial attachment and rice straw digestibility after 0, 6, and 12 h of incubation.

## Quantification of fibrolytic rumen bacteria with quantitative real-time PCR

*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 analysis of bacterial population.

*DNA extraction*: gDNA was extracted according to the method described by Purdy et al. (1996). 0.5 g of dried rice straw 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 beads (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. gDNA 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 gDNA were checked using a Biomate 5 spectrophotometer (Thermo Spectronic, USA).

*PCR primer*: Species-specific PCR primer sets for *F. succinogenes*, *R. flavefaciens* and *R. albus* were selected from a previous study (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 the 3 bacterial species were 446, 259 and 175 bp and annealing temperatures were 62, 55 and 55°C, respectively.

*Real-time PCR*: gDNA was amplified and quantified with real-time PCR (RT-PCR)(Bio-Rad Inc. USA). The iQ Syber Green Supermix (Bio-Rad INC. USA) was used for RT-PCR amplification according to the manufacturer's protocol. RT-PCR conditions were: one cycle of initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C or 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 RT-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.

The bacterial population was defined as the log copy number of 16S rDNA which was calculated from a standard curve of control plasmid. The control plasmid had an insert of a 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 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 control plasmid for each species.

### Analysis of DM digestibility and pH

The DM digestibility was calculated by the difference between dry matter of undigested feed particles before and after incubation. The undigested feed particles were filtered through filter paper (Whatman No. 4) and washed for 3 times by distilled water, and then dried at 65°C for 72 h. The pH was measured using a pH meter (Mettler Delta 340, Mettler Electronics, UK) before collection of undigested feed particles for detecting DM digestibility.

### Statistical analysis

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, 1996).
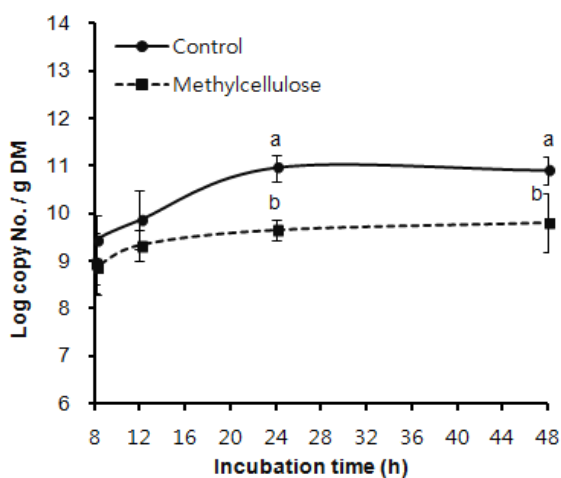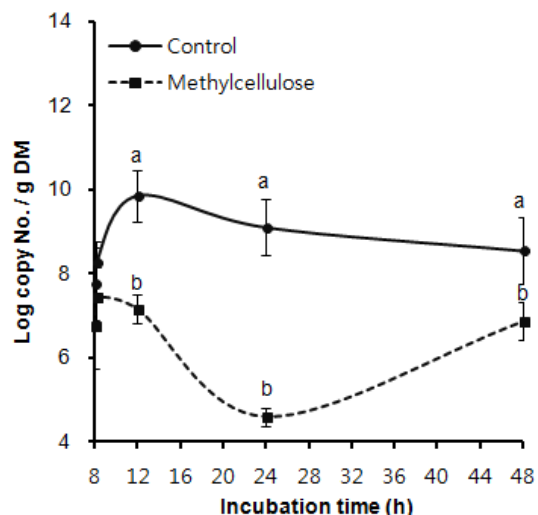


**Figure 2.** Detachment of *Ruminococcus albus* on rice straw as influenced by MC addition after 8 h of incubation. [a,b] Means with different letters differ significantly ($p<0.05$).

## RESULTS

### Effect of methycellulose addition on bacterial detachment and fiber digestibility during the *in vitro* rumen fermentation

*Bacterial detachments from fiber particles by methylcellulose addition*: The populations of *F. succinogenes*, *R. flavefaciens*, and *R. albus* attaching to rice straw substrate after addition of 0.1% methylcellulose (MC) solution and 8 h incubation are shown in Figure 1, 2 and 3, respectively. Although no variation was obtained in *F. succinogenes* attachment between the control and treatment 10 min after the addition of methyl cellulose, a significant difference ($p<0.05$) was obtained between the control and treatment 4 h after MC addition (Figure 1).
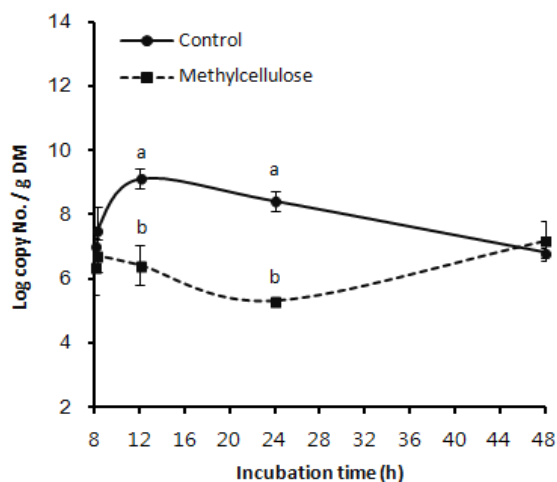


**Figure 1.** Detachment of *Fibrobacter succinogenes* on rice straw as influenced by MC addition after 8 h of incubation. [a,b] Means with different letters differ significantly ($p<0.05$).



**Figure 3.** Detachment of *Ruminococcus flavefaciens* on rice straw as influenced by MC addition after 8 h of incubation. [a,b] Means with different letters differ significantly ($p<0.05$).

The populations of *R. albus* (Figure 2) and *R. flavefaciens* (Figure 3) attachment was also not different between control and treatment 10 min after MC addition. However, the attachment of both strains abruptly dropped after 4 h following MC addition (12 h incubation), reached the minimum level at 24 h incubation and then gradually increased until 48 h incubation. The attachment of *R. flavefaciens* and *R. albus* clearly showed that bacteria detachment from rice straw was due to MC addition. Also, *R. albus* seemed to show a more sensitive response to detachment compared to *R. flavefaciens*.

*Effect of methylcellulose addition on degradation of rice straw in rumen*: The degradation of rice straw and pH in the *in vitro* rumen fermentation was affected by the addition of MC as shown in Figure 4 and 5, respectively. The degradation of rice straw gradually increased with the time of incubation and showed similar trends between control and treatment until 12 h incubation after the MC addition (Figure 4). Fiber degradation following MC addition occurred slowly compared to control after 12 h incubation and was significantly lower than the control ($p<0.05$). This result clearly indicated that MC inhibited the degradation of fiber in rumen. The pH of *in vitro* culture media gradually decreased during 48 h incubation and showed a similar trend in control and treatment. Although the pH was numerically lower in treatment compared with control after 12 h incubation, no significant difference was observed.

**Effect of methylcellulose pre-treatment on bacterial attachment and fiber degradation**

*Bacterial attachments on pre-treated rice straw by methylcellulose*: The attachment of the major species of fibrolytic bacteria on the pre-treated rice straw are presented in Figure 6, 7 and 8. The attachment of *F. succinogenes*,
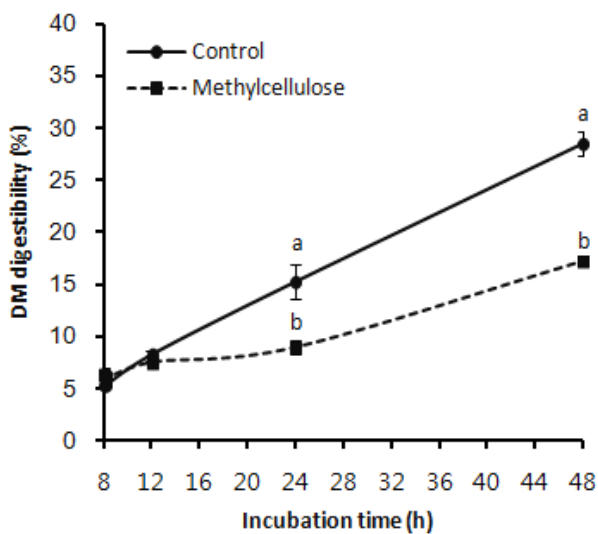


**Figure 5.** Change in pH as influenced by methylcellulose addition after 8 h of incubation.

*R. albus*, and *R. flavefaciens* on the non-treated rice straw gradually increased during the incubation, while the attachment of these species on pre-treated rice straw was clearly inhibited. In the case of *R. albus* (Figure 7) and *R. flavefaciens* (Figure 8), the attached bacterial populations were not different between the control and the pre-treatment at the initial incubation time (0 h). However, their populations in the pre-treatment groups abruptly decreased after 6 h incubation, and showed a significant difference between control and pre-treatment at 6 and 12 h incubation ($p<0.05$). The bacterial populations response to pre-treatment indicated that MC prevented the stable colonization after initial attachment, Similar patterns were also observed in the detachments following the initial attachment in *R. albus* and *R. flavefaciens*.

*Degradation of pre-treated rice straw by methylcellulose addition*: The degradation of MC pre-treated rice straw is presented in Figure 9. The degradation of rice straw gradually increased in the course of incubation



**Figure 4.** The DM digestibility of rice straw as influenced by methylcellulose addition after 8 h of incubation. [a,b] Means with different letters differ significantly ($p<0.05$).
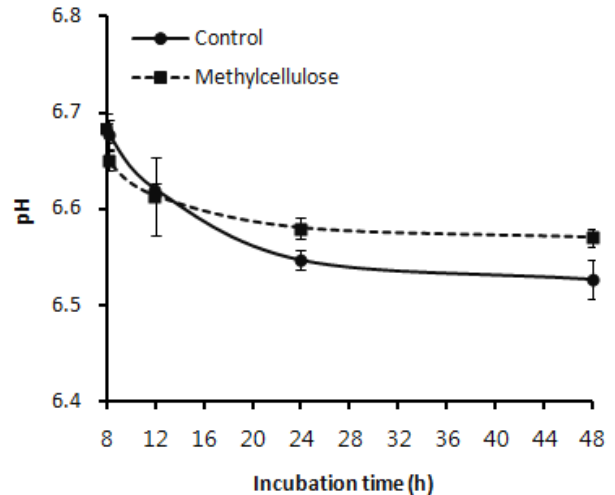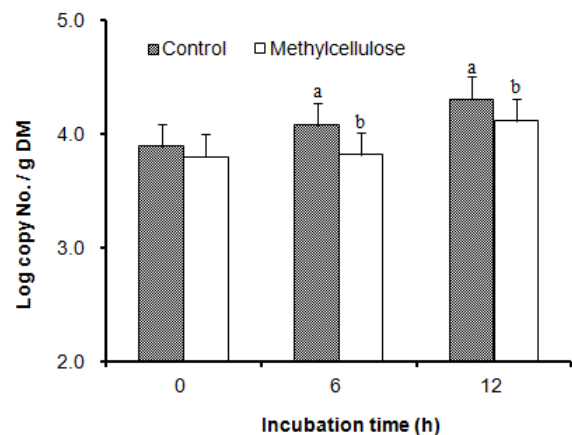


**Figure 6.** Attachment of *Fibrobacter succinogenes* as influenced by rice straw pre-treatment with MC (methylcellulose). [a,b] Means with different letters differ significantly ($p<0.05$).

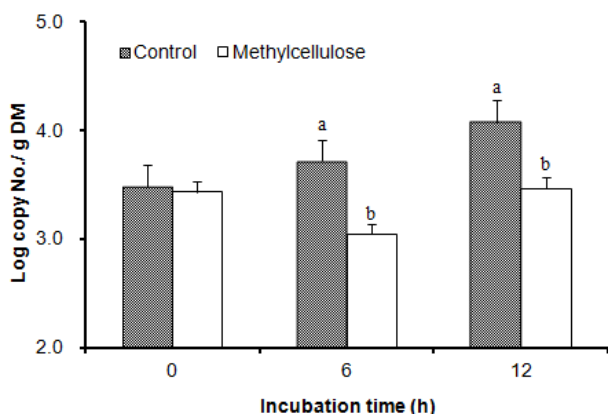**Figure 7.** Attachment of *Ruminococcus albus* as influenced by rice straw pre-treatment with MC (methylcellulose). [a,b] Means with different letters differ significantly (p<0.05).

time in both control and pre-treatment. This result indicated that fiber degradation was prevented by pre-treatment of MC the same as it was by MC addition in middle of incubation in the rumen bacterial fermentation.

## DISCUSSION

Kudo et al. (1987) reported the electron microscopic observations that MC mediated the occurrence of detachments from and the prevention of attachment to cellulose fiber by fibrolytic bacteria in the rumen. Many researchers have been interested in the phenomenon of the fibrolytic processes of microbes in the presence of various effectors including negative factors such as low temperature (Minato and Suto, 1978; Gong and Forsberg, 1989; Minato et al., 1993), low pH (Sung et al., 2007), presence of $O_2$ (Miron et al., 2001), deprivation of $Ca^{+2}$ and $Mg^{+2}$, (Roger et al., 1990) and presence of sodium-carboxymethyl-cellulose (CMC) or methylcellulose (MC) (Rasmussen et al., 1989; Bhat et al., 1990) and Tween 80 (Akin, 1980).
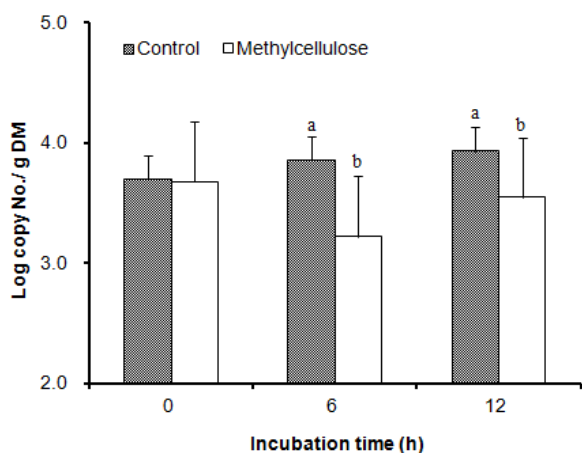
In this study, the addition of MC in the middle of incubation (Figures 1, 2 and 3) and the MC pre-treated fibrous particles (Figures 6, 7 and 8) in *in vitro* ruminal fermentation, clearly showed that major fibrolytic bacteria became detached from the substrate as well as the inhibition of bacteria attachment on fibrous particles during the digestive process. Their detachments and attachment inhibition by the addition of MC were reported in previous studies (Minato and Suto, 1978; Kudo et al., 1987; Bhat at al., 1990; Miron et al., 2001). Also, Cheng et al. (1991) reported that the attachment of fibrolytic rumen fungi was blocked by the addition of MC which did not affect the growth of these organisms with soluble substrates. Since Kudo et al. (1987) mentioned that MC was an effective agent for detaching the major species of rumen fibrolytic bacteria from their cellulosic substrate, other researchers have used MC to evaluate procedures and techniques to detach particle-associated microbes from ruminal digesta (Ranilla and Carro, 2003; Trabalza-Marinucci et al., 2006; Martínez et al., 2009). Bhat et al. (1990) reported that the degree of inhibition by MC was similar for *F. succinogenes* and *R. flavefaciens*, Our result relating to *F. succinogenes* attachment showed a different response trend to inhibition of attachment without the detachment phenomenon (Figures 1 and 4), while *R. albus* (Figures 2 and 7) and *R. flavefaciens* (Figures 3 and 8) underwent detachment with the addition of MC. Furthermore, *R. albus* showed a more sensitive response to detachment after the stable colonization (Figures 2 and 3) as well as in the initial adhesion (Figures 6, 7, and 8) compared with *R. flavefaciens* with the addition of MC. This study showed the importance of attachment in fiber digestion by the addition of MC, which inhibited degradation of rice straw with the co-occurrence of inhibition of fibrolytic bacterial attachment to the fibrous substrate (Figures 4 and 9). Minato and Suto (1978) reported that MC prevents the
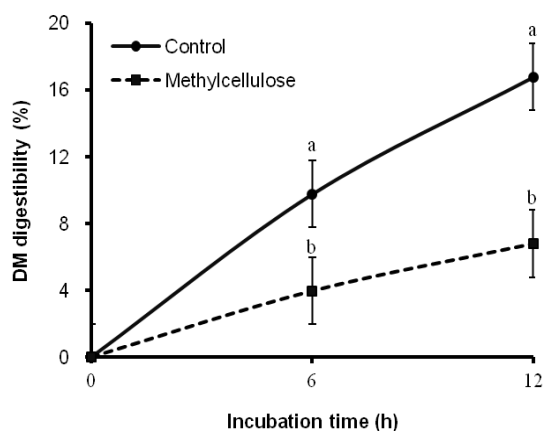


**Figure 8.** Attachment of *Ruminococcus flavefaciens* as influenced by rice straw pre-treatment with MC (methylcellulose). [a,b] Means with different letters differ significantly (p<0.05).



**Figure 9.** DM digestibility of rice straw as influenced by pre-treatment with MC (methylcellulose). [a,b] Means with different letters differ significantly (p<0.05).

attachment of fibrolytic ruminal bacteria to cellulose and inhibits cellulose digestion. Other researchers (Kudo et al., 1987; Pell and Schofield, 1993) demonstrated that MC mediated detachment of fibrolytic rumen bacteria from cellulose without affecting enzyme activity but nevertheless blocked cellulose degradation. Also, Rasmussen et al. (1988) showed that MC strongly inhibited cellulose degradation by several species of fibrolytic bacteria of ruminal origin and specifically, the growth of *R. flavefaciens* was completely inhibited at 0.1% (w/v) of MC. The necessity of adhesion for subsequent fiber digestion by fibrolytic ruminal bacteria was additionally supported by observations that mutant cells, which had lost most of their fibrolytic capability, were smoother in appearance of surface topology compared to wild type cells (Stewart et al., 1990; Miron et al., 1998; Reddy and Morrison, 1998). Furthermore, a low ruminal pH (5.7) was responsible for higher numbers of fibrolytic bacterial detachment from fibrous substrate than the optimum ruminal pH 6.7 (Sung et al., 2007).

In conclusion, the result obtained in this study showed that both addition of MC and the pre-treatment of fibrous particles by MC solution induced detachment after stable colonization as well as the initial adhesion of *F. succinogenes*, *R. flavefaciens*, and *R. albus.*

## ACKNOWLEDGEMENTS

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