

## ***In vitro* Fermentation of Rumen Microorganisms Cultured in Medium Supplemented with Bacterio-mineral Water (BMW) Produced from Bio-reacted Swine Manure\***

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**ABSTRACT :** Bacterio-mineral water (BMW) produced from manure has been known to exert a number of positive effects on animal production and odor control. An experiment was conducted to examine the effects of BMW produced from bio-reacted swine manure on *in vitro* gas production, cellulose degradation, microbial growth and fibrolytic enzyme activities of mixed rumen microorganisms. The five levels of 0, 0.001, 0.005, 0.01 and 1.0% BMW were supplemented into serum vials containing mixed rumen microorganisms. Incubations were carried out anaerobically at 39°C without shaking for 0, 12, 24, 48, 72 and 96 h. There were no significant ( $p > 0.05$ ) differences among the treatments for the initial rate of gas production. At 72 h incubation, the gas production tended ( $p < 0.1$ ) to be increased by the 0.01 and 1.0% BMW treatments compared with control and the 0.001% BMW treatment. At the end of incubation (96 h), the sample supplemented with 0.01% BMW was higher ( $p < 0.05$ ) than control (0% BMW) in the gas production. The microbial growth rate was increased by all the BMW treatments, while 0.01% BMW was most effective in stimulating the growth rate. Although the addition of BMW on the filter paper DM degradation was not significantly influenced throughout the incubation period except the 48 h incubation, DM degradation tended to be increased by all BMW treatments compared with control. The addition of both 0.005 and 0.01% BMW highly increased ( $p < 0.05$ ) CMCase activity compared with control after 24 h and 48 h incubation, while at the 72 h incubation the 0.01% BMW addition only significantly increased ( $p < 0.05$ ). After 72 h incubation, the xylanase activity was significantly ( $p < 0.05$ ) increased with the addition of 1.0% BMW compared with the addition of 0.001 and 0.005% BMW, while at the other incubation times, the xylanase activity was not different among the treatments. In conclusion, the 0.01% BMW of supplementation level would be the suitable addition level to stimulate rumen fermentation increasing microbial growth and cellulose degradation. (*Asian-Aust. J. Anim. Sci.* 2005, Vol 18, No. 10 : 1435-1439)

**Key Words :** Bacterio-mineral Water, Rumen Fermentation, Mixed Rumen Microorganisms

### INTRODUCTION

Ruminant nutritionists and rumen microbiologists have considerably devoted to manipulate the rumen environment with the aim of enhancing feedstuff utilization and improving the efficiency of ruminant production. The results of these efforts led a wide range of feed additives such as antibiotics, methane inhibitors, microbial enzymes, buffer agents, ionophores and probiotics etc (Lee and Ha, 2003). However, there is growing concern to the use of antibiotic growth promoters within the food chain. Furthermore, there is a growing awareness of the health, safety and environment issues associated with animal agriculture. Thus, the use of feed additives containing live microorganisms and/ or their metabolites to alter rumen fermentation and improve animal performance has been increased in response to demands for using more "natural"

growth-promoting substances.

Bacterio-mineral water (BMW) technology was firstly introduced in Japan based on an organic waste treatment technology (Nagasaki, 2002). Bacterio-mineral water is produced in several bio-reactors containing water, humus soil and natural rocks. During the bio-reaction, bacteria utilize manure, releasing unknown ingredients such as essential micronutrients, unknown factors, hormone-like agents, antimicrobial agents, antioxidants and immune-promoting agents, while natural rocks stimulate microbial activity in the reactors (Nagasaki, 2002). And then, finally the clean and safe BMW is produced. Thus, BMW could be used as a safe and environmental-friendly feed additive.

Bacterio-mineral water has been known to improve livestock production when it was applied with drinking water and to provide clean environment of animal house preventing odor when it was sprayed (Nagasaki, 200). However, none of research has been conducted to examine the effect of BMW supplementation on either animal performance or rumen fermentation. Only a reported effect of BMW is that supplementation of BMW originated from bio-reacted swine urine in to the culture medium increased the activities of quinine reductase and glutathione S-transferase of *Scenedesmus spp.*, a green algae which has great potential as bioresources for fishes, alternative and

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**Table 1.** Dehority's artificial medium without carbohydrate source

Ingredients	100 ml
Mineral I solution <sup>1</sup>	20.0
Mineral II solution <sup>2</sup>	20.0
Resazurine	0.1
Vitamin mixture <sup>3</sup>	1.0
VFA solution <sup>4</sup>	6.7
Casein (Acid hydrolyzed casein)	2.0
Hemin solution <sup>5</sup>	0.1
8% Na <sub>2</sub> CO <sub>3</sub>	5.0
2.5% Cystein-HCl	0.1

<sup>1</sup> Mineral I solution: KH<sub>2</sub>PO<sub>4</sub> 4.5 g in 1,000 ml distilled water (DW).

<sup>2</sup> Mineral II solution: CaCl<sub>2</sub> (anhydrous) 0.25 g, MgSO<sub>4</sub> (anhydrous) 0.25 g, NaCl 4.5 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 4.5 g, MnSO<sub>4</sub>·H<sub>2</sub>O 0.10 g, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.10 g, CoCl<sub>2</sub>·6H<sub>2</sub>O 0.01 g, ZnSO<sub>4</sub> 0.10 g in 1,000 ml DW.

<sup>3</sup> Vitamin solution: Pyridoxine HCl 0.20 g, riboflavin 0.20 g, thiamine HCl 0.20 g, nicotinic acid amide 0.20 g, Ca-d-pantothenate 0.20 g, para-amino benzoic acid 0.01 g, stock solution (folic acid 0.125 g, biotin 0.125 g, cobalamine 0.125 g in 25 ml DW) 1.0 ml in 1,000 ml DW.

<sup>4</sup> VFA solution: Acetic acid 17 ml (2.9×10<sup>-2</sup> M), propionic acid 6 ml (8.0×10<sup>-3</sup> M), and n-valeric, isovaleric and DL- $\alpha$ -methylbutyric acid, 1 ml each (9×10<sup>-4</sup> M).

<sup>5</sup> Hemin solution: Dissolve 50 mg hemin in 1 ml 1 N NaOH; and make to 100 ml with DW.

health foods, and pharmaceutical products (Shon et al., 2004). There is no scientifically proved report related to the effect of BMW supply on animal physiology and production. If BMW is added to the *in vitro* fermentation of mixed rumen microorganisms, ruminal fermentation can be improved. Therefore, the objective of the study here is to investigate the effect of BMW supplementation in the medium on *in vitro* fermentation of rumen microorganisms.

## MATERIALS AND METHODS

### Preparation of ruminal microorganisms

The mixed rumen microorganisms were obtained from a ruminally fistulated Holstein cow fed at 1.8% of body weight twice a day (06:00 and 16:00 h), a ration consisting of 50% corn silage and 45% mixed grass hay and 5% soybean meal (11% crude protein diet, as-fed basis). Ruminal contents were obtained from the bottom of the rumen, 3 h after the morning feeding. And then they were squeezed through four layers of cheese cloth and poured into a separating funnel that had been gassed with oxygen-free CO<sub>2</sub> and anaerobically incubated at 39°C for up to 60 min to allow feed particles to buoy up. The feed particles that had risen to the surface were removed by a vacuum pump, and then liquid portions were used as mixed rumen microorganisms.

### Preparation of BMW

Bacterio-mineral water was prepared through four bio-reaction steps. In the first bio-reactor containing humus soil and natural rocks, 50 kg swine manure (1.0%, w/v) and

water (<5,000 L) were added and then the bio-reaction was carried out for 10 days with air circulation. After the first step, liquid in the first bio-reactor flowed to the second bio-reactor containing natural rocks, while fresh 50 kg manure and water were added into the first bio-reactor and then the reaction carried out for another 10 days. In the third and fourth bio-reactors containing natural rocks, the similar reactions to the second bio-reaction were carried out for 10 days in each step. After 40 days, BMW from the fourth bio-reactor was finally collected and used for the experiment. Bacterio-mineral water was pH 7.0 and contained 0.51% N, 0.17% P<sub>2</sub>O<sub>5</sub>, 0.03% K<sub>2</sub>O, 0.09% Ca, 0.007% Mg, 0.0025% Cu and 0.001% Zn in the experiment.

### Culture techniques and media

The anaerobic culture techniques of Hungate (1950) with modifications (Bryant and Burkey, 1953) were used for all incubations. About 200 mg Whatman No. 1 filter paper disks (6 mm in diameter) was added to 30 ml triplicate serum vials (Miller and Wolin, 1974) and 12 ml modified Dehority's artificial medium (Dehority, 1963; Table 1) without carbon sources was transferred to each serum vial purged with oxygen-free CO<sub>2</sub>. The vials were sterilized by autoclaving at 121°C for 15 min. After then, 8 ml of ruminal fluid and 0.2 ml (1.0%) BMW were added into the vials. Various supplementation levels of 1.0, 0.01, 0.005 and 0.001% BM water were prepared by diluting BMW with distilled water, respectively. Incubations were carried out anaerobically at 39°C without shaking for 0, 12, 24, 48, 72 and 96 h.

### Determination of enzyme activities

Extracellular enzyme activity against carboxymethyl cellulose (CMC) was determined by incubating 0.5 ml of supernatants from microbial cultures with 0.5 ml of 2% (w/v) CMC in 0.05 M Na citrate buffer (pH 5.5). After 1 h incubation at 45°C, the reaction was stopped by boiling the test tubes for 5 min. Aliquots were centrifuged at 12,000×g for 5 min, and reducing sugars in the supernatants were assayed colorimetrically using the DNS (dinitrosalicylic acid) method (Miller, 1959). Xylanase activity was determined by measuring liberated reducing sugar using oat spelt xylan as a substrate using the method described above. One international unit (IU) of enzyme activity was defined as the amount of enzyme which liberated 1  $\mu$ mol of glucose (for CMCase) and xylose (for xylanase) equivalent per min under the conditions described above.

### Gas production and microbial growth determination

At the end of incubations, gas production was determined by using a water displacement apparatus (Fedorak and Hrwdey, 1983). Microbial growth was monitored spectrophotometrically by determining the

**Table 2.** Cumulative *in vitro* gas production (ml) of mixed rumen microorganisms cultured in medium containing different levels of bacterio-mineral water (BMW)

Incubation time (h)	Supplementation levels (%) of BMW					SEM
	0	0.001	0.005	0.01	1.0	
12	4.62	5.01	5.00	4.93	4.53	0.189
24	14.43 <sup>ab</sup>	14.05 <sup>ab</sup>	13.77 <sup>b</sup>	14.10 <sup>ab</sup>	14.93 <sup>a</sup>	0.162
48	32.90	32.10	32.67	31.93	32.67	0.242
72	42.60	42.65	42.90	44.47	44.93	0.383
96	45.07 <sup>b</sup>	47.60 <sup>ab</sup>	47.97 <sup>ab</sup>	48.57 <sup>a</sup>	47.96 <sup>ab</sup>	0.487

<sup>a,b</sup> Means in the same row with different superscripts differ ( $p < 0.05$ ).

**Table 3.** *In vitro* growth rate (OD value) of mixed rumen microorganisms cultured in medium containing different levels of bacterio-mineral water (BMW)

Incubation time (h)	Supplementation levels (%) of BMW					SEM
	0	0.001	0.005	0.01	1.0	
12	0.757	0.788	0.780	0.822	0.732	0.0323
24	0.901	0.900	0.954	0.934	0.920	0.0340
48	0.670 <sup>c</sup>	0.690 <sup>c</sup>	0.750 <sup>b</sup>	0.797 <sup>ab</sup>	0.814 <sup>a</sup>	0.0105
72	0.606 <sup>b</sup>	0.631 <sup>b</sup>	0.603 <sup>b</sup>	0.766 <sup>a</sup>	0.631 <sup>b</sup>	0.0197
96	0.531	0.502	0.550	0.603	0.548	0.0386

<sup>a,b,c</sup> Means in the same row with different superscripts differ ( $p < 0.05$ ).

optical densities (OD) of cultures at 660 nm. Cultures were harvested by centrifugation (10,000×g for 20 min at 4°C), and the cells were then suspended in 100 mM potassium phosphate buffer (pH 6.5) washing 3 times (Lee et al., 2003).

#### Determination of dry matter degradation

The residual pellets in cultures were used to determine dry matter degradability of filter paper. The residual cellulose pellets were treated with 1 M NaOH at 100°C to remove microbial materials, and filtrated through a filtering unit (Fisher, USA). The pellets were dried to a constant weight at 80°C. Dry matter degradation rates were calculated by the differences between filter paper weights before and after incubation.

#### Statistical analysis

The experiment was conducted with five treatments in triplicate. The data were analyzed using GLM (general linear model) procedures of SAS (1996) and statistical significance among treatment means were determined by Duncan's multiple range test (1955) and P value of  $< 0.05$  was considered significant.

## RESULTS

The cumulative *in vitro* gas production of mixed rumen microorganisms at the different incubation times for the treatments is given in Table 2. In all the treatments, the cumulative gas production of the mixed rumen microorganisms rapidly increased during the prolonged

**Table 4.** *In vitro* dry matter degradation (%) of mixed rumen microorganisms cultured in medium containing different levels of bacterio-mineral water (BMW)

Incubation time (h)	Supplementation levels (%) of BMW					SEM
	0	0.001	0.005	0.01	1.0	
12	10.84	14.06	11.01	11.79	13.15	0.953
24	14.96	19.16	17.09	17.75	16.86	0.881
48	35.01 <sup>c</sup>	44.05 <sup>ab</sup>	41.44 <sup>ab</sup>	46.68 <sup>a</sup>	39.39 <sup>bc</sup>	1.280
72	47.44	52.53	48.09	51.56	51.40	0.796
96	55.48	52.89	53.50	55.10	56.24	1.111

<sup>a,b,c</sup> Means in the same row with different superscripts differ ( $p < 0.05$ ).

incubation time. There were no significant ( $p > 0.05$ ) differences among the treatments for the initial rate of gas production, but the gas production was significantly increased ( $p < 0.05$ ) over the 0.005% BMW inclusion treatment up to 1.16 ml by the 1.0% BMW inclusion treatment at 24 h incubation, while the other treatments were not significantly different in the gas production compared with the 1.0% BMW treatment. After incubation for 48h, although the gas production for all treatments was between the ranges of 31.93 to 32.90 ml, there was no significant difference among the treatments. At 72 h incubation, the gas production tended ( $p < 0.1$ ) to be increased by the 0.01 and 1.0% BMW treatments compared with control and the 0.001% BMW treatment. At the end of incubations (96 h), the sample supplemented with 0.01% BMW was higher ( $p < 0.05$ ) up to 3.5 ml than control (0% BMW) in the gas production, while the other BMW supplementation treatments tended to increase ( $p < 0.1$ ) the gas production compared with control. Therefore, it is likely that the cumulative gas production is increased in the cultures of mixed rumen microorganisms containing the higher levels of BMW compared with the control treatment.

The growth rates of mixed rumen microorganisms at different incubation times for the treatments are shown in Table 3. Similar to the responses on the cumulative gas production in all the treatments, the growth rates also rapidly increased during the prolonged incubation time and maximized at the 24 h incubation, while there was no significant difference among the treatments. After incubation for 48 h, the 1.0% BMW treatment most highly stimulated ( $p < 0.05$ ) microbial growth except the 0.01% BMW treatment. At the 48h incubation, the growth rate was linearly increased by increasing supplementation levels of BMW. At the 72 h incubation, the growth of microorganisms in the culture of the 0.01% BMW treatment was higher ( $p < 0.05$ ) than the other treatments. In general, the growth rate was increased by the all BMW treatments, however, 0.01% BMW was most effective in stimulating the growth rate.

The effects of BMW on the filter paper dry matter degradation by mixed rumen microorganisms are shown in Table 4. The supplementation of 0.001, 0.005 and 0.01%

**Table 5.** Extracellular carboxymethyl cellulase (CMCase) and xylanase activities in the culture of mixed rumen microorganisms supplemented with different levels of bacterio-mineral water (BMW)

Incubation time (h)	Supplementation levels (%) of BMW					SEM
	0	0.001	0.005	0.01	1.0	
CMCase (glucose $\mu$ mol/min/ml)						
12	7.70 <sup>b</sup>	10.15 <sup>a</sup>	8.80 <sup>ab</sup>	7.64 <sup>b</sup>	7.66 <sup>b</sup>	0.328
24	7.34 <sup>b</sup>	10.25 <sup>ab</sup>	10.42 <sup>a</sup>	10.57 <sup>a</sup>	8.93 <sup>ab</sup>	0.392
48	17.98 <sup>c</sup>	24.14 <sup>bc</sup>	31.51 <sup>ab</sup>	35.75 <sup>a</sup>	17.80 <sup>c</sup>	2.325
72	38.14 <sup>b</sup>	38.25 <sup>b</sup>	38.66 <sup>ab</sup>	42.77 <sup>a</sup>	39.51 <sup>ab</sup>	0.685
96	28.76	29.55	28.50	27.64	25.87	0.657
Xylanase (xylose $\mu$ mol/min/ml)						
12	15.84	14.90	13.41	15.69	15.67	0.568
24	25.87	24.82	24.05	25.83	26.27	0.658
48	97.10	92.66	88.37	90.37	87.49	1.673
72	115.46 <sup>ab</sup>	109.76 <sup>b</sup>	105.77 <sup>b</sup>	113.00 <sup>ab</sup>	121.81 <sup>a</sup>	1.928
96	100.84	94.37	92.98	101.96	99.28	1.454

<sup>a,b,c</sup> Means in the same row with different superscripts differ ( $p < 0.05$ ).

BMW significantly increased ( $p < 0.05$ ) cellulose degradation by 25.8, 18.4 and 33.3%, respectively, compared with control after the 48 h incubation, though these increases were not statistically significant ( $p > 0.05$ ) in the early stage (until the 24 h incubation). Although the addition of BMW did not significantly influence the filter paper DM degradation throughout the incubation period except the 48 h incubation, the DM degradation tended to be increased by all BMW treatments compared with the control treatment except the 96 h incubation.

The time courses of CMCase and xylanase activities in the different treatments are shown in Table 5. Reflecting the effects on the cellulose degradation and microbial growth, the CMCase activity in the supernatants of medium supplemented with BMW except the highest level (1.0%) was higher than the control treatment while at the 96 h incubation there was no difference among the treatments. The CMCase activity in the culture supernatant for the 0.001% BMW treatment was significantly ( $p < 0.05$ ) higher than for the control. 0.01 and 1.0 BMW treatments at the 12 h of incubation. The addition of both 0.005 and 0.01% BMW highly increased ( $p < 0.05$ ) CMCase activity compared with the control treatment after 24 h and 48 h incubation, while the 0.01% BMW addition only significantly increased ( $p < 0.05$ ) CMCase activity compared with the control treatment after the incubation of 72 h. At 72 h after incubation, the xylanase activity was significantly ( $p < 0.05$ ) increased by the addition of 1.0% BMW compared with the addition of 0.001 and 0.005% BMW, while at the other incubation times, the xylanase activity was not different among the treatments.

## DISCUSSION

The results in the study showed that it is difficult to find

strong correlations between the parameters such as *in vitro* gas production, microbial growth, DM degradation and fibrolytic enzyme. However, a point to be pertinent in the present experiment is that most parameter means were higher in BMW supplementation treatments than in contro. Especially, 0.01% BMW supplementation was highly effective to all parameters compared with control, while there were some linear responses according to the increasing supplementation levels ranging from 0.001 to 0.01% BMW in DM degradation and cellulase activity at the 48 h incubation and from 0.001 to 1.0% BMW in microbial growth rate at the 48 h incubation. Although the highest supplementation level of 1.0% seems not to stimulate *in vitro* fermentation of mixed rumen microorganisms, the level did not have any negative effect on all parameters.

Since the late 1970s, measurement of *in vitro* gas production has become increasingly popular for determining forage digestion characteristics and the kinetics of fermentation (Theodorou et al., 1994, 1998). The amount of gas produced depends on the amount of substrate fermented and the amount and molar proportions of the VFA produced (Beuving and Spoelstra, 1992). Rymer and Givens (2002) suggested that the gas production technique predicts dynamic parameters of rumen fermentation, particularly the short chain fatty acids (SCFA). Again, Theodorou et al. (1995) demonstrated that measuring the accumulation of fermentation gases during rumen anaerobic fungal growth is a useful method for the rapid and precise determination of microbial growth on soluble and particulate substrates. However, it is unlikely that there is a relationship between gas production and other parameters in the present experiment because the gas production technique seems more useful in complex substrate characterization such as forage characterization than pure cellulose substrate (Rymer and Givens, 2002).

A preliminary experiment showed that the supplementation of BMW in the medium did not have antibacterial effects on *Bacillus subtilis* and other pathogens such as *Salmonella typhimurium*, *S. enteritidis*, *Staphylococcus dysgalactiae* and *E. coli* k-99 (C.-H. Kim and D. H. Choi, unpublished data). Thus, BMW used in the present experiment and originated from swine manure seems to increase microbial growth rather than to inhibit (Table 3). In the present study, since the carbohydrate source was only filter paper (pure cellulose), growth of cellulolytic bacteria including *Fibrobacter succinogenes*, *Ruminococcus albus* and *R. flavefaciens*, which are predominant cellulolytic bacteria in the rumen (Hungate, 1950), could be improved by the BMW addition to medium. These are also highly correlated to higher DM degradation and cellulase activity, especially at the 48 h incubation, by BMW supplementation. Although it is not known the exact mechanism involved in the current response of BMW.

which it should be defined in future, the assumption is that because BMW is originated from swine manure, thus it could be a good source of some nutrients such as nitrogen and minerals, enzymes and unknown growth factors. Shon et al. (2004) reported that BMW could increase activities of certain enzymes (quinine reductase and glutathione S-transferase) in culture of *Scenedesmus spp.*, a green algae, which suggests that BMW could also highly stimulate mixed rumen microorganisms to produce cellulolytic enzyme activity rather than xylanase activity in the present study.

In conclusion, the supplementation level of 0.01% BMW would be a suitable addition level to stimulate rumen fermentation increasing microbial growth and cellulose degradation. Again, it is very important that the manure treated water could be a useful probiotics for animal production under the current circumstances in which most governments in the world have tried to reduce pollution factors such as global warming gases and animal wastes. Therefore, further research is required to find out the effects of BMW on animal performance and productivity when BMW originated from bio-reacted manure is supplemented to feedstuffs or drinking water. And, also, it is needed for the more research to define the optimum application rates of BMW under different feeding regimes.

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