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Effects of a Bacteria-Based Probiotic on Ruminal pH, Volatile Fatty Acids and Bacterial Flora of Holstein Calves

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ABSTRACT.   Twelve ruminally cannulated Holstein calves (age, 12 ± 3 weeks) were used to identify the effect of a probiotic comprised of *Lactobacillus plantarum*, *Enterococcus faecium* and *Clostridium butyricum* on ruminal components. The calves were adapted to a diet containing a 50% high-concentrate (standard diet) for 1 week, and then, the probiotic was given once daily for 5 days (day 1–5) at 1.5 or 3.0 g/100 kg body weight to groups of four calves each. Four additional calves fed the standard diet without probiotic served as the corresponding control. Ruminal pH was measured continuously throughout the 15-day experimental period. Ruminal fluid was collected via a fistula at a defined time predose and on days 7 and 14 to assess volatile fatty acid (VFA), lactic acid and ammonia-nitrogen concentrations, as well as the bacterial community. The probiotic at either dose improved the reduced 24-hr mean ruminal pH in calves. The circadian patterns of the 1 hr mean ruminal pH were identical between the probiotic doses. In both probiotic groups, ruminal lactic acid concentrations remained significantly lower than that of the control. Probiotic did not affect ruminal VFA concentrations. *L. plantarum* and *C. butyricum* were not detected in the rumen of calves given the high-dose probiotic, whereas *Enterococcus* spp. remained unchanged. These results suggest that calves given a probiotic had stable ruminal pH levels (6.6–6.8), presumably due to the effects of the probiotic on stabilizing rumen-predominant bacteria, which consume greater lactate in the rumen.
KEY WORDS: calf, probiotic, ruminal bacteria, ruminal pH.

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Bacteria-based probiotics are comprised of a variable number of species and strains of beneficial bacteria known to have positive implications on animal health and performance. In cattle, bacterial probiotic has shown to improve the rumen-predominant microorganisms [8, 12] and ruminal performance [27]. Probiotic consisting lactic-acid-producing bacteria (LAB) promote the stability of the rumen flora [3, 8, 12, 40], which result in increased dry matter intake and weight gain and improved health in cattle [6, 38]. Among LAB, the species used most frequently as probiotics are *Lactobacillus plantarum* and *Enterococcus faecium* [39]. Nocek *et al.* [27] reported the decreased risk of acidosis for dairy cows receiving a combination of probiotic including *Lactobacillus* and *Enterococcus*. Furthermore, steers receiving a probiotic including both lactate-utilizing *Propionibacterium* and lactate-producing *Enterococcus* had higher ruminal concentration of acetate, and the blood variables indicated a reduced risk of metabolic acidosis [12]. It has been hypothesized that the functionality and efficacy of bacterial probiotics can be determined by their effect on rumen microbial populations [8, 12]. This thinking is consistent with previous reports that probiotics improve the gastrointestinal tract microbiota in calves [6, 38].

Ruminal components, such as pH and amounts of volatile fatty acids (VFA) and lactic acid, are important environmental factors for survival of ruminal microorganisms [19, 25, 32]. Decreased pH and overgrowth of acidophilic bacteria in ruminal fluid are synchronized with an increased level of rapidly fermentable carbohydrate, which results in prompt production and accumulation of ruminal VFAs and lactic acid, leading to acidosis [5, 25]. Likewise, a number of ruminal bacteria, such as fibrolytic bacteria, decrease when the ruminal pH remained under 5.5 [17, 24, 32]. Orderly shifts occur among the rumen-predominant amylolytic and lactate-utilizing bacterial populations in response to gradually decreasing ruminal pH [27]. Similarly, when quantities of rapidly fermentable carbohydrates exceed the buffering capacity of the rumen [34], accumulated VFA and lactic acid are associated with a decreased ruminal pH [2, 32], even in newborn calves [15].

Highly fermentable diets stimulate ruminal microbial proliferation and VFA production in pre-weaned calves, followed by initiation of ruminal development [18]. Rapid fermentation of ingested calf starter causes an increased

concentration of VFA and decreased pH in the incompletely developed rumen [15, 30]. Furthermore, consumption of a highly fermentable diet results in increased lactic acid levels and ruminal acidosis in weaned calves [1, 30]. Challenges, such as the transitive ruminal microbiome and high-concentrate feeding strategies, are related to the incidence of ruminal acidosis in young ruminants [15]. Methods, such as terminal-restriction-fragment length polymorphism (T-RFLP), PCR-denaturing gradient gel electrophoresis and real-time analyses of the ruminal flora, show that the most complex interactions occur between the ruminal bacteria and other bacterial products [11, 14, 19]. However, little information on the effect of probiotics containing LAB on ruminal pH, VFA and the properties of rumen bacterial flora in calves is available. Furthermore, the mechanism underlying the effects of probiotics on ruminal components remains unclear. The objective of this study was to determine whether administration of a probiotic containing LAB affects the ruminal pH, VFA, lactic acid and ammonia-nitrogen (NH_3-N) levels in conjunction with the levels of a number of ruminal bacteria in weaned Holstein calves.

MATERIALS AND METHODS

Animals and treatment: This experimental design was approved by the Iwate University Laboratory Animal Care and Use Committee (A201026). Twelve ruminally cannulated Holstein bull calves aged 12 ± 3 weeks [95 ± 2 kg body weight (BW); mean $\pm$ SE] were housed in a 4×5-m^2, open-sided straw bed and naturally ventilated barn at the Cattle Research Center of Iwate University. The calves had been weaned within 4 weeks after birth and fed starter pellets containing ground corn. The ruminal cannula (diameter; 5 cm) was applied 4 weeks before starting the experiment. One week before start of the experiment, the calves were gradually adapted to the high-grain diet containing steam-rolled corn mixed with timothy hay (standard diet). The concentrate: hay ratio was 1:1. The percentages of ingredients and chemical composition of the total mixed diet are shown in Table 1. During the 15-day experimental period, each calf received 3.2 kg of diet twice per day at 0800 hr and 1700 hr and had access to fresh water *ad libitum*.

A probiotic (Miyarisan Pharmaceutical Co., Ltd., Tokyo, Japan), which included *L. plantarum* strain 220 [9×10^6 colony forming units (CFU)/g], *E. faecium* strain 26 (9×10^5 CFU/g) and *Clostridium butyricum* strain Miyari (9×10^4 CFU/g), was administered daily as a single dose of 1.5 or 3.0 g/100 kg BW to each group of four calves for 5 consecutive days. Four additional calves fed the standard diet without probiotic served as the control. The probiotic was stored at 4°C, each dose was mixed with 50 m*l* of tap water in a beaker, and the probiotic suspension was orally administered to each calf with a 50-m*l* drencher the morning prior to feeding. No changes in the health of the calves were observed during the experimental period.

Ruminal pH measurement: Ruminal pH was measured using a radio transmission system, as reported previously [36]. Briefly, the system consisted of a pH sensor attached

Table 1.   Ingredient and chemical components of diet

Item	Quantity
Ingredients, % (DM)	
Steam-rolled corn	50.0
Alfalfa pellet	7.5
Corn grain	18.0
Wheat bran	13.5
Soybean meal	10.0
Dried whey	1.0
Nutrient components[a]	
DM, %	87.8
CP, %	17.7
NDF, %	40.5
NFC, %	28.7
Calcium, %	0.50
Phosphorus, %	0.35

a) All except DM presented on DM basis. DM presented as percent fed basis.

to a transmitter through a 60-cm wire, a data measurement receiver and a personal computer with special software (YCOW-S; DKK-Toa Yamagata, Yamagata, Japan). The pH sensor was inserted through the ruminal cannula and located in the ventral sac of the rumen, and a small data transmitter was mounted at the back of each calf. The standardized site for measuring ruminal pH is the (cranial-) ventral sac, because most mixing of ruminal contents occurs at this site, and the pH values are more stable compared to at other ruminal sites [10]. The pH sensors were calibrated with pH 4 and 7 buffer solutions before insertion and within two weeks interval during the experimental period. Ruminal pH was recorded every 10 min at 1 week before experiment and during the 15-day experimental period. The location of the pH sensor in the rumen was checked each morning.

Ruminal fluid sampling and VFA, lactic acid and NH_3-N assays: Ruminal fluid was collected from the ventral sac of the rumen, adjacent to the pH sensor. A manual vacuum pump was used to collect samples through the ruminal cannula, the morning prior to feeding, at predose and on days 7 and 14. Ruminal fluid was filtered immediately through two layers of cheesecloth and collected into a sterile plastic tube to analyze ruminal bacteria, total and individual VFA components (acetic acid, propionic acid and butyric acid), lactic acid and NH_3-N. Two milliliters of ruminal fluid were immediately stored at −80°C for assessment of the bacterial population. Ten milliliters of ruminal fluid were added to 2 m*l* of 25% metaphosphoric acid in 3 N H_2SO_4 for assay of VFA. Total and individual VFA components were separated and quantified by gas chromatography (model GC-2014, Shimazu, Kyoto, Japan) using a packed-glass column (Thermon-3,000; 3%) on a Shimalite TPA 60–80 support (Shinwa Chemical Industries Ltd., Kyoto, Japan). For assay of lactic acid, the ruminal fluid was centrifuged immediately at $2,000 \times g$ for 15 min, and concentrations in the supernatant were determined using a commercial kit (F-kit; D-lactate/L-lactate, J. K. International, Tokyo, Japan). NH_3-N levels in ruminal fluid were determined by the steam distillation

Table 2. Species- and genus-specific primers for the quantification of ruminal bacteria using real-time PCR assay

Bacteria	Primer	Sequence (5′- 3′)	Source
Lactobacillus plantarum	Sg-Lpla-F	CTCTGGTATTGATTGGTGCTTGCAT	[22]
	Sg-Lpla-R	GTTCGCCACTCACTCAAATGTAAA	
Enterococcus spp.	g-Bfra-F	CCCTTATTGTTAGTTGCCATCATT	[31]
	g-Bfra-R	ACTCGGTTGTACTTCCCATTGT	
Clostridium butyricum	209F25	AGTGATTGTCAGTAGTAGACGAGCG	[26]
	R221	CATGCGCCCTTTGTAGC	
Clostridium coccoides	g-Coc-F	AAATGACGGTACCTGACTAA	[23]
	g-Coc-R	CTTTGAGTTTCATTCTTGCGAA	
Megasphaera elsdenii	MegEls1F	GACCGAAACTGCGATGCTAGA	[13]
	MegEls1R	CGCCTCAGCGTCAGTTGTC	
Selenomonas ruminantium	SelRum1F	GGCGGGAAGGCAAGTCAGTC	[13]
	SelRum1R	CCTCTCCTGCACTCAAGAAAGACAG	

method using an automatic-N analyzer (Kjeltec auto sampler system 1035 Analyzer, Tecator, Sweden).

Rumen bacterial flora assay: Bacterial composition of ruminal fluid was assessed using T-RFLP and real-time PCR. The rumen bacterial flora was evaluated in ruminal fluid samples collected at predose and on days 7 and 14 from both the group given 3.0-g probiotic and the control group. Bacterial DNA was extracted from 1 m*l* of ruminal fluid using the bead–phenol method [23]. Extracted DNA was dissolved in TE buffer and stored at −80°C until T-RFLP and real-time PCR analyses. T-RFLP measurements were performed according to the method of Sakamoto *et al*. [35]. In brief, the complete 16S rDNA was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) for PCR amplification of the 16S rRNA gene sequences [16]. The forward primer was labeled at the 5′ end with 6′-carboxyfluorescein (Applied Biosystems, Tokyo, Japan). The following program was used to amplify 16s rDNA: 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec and 72°C for 1.5 min, with a final extension at 72°C for 10 min. The PCR products were purified using a High Pure PCR Product Purification kit (Roche, Indianapolis, IN, U.S.A.) and digested using either *Hha*I or *Msp*I at 37°C for 3 hr. A standardized marker (1,200 LIZ) was then added to each sample. The length of the terminal restriction fragment was determined on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Fragment sizes were estimated using GeneMapper Software (Applied Biosystems). The predicted T-RFLP patterns of the 16S rDNAs of known bacterial species were obtained using InfoCom software (Microbiota Profiler; Infocom Co., Tokyo, Japan).

Bacterial species, including the probiotic and some ruminal bacteria, were quantified by real-time PCR according to a previous method [23]. Briefly, DNA extraction from ruminal fluid samples was performed using a method similar to that for the T-RFLP measurements. Real-time PCR was carried out using a Thermal Cycler Dice TP800 device (Takara, Otsu, Japan), using the primers shown in Table 2. The quantification of DNA for each bacterial species in ruminal fluid was performed using the SYBR Green intercalation procedure. Standards and samples were assayed in a 25-*μl* reaction mixture containing 12.5 *μl* of SYBR Premix EX

Taq II (Tli RNase H plus), 0.5 *μl* of each primer, 10.5 *μl* of nuclease-free water and 1 *μl* of DNA template. The amplification program included an initial denaturation step at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 5 sec, annealing/extension at 60°C for 30 sec, with a final extension step at 72°C for 5 min.

Statistical analysis: The calf was the experimental unit for all statistical analyses, and the random effect was the calf within a treatment. The main effects included challenge treatment, day of the experiment (predose day=24 hr before treatment, day 1=0–24 hr, followed until day 14) and after 0800 hr treatment and feeding. Diurnal measurements of ruminal pH were analyzed as 24-hr mean pH from 1 day before to 14 days after treatment. Each 10-min interval of the pH data was summarized as a 1-hr mean from 0800 hr to 0700 hr the following day to assess circadian changes. Minimum and maximum pH values during a single day were determined on the predose day and days 7 and 14. Minimum and maximum pH values were analyzed by the Mann–Whitney *U*-test. Quantities of 16S rRNA as determined by real-time PCR were analyzed to determine significant differences in copy number between treatment and control groups for each targeted bacterial species. Ruminal parameters and the number of bacteria data are presented as means ± SE. T-RFLP analyses of rumen bacterial flora were expressed on an electropherogram based on the size of the intergenic spacer and the fluorescence intensities of the fragments. Graph Pad Prism ver. 5.01 software (La Jolla, CA, U.S.A.) was used for the statistical calculations, and one-way repeated-measures analysis of variance followed by the Tukey's multiple comparison method was used to evaluate differences among the groups. A *P*-value <0.05 was considered significant.

RESULTS

Ruminal pH: Ruminal pH in calves received the probiotic at either dose exhibited a constant 24-hr mean pH during the experimental period. A difference (*P*<0.01) in ruminal pH was found between the probiotic and control groups on days 4 to 14. In addition, the 24-hr mean ruminal pH decreased (*P*<0.01) in the control group on days 5, 9, 10 and 11 compared to predose values. Considerable disparities in
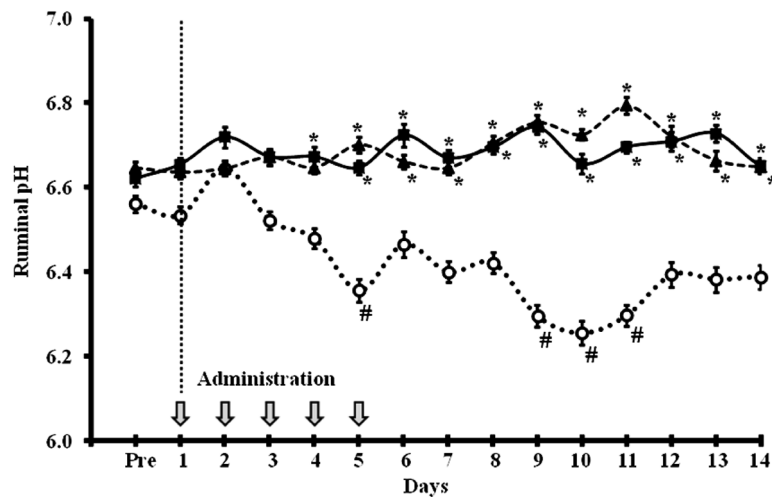
Fig. 1. Changes in 24-hr mean pH in the ruminal fluid of calves given 1.5 g (n=4; ▲) or 3.0 g/100 kg BW (n=4; ■) probiotic for 5 consecutive days. Additional calves without probiotic served as control (n=4; ○). Values represent the means ± SE. * Ruminal pH in the probiotic groups compared to control on the same day (*P*<0.01). # Ruminal pH in the control group compared to the predose day (Pre; *P*<0.01). The first day of probiotic administration was regarded as day 1.

the ruminal pH values among the probiotic groups and control were observed 3 day after initial administration of the probiotic and continued throughout the experimental period (Fig. 1). The circadian pattern of 1-hr mean pH was almost identical among the probiotic groups at predose to day 14. However, a difference (*P*<0.01) between the pH values of the probiotic groups and control was observed at similar times on days 7 and 14. The ruminal pH decreased approximately 1-hr after feeding of a standard diet in the morning and evening. The 1-hr mean pH curve in the control on days 7 and 14 differed (*P*<0.01) from the values at 0800 hr on the same day (Fig. 2). The minimum ruminal pH was greater (*P*<0.05) in probiotic groups than that in the control on day 7. On the same day, the minimum pH in the control calves was different (*P*<0.05) from that predose. The maximum ruminal pH values approached 7.0 in the probiotic groups and did not differ from that of control during the experimental period (Fig. 3).

Ruminal VFA, lactic acid and NH₃-N: No difference in the concentrations of total VFA between the probiotic groups and the control was observed. Concentrations of none of the individual VFA were altered among the groups. The acetic acid and propionic acid ratios were almost identical in all probiotic groups. Lactic acid concentrations remained unchanged in both probiotic groups. However, the lactic acid concentration increased (*P*<0.01) in the control on day 7 compared to that predose and differed (*P*<0.01) from the values in the probiotic groups on the same day. No difference in rumen fluid NH₃-N concentration between the probiotic groups was observed. However, the NH₃-N concentration was significantly high (*P*<0.01) in the control group on days 7 and 14, compared to predose day and also the values in the probiotic groups on the same days (Table 3).

Rumen bacterial flora: Ruminal cellulolytic bacteria, such as *Butyrivibrio fibriosolvens* and *Eubacterium ruminantium*, showed greater fluorescence intensity peaks in most samples from calves given 3.0-g probiotic on day 7. The *Bacteriodetes* group bacteria had greater peaks in the control group on days 7 and 14. However, most bacteria were not detected by T-RFLP measurement in the probiotic group samples on day 14 (Fig. 4). The mean number of *L. plantarum* by real-time PCR was less than 2×10^3 cells/m*l* in ruminal fluid of probiotic group on days 7 and 14. In contrast, the number of *L. plantarum* was unchanged in the control samples on days 7 and 14. The number of *Enterococcus* spp. remained unchanged in the probiotic and control groups, while *C. butyricum* was not detected (less than 2×10^3 cells/m*l* of ruminal fluid) in the probiotic groups on days 7 and 14. The numbers of *Clostridium coccoides* were similar among the groups. The number of *Megasphaera elsdenii* and *Selenomonas ruminantium* did not differ among calves given probiotic on days 7 and 14. However, *M. elsdenii* was slightly changed in the controls on days 7 and 14 (Table 4).

DISCUSSION

Probiotics have been shown to improve anaerobiosis, stabilise pH and supply nutrients to ruminal microbes in their microenvironment [3, 7, 8, 12, 21]. A probiotic consisting of *L. plantarum* and *E. faecium* induced a change in the ruminal pH in cows fed a high grain diet [27]. Ruminal pH decreases immediately after feeding of a high concentrate to adult cattle and newborn calves [15, 34]. The decrease in ruminal pH depends on feeding frequency, effective neutral detergent fiber, ruminal digestion rate and absorption of VFA through the ruminal epithelium [2, 29]. Furthermore, changes in
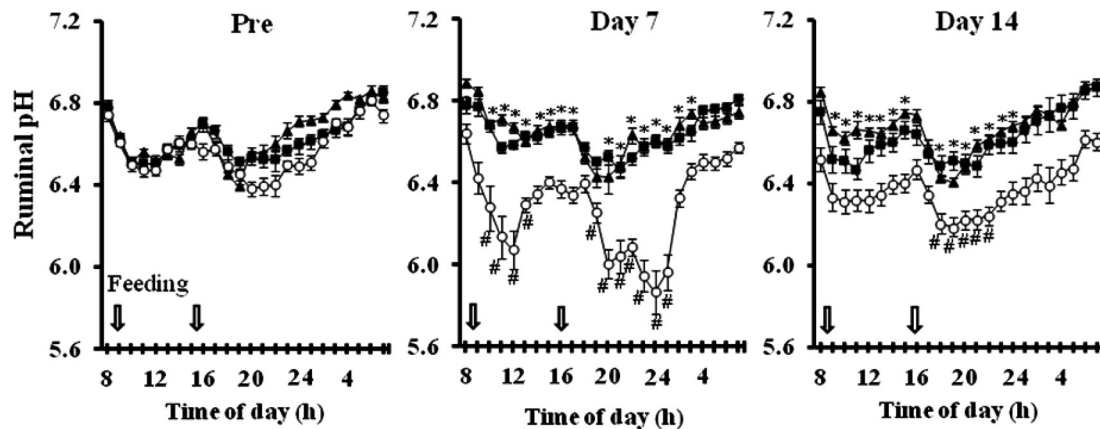
Fig. 2. Circadian changes in 1-hr mean pH at predose day (Pre) and days 7 and 14 in the ruminal fluid of calves given 1.5 g (n=4; ▲) or 3.0 g/100 kg BW (n=4; ■) probiotic for 5 consecutive days. Additional calves without probiotic served as controls (n=4; ○). Values represent the means ± SE. * Ruminal pH in the probiotic groups compared to the controls at the same time (*P*<0.01). # Ruminal pH in the control group compared to time before feeding (0800 hr; *P*<0.01).
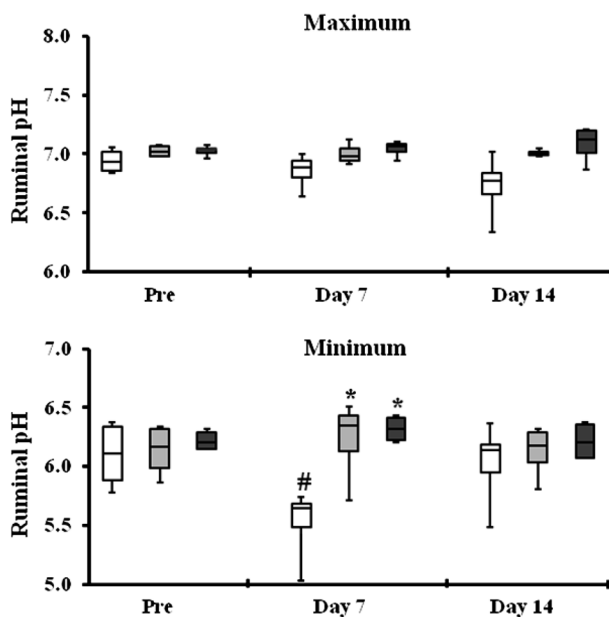

Fig. 3. Box plots showing maximum and minimum ruminal pH on the predose day (Pre) and days 7 and 14 in calves given 1.5 g (n=4; grey boxes) or 3.0 g/100 kg BW (n=4; dark boxes) probiotic for 5 consecutive days. Additional calves not given probiotic served as control (n=4; white boxes). Median and quartiles are displayed in the box. Upper and lower bars represent maximum and minimum values, respectively. * Ruminal pH in the probiotic groups compared to the controls on the same day (*P*<0.05). # Ruminal pH in the control group compared to the predose day (Pre; *P*<0.05). The first day of probiotic administration was regarded as day 1.

rumen bacterial composition, increased activities of lactate-utilizing bacteria and greater lactate consumption also affect ruminal pH [13, 20, 21]. A stable ruminal pH may result from decreased fermentation by LAB and other carbohydrate fer-

menters, which inhabit low-pH environments [39]. Nocek *et al.* [27] reported that cows fed fewer probiotic maintained a greater pH than cows fed high concentration without probiotic, and they suggested that a probiotic consisting of LAB produced sufficient acid to stimulate acid utilizers. By contrast, our results indicate that a LAB-containing probiotic had a marked effect on the ruminal pH of high-concentrate-fed calves at either dose. In the present study, the ruminal VFA concentration was not affected by the probiotic treatment. This is in agreement with previous reports, in which ruminal VFA was not affected by a probiotic included LAB [7, 12]. In our study, the VFA concentration was likely affected by sampling time because the total ruminal VFA concentration in calves increases soon after feeding [4], and the majority of VFA present have been absorbed by the following morning [29, 30, 34]. This explains the greater ruminal pH in calves in the control group in the morning. In addition, the size of the rumen and the effectiveness of ruminal absorption may also affect the ruminal VFA concentration in calves [18].

The lactic acid concentration increased in the control but not in the probiotic groups, and was correlated with a significantly lower mean ruminal pH in the control group. This indicates that control calves had a normal ruminal fermentation capacity during feeding of high-concentrate, which was supported by the decrease in ruminal pH and growth of LAB [15, 39]. However, the circadian ruminal pH increased gradually in control calves, which may be attributable to the effects of time and adaption to diet [9]. A decrease in ruminal pH is known to be related to decreased absorption of VFA and the accumulation of lactic acid in the few hours after high grain feeding [2, 13, 29]. Probiotics including LAB affect ruminal pH by altering the growth of lactate-utilizing bacteria in the rumen [8]. In contrast, the number of lactate-utilizing bacteria increases only when lactic acid accumulates and ruminal pH decreases [13, 21]. A common theory is that probiotic may prevent a decline in rumen pH by decreasing lactic acid production and increasing the utilization of lactic acid by

Table 3.   Ruminal VFA, lactic acid and NH$_3$-N concentrations in probiotic-treated and control calves

Item	Treatment [a]						Control		
	Probiotic (1.5 g)			Probiotic (3.0 g)					
	Pre [b]	Day 7	Day 14	Pre	Day 7	Day 14	Pre	Day 7	Day 14
VFA, mmol/dl									
Total	7.5 ± 0.6	8.1 ± 0.6	8.0 ± 0.4	6.8 ± 0.4	7.6 ± 0.4	7.4 ± 0.3	7.7 ± 0.2	9.2 ± 0.5	7.6 ± 0.3
Acetic acid	5.3 ± 0.5	5.8 ± 0.4	5.6 ± 0.2	5.0 ± 0.3	5.3 ± 0.2	5.1 ± 0.2	5.3 ± 0.1	6.5 ± 0.3	5.2 ± 0.2
Propionic acid	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.8 ± 0.1	1.5 ± 0.1
Butyric acid	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
A:P [c]	3.8 ± 0.2	4.0 ± 0.1	3.8 ± 0.1	3.9 ± 0.1	3.5 ± 0.1	3.3 ± 0.1	3.8 ± 0.2	4.1 ± 0.1	4.1 ± 0.2
Lactic acid, mg/dl	2.3 ± 0.3	3.0 ± 1.1*	2.1 ± 0.4	2.5 ± 0.5	2.0 ± 0.2*	2.2 ± 0.0	2.3 ± 0.1	10 ± 2.4#	3.1 ± 0.1
NH$_3$–N, mg/dl	7.8 ± 0.8	7.5 ± 0.6*	8.0 ± 0.3*	6.2 ± 0.8	8.1 ± 0.7*	7.0 ± 1.0*	6.8 ± 1.0	13 ± 1.3#	12 ± 1.6#

a) Calves of each group received probiotic at 1.5 or 3.0 g/100 kg BW per day for 5 days. b) Predose day (day before administration). c) Acetic acid:Propionic acid. Values represent mean ± SE (n=4). * Compared to the control values on the same day ($P<0.01$). # Compared to the predose (Pre) values in the same group ($P<0.01$).
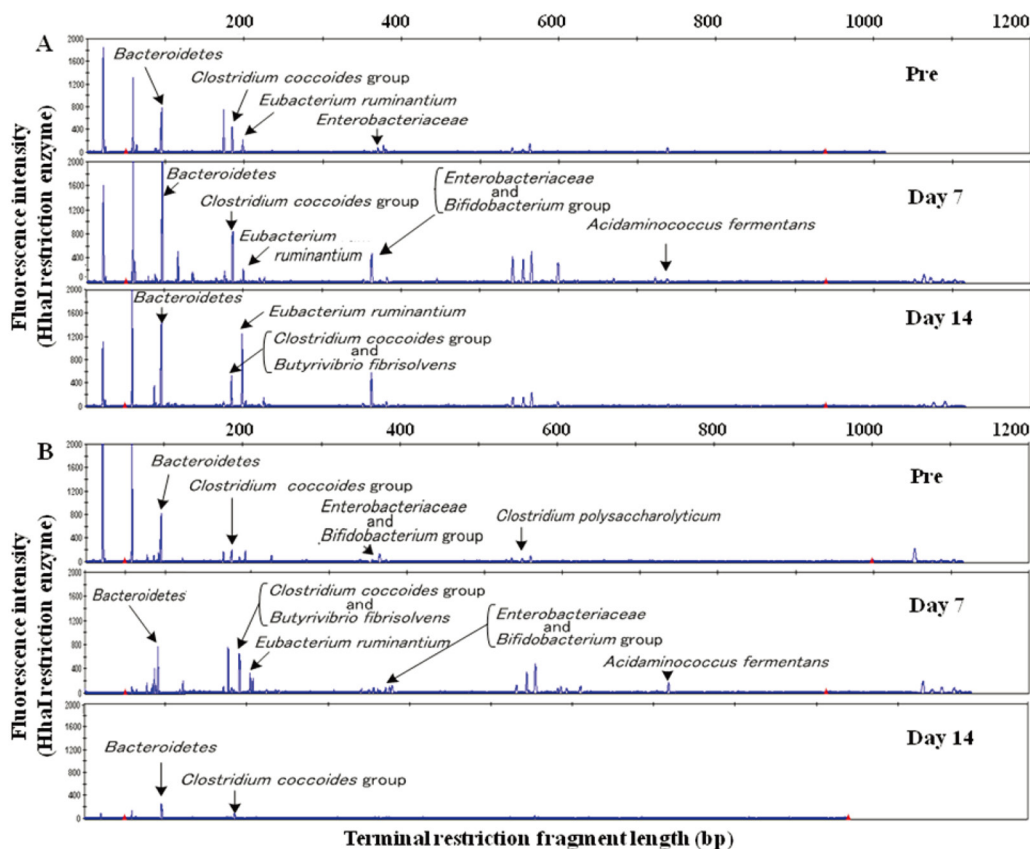

Fig. 4.   Representative terminal-restriction-fragment length polymorphism (T-RFLP) analysis of rumen microbiota composition on the predose day (Pre) and days 7 and 14 in control calves given standard diet without probiotic (A) and the calves given 3.0 g/100 kg BW probiotic (B). Each peak represents a terminal restriction fragment of a specific length that corresponds to a bacterial phylotype, usually a genus.

some microbes [3, 7, 27]. It is possible that certain probiotic combinations, which synthesize lactic acid, may sustain a tonic level of lactic acid in the rumen. This would stimulate the rumen predominant microbial communities, which consume lactic acid and reduce total acidity, as a consequence the ruminal pH would remain stable. In the present study, the ruminal concentration of lactic acid and the number of lactate-utilizing bacteria (*M. elsdenii*) were not affected by probiotic treatment, whereas the number of *M. elsdenii* was greater in the controls. Compared to predose day, the

Table 4. Enumerated eubacteria 16S rRNA genes of some rumen bacterial species in probiotic-treated and control calves

Bacteria	Treatment [a]			Control		
	Pre [b]	Day 7	Day 14	Pre	Day 7	Day 14
Lactobacillus plantarum	2.5 ± 0.8	ND [c]	ND	2.1 ± 0.7	2.8 ± 0.6	2.3 ± 0.7
Enterococcus spp.	4.8 ± 0.1	4.3 ± 0.1	4.5 ± 0.1	4.5 ± 0.1	4.8 ± 0.1	5.0 ± 0.2
Clostridium butyricum	2.1 ± 0.8	ND	ND	2.9 ± 0.6	2.4 ± 0.8	ND
Clostridium coccoides group	8.8 ± 0.1	8.7 ± 0.1	8.9 ± 0.3	8.8 ± 0.1	8.7 ± 0.1	9.0 ± 0.1
Megasphaera elsdenii	4.5 ± 0.1	5.0 ± 0.1	5.6 ± 0.8	4.7 ± 0.1	6.4 ± 0.1	6.1 ± 0.1
Selenomonas ruminantium	8.2 ± 0.2	8.1 ± 0.2	8.1 ± 0.1	8.0 ± 0.3	8.2 ± 0.3	8.5 ± 0.1

a) Calves of group received probiotic at 3.0 g/100 kg BW per day for 5 days, and additional calves fed the standard diet without probiotic served as the control. b) Predose day (day before administration). c) Not detected (less than 2×10^3 cells/m*l*). Values represent number of bacteria ($\times 10^3$ cells/m*l*) in ruminal fluids (Mean ± SE; n=4).

NH_3-N level remained unchanged in the probiotic groups on days 7 and 14, however, the values were significantly lower compared to that of the control group. Probiotics have been reported to have no effects on the NH_3-N concentration in the rumen [8, 12]. It was also reported that many cellulolytic bacteria used NH_3 as their only source of N [33]. Therefore, a lower NH_3-N concentrations and stable ruminal pH in probiotic treated calves might imply the greater growth of cellulolytic bacteria in the rumen.

The numbers of *L. plantarum* and *Enterococcus* spp. did not increase in the ruminal fluid of the probiotic groups. This was likely because growth of LAB is inhibited at pH values >6.0 [5, 39]. It has also been suggested that orderly shifts occur among the predominating amylolytic and lactate-utilizing bacteria in response to changes in ruminal pH [20]. Likewise, Russell *et al.* [33] reported that LAB growth and carbohydrate fermentation are more favorable at lower ruminal pH values, whereas fiber digestion is enhanced at a ruminal pH >6.2 [32]. According to circadian measurements, ruminal pH was <6.6 during the morning when the probiotic was administered, which is ideal for cellulolytic bacteria [24]. In contrast, the number of *B. fibrisolvens* peaked at a greater level in the most calves given 3.0-g probiotic, based on the T-RFLP measurements; thus, *B. fibrisolvens* is the most active and numerous cellulolytic and acid-sensitive bacterium in the rumen [24, 34]. *Bacteroidetes*-group organisms exhibited high growth rates only in the control group; indeed, these bacteria exhibit a distinctive ability to survive at a range of ruminal pH [33]. Recently, it was reported that increased amounts of high-concentrate diet and a reduction in ruminal pH were less favorable to fibrolytic bacteria in the rumen [28]. Furthermore, earlier studies noted that a ruminal pH of 6.0 to 7.0 facilitated the growth of acid-sensitive ruminal bacteria [14, 33]. A maintaining effect of bacteria-based probiotic on rumen-predominant microorganisms has been reported [12, 35]. Sharp *et al.* [37] reported that *L. plantarum* and starch-utilizing bacteria were rapidly lost from the rumen due to protozoan predation. Although probiotic products contain fewer surviving organisms, the recommended probiotic composition and dose remain obscure [11]. Such products likely enhance the effects of rumen-predominant microorganisms [40]. However, a possible antagonistic interaction and inhibitory effects among the rumen-predominant bacteria and inoculated probiotic strains have also been reported [8, 12]. Although the mode of action of probiotic in the rumen is not completely understood, the administration of LAB probiotics is thought to help the rumen microflora adapt to the presence of lactic acid [12] and prevent lactate accumulation in rumen [6, 32].

In summary, a repeated 5 days administration of a bacterial probiotic resulted in reducing the decrease in ruminal pH in Holstein calves fed high concentrates. High 24-hr mean ruminal pH was observed after the probiotic treatment. Although the numbers of bacteria in the probiotic (*L. plantarum*, *Enterococcus* spp. and *C. butyricum*) did not increase in the rumen of calves during the treatment, the numbers of the other rumen-predominant bacterial species were stable. These findings demonstrate the beneficial effects of a probiotic, which are mediated by their influence on the rumen-predominant bacteria. Probiotic supplementation may affect ruminal lactic acid concentration due to increasing lactate consumption, and therefore, the decreased lactic acid concentration resulted in stable ruminal pH in calves. Further studies using more animals are needed to confirm the effects of such a probiotic on rumen bacterial composition, VFA proportions and lactic acid concentrations in cattle.

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