Influences of Prebio Support™ (Mixture of Fermented Products of Lactobacillus gasseri OLL2716 and Propionibacterium freudenreichii ET-3) on the Composition and Metabolic Activity of Fecal Microbiota in Calves

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The influence of Prebio Support™ (PS), which is a mixture of fermented products of Lactobacillus gasseri OLL2716 and Propionibacterium freudenreichii ET-3, on the fecal microbiota and fecal metabolites in calves were investigated. During the intake of PS, the number of bifidobacteria was significantly higher ($p<0.05$), and the fecal water content ($p<0.05$) and fecal ammonia ($p<0.05$) were significantly lower in the PS intake group than in the control group. Furthermore, fecal concentrations of sulfide tended to decrease and short-chain fatty acids (acetic, butyric, and propionic acids) tended to increase through the intake of PS. The numbers of other fecal bacteria and the fecal pH of the PS intake group did not differ from those of the control group. The fecal condition, such as hardness, in calves given PS was better than that of the control group. These findings indicate that PS intake effectively improves the fecal environment, and there is a possibility of it alleviating clinical symptoms.

Key words: Prebio Support™; Lactobacillus gasseri; Propionibacterium freudenreichii; fermented products; calf; fecal microbiota; fecal metabolites

INTRODUCTION

It is known that several factors, including the physiology of the host, aging, diet, disease, drugs, and stress affect the composition of intestinal microbiota, and the predominance of harmful bacteria such as clostridia in the intestines is related to various disturbances (10). Harmful bacteria may produce putrefactive substances such as ammonia, hydrogen sulfide, amines, phenols, indoles, and secondary bile acids (10). Putrefactive substances are harmful to the host (15). Kimura et al. (8) reported that disturbances in intestinal microbiota during scouring were only seen in some limited bacteria, and that the oral administration of a bifidobacteria preparation seemed to reinforce the recovery of normal intestinal microbiota and alleviate the clinical symptoms of animals being scourcd. Therefore, it appears that improvement of the composition of intestinal microbiota is important for the host’s health.

Prebio Support™ (PS; Meiji Feed Co., Ltd., Tokyo, Japan) was developed to improve the intestinal environment and protect calves against diarrhea. PS is a mixture of fermented products of Lactobacillus gasseri OLL2716 and Propionibacterium freudenreichii ET-3. PS contains 2 x $10^8$ killed Lactobacillus cells per gram; L. gasseri and P. freudenreichii are incubated separately using medium containing whey treated with enzymes. Although there have been several reports concerning the influences of nutrients (14, 16) and a viable bifidobacteria preparation (8) on the microbiota of calves, studies are still insufficient for confirmation of effect.

In the present study, an investigation of the influences of PS on intestinal microbiota and fecal metabolites was carried out using calves as subjects.

MATERIALS AND METHODS

Animal

The animals used in this study were 12 male calves bred at the Mito Experimental Dairy Farm of Meiji Feed Co., Ltd., Ibaraki-machi, Ibaraki, Japan. The calves were...
aged 4 days, and they were divided into two groups of six animals each. Calves used in this experiment were treated in accordance with Meiji Feed’s ethical guidelines for animal care and handling.

**Experimental design and diets**

The experimental schedule is shown in Table 1. A total of 500 g of a commercial milk replacer (Meiji Feed Co., Ltd., Tokyo, Japan) was given per day to each animal in both test groups. Two hundred fifty grams of the commercial milk replacer was dissolved in 1,800 ml of hot water and was given twice per day to each animal. Starter (Meiji Feed), which is a pellet containing grains as the main ingredients and is given in the shift from suckling to weaning, dried grass, and water were available ad libitum during the experimental period. Furthermore, a total of 20 g of PS was also given per day to each animal in the PS intake group between 4 and 42 days old. PS dissolved in water together with the milk replacer was administered. The number of fecal bifidobacteria at 21 days old was also measured using the real-time PCR method. Bacterial DNA was extracted from approximately 0.1 g of feces according to Godon et al. (2). Real-time PCR was performed with the MyiQ real-time PCR system (BIO-RAD, Tokyo, Japan). The reaction mixture (20 µl) contained 10 µl of the IQ SYBER Green Supermix (BIO-RAD), 0.5 µl of fecal DNA, and 400 µmol/l of each primer. For the quantification of *Bifidobacterium* genus-specific 16S rDNA, the primers g-Bifid-F (5’-CTCCTGGGAAACGGGTGG-3’) and g-Bifid-R (5’-GGTGTTCTTCCCGATATCTACA-3’) were used (9).

The thermal program consisted of an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec and 55°C for 30 sec, and elongation at 72°C for 40 sec. Melting curve analyses of the product were performed after the completion of amplifications to determine the specificity of the PCR. Dilutions of the genomic DNA from a known amount of *Bifidobacterium longum* ATCC15707T were used to construct calibration curves for calculation of the *Bifidobacterium* genus-specific 16S rDNA concentration in total fecal digesta DNA.

Fecal concentrations of short-chain fatty acids (acetic, butyric, and propionic acids) were analyzed using a high-performance liquid chromatography organic acid analysis system (HPLCOA, Shimadzu Co., Ltd., Kyoto, Japan) employing the method of Hara et al. (3).

### Table 1. Experimental schedule

| Items investigated: | Microbiota | Short-chain fatty acids | Ammonia | Sulfide | pH | Water content | Fecal hardness |
|---------------------|-----------|-------------------------|---------|---------|----|---------------|---------------|
|                      | ○         | ○                       | ○       | ○       | ○  | ○             | ○             |

*Intake of Prebio Support™

| Age of calf | 4 days | 21 days | 42 days |
|-------------|--------|---------|---------|
| Intake periods of Prebio Support™ | 1 day | 18 days | 39 days |
| Prebio support™ group | ← | Intake | → |
| Control group | ← | No intake | → |

*Investigation was performed.*
meter, IM-55g (DKK, TOA Corporation, Tokyo, Japan) with a sulfide electrode, S-2021 type (DKK, TOA), following the methods of Terada et al. (17). Fecal moisture, fecal ammonia, and fecal pH were measured, as described previously (1).

Observation of fecal hardness

During the experiment, the fecal hardness of all the calves was assessed. Fecal hardness was monitored using the following score: 1, diarrhea; 2, muddy to soft; 3, normal; 4, slightly hard; and 5, hard. Moderate hardness generally noted in healthy cattle and excreted smoothly was classed as normal.

Statistical analysis

Student’s t-test was used for statistical analysis of the fecal microbiota, pH, water content, and metabolic products, and the Mann-Whitney U test was also employed for analysis of the data on fecal hardness. A probability value of $p<0.05$ was considered significant.

RESULTS

Fecal microbiota analysis

The results regarding fecal microbiota are shown in Table 2. Through the intake of PS, the number of Bifidobacterium increased significantly at 21 days old. However, no significant changes in the numbers of other bacteria were noted between the two groups. According to the DNA analysis of feces, the number of Bifidobacterium in the PS group was also increased significantly compared with that of the control group at 21 days old.

Fecal metabolites

The influence of PS on the fecal metabolites is shown in Table 3. No significant changes in short-chain fatty acids (acetic, butyric, and propionic acids), fecal pH, and fecal sulfide were observed between the two test groups during the experimental period. On the other hand, the fecal ammonia concentration and fecal water content showed a significant difference between the two groups.

Fecal hardness

The fecal hardness of the calves administered PS differed from that of the control group, as shown in Table 3. The fecal characteristics of the PS group were more normal than those of the control group at 21 days old.

DISCUSSION

Our results show that the number of Bifidobacterium was higher in the PS group than in the control group.

| Age of calf | Prebio Support™ group | Control group | Prebio Support™ group | Control group |
|------------|-----------------------|---------------|-----------------------|---------------|
| 21 days    | 10.7 ± 0.1            | 10.6 ± 0.2    | 10.6 ± 0.2            | 10.6 ± 0.2    |
| 42 days    | 9.8 ± 0.5* (5/6)      | 9.3 ± 0.3 (4/6) | 9.6 ± 0.4 (4/6) | 8.9 ± 0.5 (4/6) |
| 21 days    | 9.7 ± 0.5*            | 8.6 ± 0.5     | Not done              | Not done      |
| 42 days    | 10.3 ± 0.3 (6/6)      | 10.2 ± 0.3 (6/6) | 10.3 ± 0.3 (6/6) | 10.5 ± 0.2 (6/6) |
| 21 days    | 9.8 ± 0.3 (6/6)       | 9.8 ± 0.3 (6/6) | 9.5 ± 0.4 (6/6) | 9.6 ± 0.6 (6/6) |
| 42 days    | 9.7 ± 0.3 (6/6)       | 9.6 ± 0.7 (6/6) | 9.4 ± 0.4 (6/6) | 9.4 ± 0.2 (6/6) |

*Values are expressed as the mean of the log number ± SD (CFU)/g wet feces (Number of samples detected/Number of samples tested).

bThe number of Bifidobacterium was measured using real-time PCR. Measurement was carried out using samples in which Bifidobacterium was detected employing the methods of Mitsuoka et al. (11–13). Values are expressed as the mean of the log number ± SD (cell)/g wet feces.

Signed significantly different ($p<0.05$) from the value of the control group at the same age.
Kimura et al. (8) reported that the changes seen in populations of lactobacilli, bifidobacteria, and Enterobacteriaceae in the lower intestine may be closely related to clinical symptoms. Kimura et al. (8) also reported that a viable bifidobacteria preparation has an anti-scouring effect in early weaned calves.

The intestinal microbiota play an important role in the host’s health, and it has reported that the maintenance of an optimal balance of intestinal microbiota is important for the health of the host (10). It has also been reported that bifidobacteria and lactobacilli, which are members of the intestinal flora family, are beneficial bacteria (10). Bifidobacterium and/or Lactobacillus are predominant members of the intestinal flora of humans and animals. In the present study, the administration of PS induced an increase in the numbers of bifidobacteria, and was related to the normalization of fecal hardness. These findings indicate that the administration of PS effectively improves the fecal microbiota, and there is a possibility of it alleviating clinical symptoms. The high number of bifidobacteria found in the calves of the PS intake group may be related to the administration of the fermented products of \textit{P. freudenreichii}. It is known that \textit{P. freudenreichii} produces bifidogenic growth stimulator (BGS) (6, 7). BGS is present in the cell-free filtrate and in the methanol extract fraction of starter (\textit{P. freudenreichii}) cells used in the manufacture of Swiss-type cheese (5). The chemical structure of BGS is 1,4-dihydroxy-2-naphthoic acid (DHNA), and DHNA stimulates the growth of bifidobacteria (4). PS contains DHNA.

Although the administration of PS did not help to decrease the numbers of clostridia and Enterobacteriaceae, the fecal water content and hardness of calves given PS were more normal than those in the control group at 21 days old. Since the level of fecal ammonia was lowered in the PS intake group, it seems that the metabolic activities of bacteria producing ammonia were reduced by the intake of PS.

Based on the results obtained from the present study, the oral administration of PS helps improve the fecal environment and normalize fecal hardness in calves. Further studies using larger numbers of animals are needed to confirm these results.

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