Running title: Fecal microbiota of calves fed trehalose

Effect of trehalose supplementation in milk replacer on the incidence of diarrhea and fecal microbiota in preweaned calves[1]

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ABSTRACT

Trehalose, a nonreducing disaccharide consisting of D-glucose with α,α-1,1 linkage, was evaluated as a functional material to improve the gut environment in preweaned calves. In Experiment 1, 173 calves were divided into two groups; the trehalose group was fed trehalose at 30 g/animal/day with milk replacer during the suckling period, and the control group was fed nonsupplemented milk replacer. Medication frequency was lower in the trehalose group ($P < 0.05$). In Experiment 2, calves (n = 20) were divided into two groups (control group, n = 10, and trehalose group, n = 10) based on their body weight and reared under the same feeding regimens as in Experiment 1. Fresh feces were collected from individual animals at the beginning of the trial (average age 11 days), 3 weeks after trehalose feeding (Experimental Day 22), and one day before weaning, and the fecal score was recorded daily. Fecal samples were analyzed for fermentation parameters and microbiota. Fecal score was significantly lower in the trehalose group than in the control group in the early stage (at an age of 14–18 days; $P < 0.05$) of the suckling period. Calves fed trehalose tended to have a higher proportion of fecal butyrate on Day 22 than calves in the control group ($P = 0.08$). Population sizes of *Clostridium* spp. were significantly lower ($P = 0.036$), whereas those of *Dialister* spp. and *Eubacterium* spp. tended to be higher in the feces of calves in the trehalose group on Day 22 ($P = 0.060$ and $P = 0.083$). These observations indicate that trehalose feeding modulated the gut environment and partially contributed to the reduction in medication frequency observed in Experiment 1.

Key words**:** calf, fecal microbiota, diarrhea, trehalose

List of abbreviations

VFA, volatile fatty acid

CP, crude protein

PCoA, principal coordinate analysis

OTU, operational taxonomic unit

INTRODUCTION

Calf health in the suckling period is an important aspect for future production of cattle. Prevention of infectious diseases, particularly diarrhea (Uetake, 2013), is one of the major concerns in calf management because innate and adaptive immune systems in preweaned calves are not sufficiently established (Weaver et al., 2000) and develop gradually by weaning (Jami et al., 2013; Steele et al., 2016).

Gut functions play a vital role not only in nutrient acquisition but also in defense against infectious pathogens (Bischoff, 2011). Gut microbiota is a key factor involved in the maintenance of the gut function (Yeoman and White, 2014). Oikonomou et al. (2013) suggested that the gut microbiota of preweaned calves were related to susceptibility to enteric infections. Therefore, appropriate management focusing on gut microbiota is considered effective for the healthy growth of preweaned calves. In this regard, feeding oligosaccharides is one of the useful approaches to maintain the stability of gut microbiota in calves (Ghosh and Mehla, 2012; Uyeno et al., 2015).

In this study, we focused on trehalose, a nonreducing disaccharide consisting of D-glucose with an α,α-1,1 bond. Studies have shown that trehalose feeding exhibited beneficial effects in poultry and dairy cattle, increased the growth of juvenile chicks by improving their intestinal innate immunity (Kikusato et al., 2016) and improved the antioxidative activity in the rumen fluid, blood, and milk of dairy cattle (Aoki et al., 2010; 2013) through the alteration of rumen microbiota rather than a direct effect of trehalose as an antioxidant. In addition to these beneficial effects on the poultry and dairy cattle, trehalose can be used as a functional material for preweaned calves through modification of the gut microbiota since trehalose has slower digestion and absorption rate in the human small intestine (Oku and Nakamura, 2000). On the other hand, our preliminary in-vitro experiment suggests that trehalose increases lactate production in calf small intestine (unpublished data). We hypothesized that the excess lactate that flowed into the large intestine is converted into butyrate by lactate-utilizing bacteria. Increased butyrate could exhibit beneficial effects contributing to the reduction of diarrhea incidences, such as the increase of mucin production (Canani et al., 2011) and attenuation of pathogenic bacteria (Namkung et al., 2011) in the calf gut. Therefore, we conducted this study to investigate the effects of trehalose feeding on the incidence of diarrhea in preweaned calves (Experiment 1), followed by an investigation of the fecal microbiota and fermentation parameters of preweaned calves to clarify the mode of action of trehalose as a functional material (Experiment 2).

MATERIALS AND METHODS

The calves (crossbreed of Japanese Black and Holstein) used for the two feeding trials in this study were raised in the same farm located in the northern part of Japan (Hokkaido, 44°06 N, 143°79 E). The farm was selected based on two criteria: a sufficient number of calves (c.a. 6,000 calves per year) were available for the experiment, and the experienced professional farmers keep detailed records about feeding management and health condition of individual calves. The animal protocol was conducted in accordance with the Guidelines for Animal Experiments and the Act on Welfare and Management of Animals, Hokkaido University.

Feeding trial 1 (Experiment 1)

A total of 173 crossbred calves with similar age and body weight were chosen and used in Experiment 1. The animals were blocked by birthdate and body weight and randomly assigned to either the control (n = 83) or the trehalose (n = 90) group (Table 1). The number of animals used in the present study was determined following the previous reports in which the effect of feeding oligosaccharides on calf performance was evaluated (Heinrich et al., 2003; Ghosh and Mehla., 2012). To maximize the animal number for the feeding trial, all calves raised in the experimental period (January 2012 to April 2012) at the same feeding area on the farm were subjected to the trial. Because of this technical reason, there was a difference in the number of calves between the control and trehalose groups, but as mentioned above, the animals were randomly allocated to each group. Feeding regimens were similar in both calf groups, with the exception of trehalose feeding. All calves were housed in individual calf hutches and fed 2.5 L of milk replacer (38 to 40°C) [27.0% crude protein (CP), 15.5% fat] by feeding bottle once a day at 0800 h. Trehalose was dissolved in a milk replacer, and calves in the trehalose group received 30-g trehalose per day via milk replacer throughout the suckling period. Based on the preliminary results, where feeding 5- or 10-g trehalose per day showed no remarkable beneficial or adverse effects on calf health and growth performance (unpublished data), the feeding level of trehalose (30 g/animal/day) in the present study was determined. Calf starter (20.0% CP, 10.0% crude fiber, 9.0% crude ash, and 2.0% crude fat), timothy hay (10.1% CP, 64.8% neutral detergent fiber, and 39.7% acid detergent fiber), and water were provided on an *ad libitum* basis. Calves were weaned when more than 1.5 kg of calf starter consumption was observed for three consecutive days. The date of weaning was recorded for each calf. As a part of health indices, the incidences of diarrhea and medications were recorded daily for individual animals. Fecal consistency was monitored daily by the experienced professional farmers as a part of routine work on the farm, and the feces showing primarily liquid appearance was recorded as diarrhea. Regarding the medications, when the animals displayed any symptoms of diseases such as diarrhea and respiratory disorder, a combination of antibiotics and antipyretics was administered. Similar to the monitoring of fecal consistency, the necessity of medication was carefully judged by the experienced professional farmers; blinding was not conducted in this experiment, though. The number of calves that experienced diarrhea (%), the average number of days with diarrhea (days/animal), the average frequency of medications (times/animal), and calf mortality (%) were calculated for the control and trehalose groups. In the calculation of medication frequency and mortality, not only diarrhea-associated causes but also other causes, including respiratory diseases, were counted.

Feeding trial 2 for elucidating the mode of action of trehalose (Experiment 2)

Experimental design

Male crossbred calves (n = 20) were used in Experiment 2. To perform precise fecal scoring and sampling, Experiment 2 was scaled-down compared to Experiment 1. The number of animals used in Experiment 2 was determined following the previous reports in which the effect of feeding oligosaccharides on the fecal microbiota of calves was evaluated (Uyeno et al., 2013). Calves were divided into two groups (control group, n = 10, and trehalose group, n = 10) on the day before the beginning of the trial (at the age of 10.6 ± 2.0 days) based on their body weight (Table S1). Calves were reared under the same housing condition and same feeding regimens as in Experiment 1. Body weight was measured at the beginning of the trial (Experimental Day 1) and one day before weaning (Experimental Day 55) using a digital body weight scale. Fresh feces were collected from individual animals on Day 1, three weeks after trehalose feeding (Experimental Day 22) and Day 55. Fecal samples were obtained by rectal stimulation of calves to defecate and collected directly into 50 mL tubes. Tubes containing the feces were immediately placed on the ice and then stored at −30°C until use. In Experiment 2, the fecal score was recorded daily by the experienced professional farmers according to the following criteria, as reported in the previous studies (Heinrichs et al., 2003; Ghosh and Mehla, 2012): 1 = normal feces; 2 = slightly liquid feces; 3 = moderately liquid feces; and 4 = primarily liquid feces. Blinding was not conducted in this experiment.

Measurement of fermentation parameters

For measuring pH and volatile fatty acid (VFA), 0.3-g feces was mixed with 800 μL of saline and centrifuged at 16,000 × *g* for 5 min at 4°C. The supernatant was subjected to pH measurement using a pH electrode (LAQUAtwin B-712; HORIBA, Kyoto, Japan) and VFA measurement using a gas chromatograph (GC-14B; Shimadzu, Kyoto, Japan), as described previously (Oh et al., 2017).

For measuring ammonia nitrogen (NH_3-N) and D-/L-lactate, 0.1-g feces was mixed with 500 μL of saline and centrifuged at 16,000 × *g* for 5 min at 4°C. NH_3-N levels were measured using the phenol–hypochlorite reaction method (Weatherburn, 1967). The levels of D-/L-lactate were measured using a commercial assay kit (Megazyme, Wicklow, Ireland). For measuring indole and skatole, 0.1-g feces was mixed with 1-mL 0.1 M phosphate-buffered saline and centrifuged at 16,000 × *g* for 5 min at 4°C. The assays for indole and skatole were conducted using colorimetric methods (Walstra et al., 1999).

Analysis of microbiota

Total DNA was extracted and purified from 0.3-g fecal sample using the repeated bead-beating plus column method (Yu and Morrison, 2004) using a commercial kit (QIAmp DNA Stool mini kit; Qiagen, Hilden, Germany). The DNA concentration was quantified by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, USA).

Total DNA was diluted to a final concentration of 5 ng/μL for all samples and subjected to the amplification of the V3–V4 region of 16S rRNA gene using the primer set of S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) (Herlemann et al., 2011). The PCR mixture consisted of 12.5 μL of 2 × KAPA HiFi HotStart Ready Mix (KAPA Biosystems, Wilmington, USA), 0.1 μM of each primer, and 2.5 μL of DNA (5 ng/μL). The PCR steps were performed according to the following program: initial denaturation at 95°C for 3 min, 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 5 min. Amplicons were purified using AMPure XP beads (Beckman-

Coulter, Brea, USA) and subjected to sequencing on the Illumina MiSeq platform (Illumina, San Diego, USA). Raw sequences have been deposited in the NCBI Sequence Read Archive under the accession no. DRA010638.

Data obtained from MiSeq sequencing were analyzed using the QIIME tool kit version 1.9.1 (Caporaso et al., 2010). Paired-end reads were merged and filtered to remove low-quality reads based on the quality scores (sequences with a quality score of >20 were retained for further analysis). The high-quality reads were assigned to operational taxonomic units (OTUs) at a 97% identity threshold using the UCLUST algorithm (Edgar, 2010), and taxonomy was assigned using the latest Greengenes database (v.13_8). The taxonomic identification was performed at the genus level. Alpha diversity indices, including Chao1, Shannon, ACE, and Simpson, were calculated using the QIIME. The relative abundance of each bacterial taxon was calculated by dividing the number of reads assigned to each bacterial taxon by the total number of reads. Only taxa with a relative abundance of >0.1% in at least 5 individuals in either control and trehalose groups on Day 22 or Day 55 were considered as being observed and used for the analysis. Principal coordinate analysis (PCoA) was used to determine the differences in the microbial community structure based on the Bray–Curtis dissimilarity matrices at the genus level using the vegan package version 2.5.6 (Oksanen et al., 2019) in R.

Quantitative real-time PCR was performed to determine the population sizes of major gut bacterial groups, including Ruminococcaceae, *Bacteroides–Prevotella–Porphyromonas* group, *Prevotella* spp., *Faecalibacterium prausnitzii*, *Lactobacillus* spp., *Clostridium coccoides-Eubacterium rectale* group, *Bifidobacterium* spp., *Escherichia* *coli*, *Akkermansia muciniphila*, *Clostridium perfringens*, *Eubacterium hallii*, *Streptococcus bovis*, and *Megasphaera elsdenii*, using a LightCycler system with a KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Charlestown, USA) with the respective primer sets (Table S2). The PCR thermal conditions and the PCR mixture were the same as those reported earlier (Myint et al., 2017). The population size of each bacterial target was expressed as the proportion (%) of the abundance of 16S rRNA genes of each bacterial target against that of the total bacteria.

Statistical analysis

The ages of calves, body weight, number of days with diarrhea, frequency of medications, fecal score, fecal fermentation parameters, alpha diversity indices in fecal microbiota, and the bacterial abundance quantified by MiSeq and real-time PCR were compared between the control and trehalose groups by Welch's t-test using R version 3.6.2 (R Core Team, 2019). Prior to performing Welch's t-test, the same data set was analyzed with a repeated measures model with the fixed effects of treatment, day, and treatment × day interaction and the random effect of calf within the groups. Results showed the greater effect of day in all data, while the effects of treatment and interaction between treatment × day were less apparent (Table S3). To clarify the effect of feeding trehalose at respective sampling points (i.e., Day 22 and Day 55), the values were compared between the control and trehalose groups using Welch's t-test. The number of calves that experienced diarrhea and calf mortality were compared (control vs. trehalose groups) by the Chi-squared test using R. *P*-values of <0.05 and <0.10 were considered to be statistically significant and trend, respectively. The Pearson correlation between the molar ratio of each VFA and the bacterial abundance quantified by MiSeq was analyzed using the corrplot package version 0.84 (Wei and Viliam, 2017) in R.

RESULTS

Analysis of the effects of trehalose feeding on the incidence of diarrhea in preweaned calves (Experiment 1)

Table 1 shows the results obtained from Experiment 1. There were no differences in the weaning age, number of calves that experienced diarrhea, and mortality between the feeding groups. However, medication frequency was significantly lower in the trehalose group than in the control group ($P < 0.05$).

Feeding trial 2 for elucidating the mode of action of trehalose (Experiment 2)

Growth and fecal fermentation parameters

The growth of calves and the medication frequency observed in Experiment 2 were not different between the feeding groups (Table S1). The average fecal score during the suckling period is depicted in Figure 1. In the trehalose group, the average fecal score was significantly lower at Experimental Days 4 and 8 ($P < 0.05$) and tended to be lower at Experimental Days 5 and 6 than those in the control group ($P < 0.10$). The repeated-measures model analysis also supported this time-dependent effect; there was a significant interaction between treatment and day ($P = 0.001$).

The fecal fermentation parameters are presented in Table 2. There were no significant differences in fecal pH, total VFA, ammonia, total lactate, indole, and skatole concentrations between the control and trehalose groups. The proportion of butyrate in the trehalose group tended to be higher on Day 22 (P = 0.080), whereas that of valerate was significantly lower on Day 55 ($P < 0.05$).

Community structure and composition of fecal microbiota

Amplicon sequencing of the fecal samples yielded a total of 878,751 high-quality reads, with an average of 21,968 ± 5,532 reads per sample. No differences were observed in the alpha diversity indices (Chao1, ACE, Shannon, and Simpson) between the two groups on Day 22 and Day 55 (Table S4). Although the PCoA revealed a distinction between Day 22 and Day 55, there was no clear difference in the microbial composition between the control and trehalose groups (Figure S1).

A total of 43 OTUs at the genus level were detected from the fecal samples (Table S5). The bacterial taxa exhibiting statistical significance or trend in the relative abundance are listed in Table 3. On Day 22, the relative abundance of *Clostridium* spp. was lower, whereas those of *Dialister* spp. and putative [Eubacterium] were higher in the trehalose group than in the control group. On Day 55, the proportions of unclassified S24-7, putative [Prevotella], *Coprococcus* spp., *Odoribacter* spp., *Dialister* spp., and *Lachnospira* spp. were higher in the trehalose group, whereas the proportion of unclassified Coriobacteriaceae was lower in the trehalose group. A correlation analysis was conducted between the molar ratio of each VFA and bacterial abundance quantified by MiSeq to explore the bacterial taxa related to butyrate proportion (Figure 2). The results showed that the butyrate proportion correlated negatively with the relative abundance of *Clostridium* spp. but positively with *Bifidobacterium* spp..

The population sizes of major gut bacterial groups quantified by real-time PCR are depicted in Figure 3. On Day 22, the population sizes of targeted bacterial groups were not significantly different, whereas the prevalence of *Clostridium perfringens* was lower in the trehalose group; two calves in the control group harbored this species, whereas it was not detected in calves in the trehalose group (Figure 3). On Day 55, the population size of Ruminococcaceae tended to be lower in the trehalose group than in the control group (P = 0.09).

DISCUSSION

Existing literature reports the beneficial effects of trehalose feeding in poultry (Kikusato et al., 2016) and dairy cattle (Aoki et al., 2010; 2013). In the present study, we evaluated trehalose as a functional material for preweaned calves. Lower medication frequency in the trehalose group was confirmed in Experiment 1. Although this result suggests the improvement of calf health by trehalose feeding, the mode of action needs to be further investigated. It has been suggested that the severe disease which requires antibiotic treatment during the preweaning period of calves leads to less milk production in their lifetime (Soberon et al., 2010). Therefore, reducing the medication frequency (i.e., less antibiotic administration) via trehalose feeding could be beneficial in terms of the lifetime productivity of cattle. The calves in the present study were fed milk replacer once a day, and this feeding management is not common. Although the farm used in the present study has been adopting this feeding management without adverse effects (i.e., comparable growth and health conditions with multiple feedings), the optimum amount of trehalose under different feeding management needs to be considered.

Although the average number of days with diarrhea was numerically higher in the trehalose group in Experiment 1, we speculated that diarrhea in the trehalose group was a mild symptom that did not require medications. In fact, improved fecal score was confirmed in Experiment 2 of this study. Earlier studies have reported that feeding with mannan oligosaccharides (MOS) improves the fecal score of calves (Heinrichs et al., 2003; Ghosh and Mehla, 2012). MOS is known to inhibit the attachment of infectious pathogens to the gut epithelium, thus leading to the improvement of animal health (Spring et al., 2000). In contrast, Uyeno et al. (2013) suggested that cello-oligosaccharides primarily composed of cellobiose modulate the gut environment with higher population size of *Clostridium coccoides–Eubacterium rectale* group and butyrate concentration. Similar to cellobiose, trehalose is a dimer of D-glucose, and the improvement in fecal score by trehalose is probably due to the modulation of gut microbiota rather than due to the direct inhibition of infectious pathogens.

To elucidate the mechanism of modulation of the gut environment by trehalose feeding, a further feeding trial was conducted as Experiment 2 for fecal analysis. The trehalose group showed an increase in butyrate levels at 3 weeks after trehalose feeding (Day 22). Butyrate is the primary energy source of gut epithelial cells, which mediate the absorption of water, mineral, and nutrients (Bedford and Gong, 2018). Moreover, butyrate increases the expression of mucin protein in the gut epithelia (Canani et al., 2011). The mucosal layer in the gut plays a key role in preventing the invasion of infectious pathogens (Johansson et al., 2011). Therefore, the increase in butyrate levels by trehalose feeding may contribute to the maintenance of the gut function, which reduces the severity of diarrhea.

The alpha diversity indices and the PCoA plot of fecal microbiota indicated that there were no remarkable alterations in the microbial community structures by trehalose feeding. However, some bacterial groups changed their abundances, and a decrease in *Clostridium* spp. abundance and an increase in *Dialister* spp. and putative [Eubacterium] abundances were observed in the trehalose group on Day 22. It has been reported that *Clostridium* spp. was detected at a higher abundance in the feces of diarrheic cattle (Zeineldin et al., 2018). Some *Clostridium* species are considered as pathogenic (e.g., *C. perfringens* and *C. difficile*) (Blanchard, 2012; Cho and Yoon, 2014). Therefore, the decrease in *Clostridium* spp. abundance by trehalose feeding can contribute to the prevention of infectious diseases and may partially explain the lowered medication frequency in Experiment 1. A negative correlation between the abundance of *Clostridium* spp. and the butyrate molar ratio was observed in Experiment 2, and the reduction in *Clostridium* spp. abundance by trehalose feeding might be attributed to butyrate enhancement. Namkung et al. (2011) reported that butyrate attenuated *C. perfringens in vitro*. In Experiment 2 of the present study, none of the calves fed trehalose harbored *C. perfringens*, whereas this bacterium was detected from two calves in the control group.

Altogether, an increase in butyrate level by trehalose feeding can be one of the factors contributing to the beneficial effect of trehalose.

Eubacterium spp. are considered as beneficial bacteria producing butyrate from lactate in the animal gut (Rivière et al., 2016). Therefore, an increase in *Eubacterium spp.* abundance by trehalose feeding probably contributed to butyrate enhancement on Day 22. Judging from the positive correlation between the relative abundance of *Bifidobacterium* spp. and the butyrate proportion observed in Experiment 2, lactate produced by *Bifidobacterium* spp. could act as a substrate for butyrate production by *Eubacterium* spp. However, the elevation of *Bifidobacterium* spp. abundance in the trehalose group was not observed in both the MiSeq sequencing and real-time PCR quantification results. Therefore, we hypothesized that lactate was produced in the small intestine where lactic acid bacteria are abundantly colonized (Malmuthuge et al., 2014). Recently, it is reported that trehalose supplementation increased the abundance of *Lactobacillus* spp. in the duodenum and jejunum of broilers (Wu et al., 2020). We conducted batch culture tests using the jejunum content of Holstein calves and confirmed a remarkable increase in lactate levels (unpublished data). This increase in lactate levels indicates that trehalose was used by lactic acid bacteria in the small intestine of calves. These findings suggest that lactate production in the small intestine was stimulated by trehalose, and the excess lactate that flowed into the large intestine was metabolized by lactate-utilizing bacteria such as *Eubacterium* spp.

Previous studies indicate the positive correlation between ruminal or fecal abundance of *Dialister* spp. and the calf performance such as body weight, feed intake and feed efficiency (Myer et al., 2015; Meale et al., 2017). Wang et al. (2016) reported that *Dialister* spp. in the rumen might be involved in starch and fiber digestion and contribute to VFA production. Although the role of *Dialister* spp. in the calf gut is not completely understood, previous studies have reported a higher abundance of *Dialister* spp. in healthy individuals than in patients with colorectal cancer or Crohn’s disease (Joossens et al., 2011; Weir et al., 2013). Therefore, *Dialister* spp. is considered to contribute to host health, and an increased abundance of this bacterial genus by trehalose feeding might be beneficial for the gut environment of calves.

In this study, on Day 55 (one day before weaning), there were increases in the abundance of unclassified S24-7, putative [Prevotella], *Coprococcus* spp., *Odoribacter* spp., *Dialister* spp., and *Lachnospira* spp. in the trehalose group. Studies have shown the involvement of unclassified S24-7 in cellulose digestion in the gastrointestinal tract (Naas et al., 2014; de Mulder et al., 2017). *Prevotella* and *Lachnospira* genera are also considered to be involved in fiber digestion (Bryant and Small, 1956; Matsui et al., 2000). The increase in the abundance of fiber-degrading bacteria by trehalose feeding on Day 55 potentially stimulates the utilization of dietary fiber in the large intestine after weaning.

There are some limitations in the present study; the feeding trials were conducted in the commercial farm, and experienced professional farmers monitored fecal consistency as a part of routine work on the farm without blinding. Since early detection of diarrhea in the calves is one of the most important tasks of the farm manager to prevent severe illness that directly impacts animal productivity, fecal scoring was performed with stringent criteria. However, the beneficial effect of feeding trehalose to the calves (reduction of medication frequency and fecal score improvement) needs to be further evaluated by a blinding test with a trained person for the research purpose. Although the effect of feeding trehalose was detected at respective sampling points, the treatment effect was not clearly observed in the statistical analysis with the repeated measure model. This was probably due to the individual differences in fermentation parameters and microbiota of the calves. Improvement of the gut environment by feeding trehalose needs to be further clarified by the studies with larger sample size.

In conclusion, trehalose feeding reduced the medication frequency of preweaned calves, and the growth inhibition of pathogenic bacteria through butyrate enhancement might be a possible mode of action causing these beneficial effects. Although further investigation using pure culture of gut bacteria is necessary to elucidate the details, trehalose can be used as a functional material for calf production.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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TABLES AND FIGURES

Table 1. Effect of trehalose feeding on the incidence of diarrhea, medication frequency, and mortality in the calves (Experiment 1)

Item	Control	Trehalose	*P*-value [1]
Number of calves	83	90	NA
Age at the beginning of trial, days	19.19 ± 7.68	19.80 ± 8.69	0.628
Body weight at the beginning of trial, kg	47.51 ± 5.54	48.20 ± 5.88	0.427
Age at weaning, days [2]	72.96 ± 14.30	71.51 ± 14.08	0.511
Number of calves that experienced diarrhea (%) [3]	16/83 (19.2)	22/90 (24.4)	0.524
Average number of days with diarrhea, days/animal [3,4]	2.81 ± 1.38	4.59 ± 4.62	0.108
Average frequency of medications, times/animal [5,6]	3.68 ± 4.70	2.38 ± 3.54	0.049
Mortality (%) [6]	3/83 (3.6)	2/90 (2.2)	0.926

Values are shown as mean ± SD.

NA: not applicable

[1] *P*-values were calculated based on Welch's t-test.

[2] Calves were weaned when more than 1.5 kg of calf starter consumption was observed for 3 consecutive days.

[3] Fecal consistency was monitored daily by experienced professional farmers as a part of routine work on the farm, and the feces showing primarily liquid appearance was recorded as diarrhea.

[4] The sum of the number of days with diarrhea in the calves that experienced diarrhea was normalized by the number of calves that experienced diarrhea.

[5] Total frequency of medications in each herd was normalized by total animals in each herd. The necessity of medication was carefully judged by the experienced professional farmers.

[6] Not only diarrhea-related causes but also other causes, including respiratory diseases, were counted.

Table 2. Effect of trehalose feeding on fecal fermentation parameters in the calves at Day 22 and Day 55 (Experiment 2)

Item	Day 22			Day 55		
	Control	Trehalose	*P*-value [1]	Control	Trehalose	*P*-value [1]
pH	7.15 ± 0.49	7.23 ± 0.48	0.734	7.66 ± 0.30	7.55 ± 0.43	0.553
Total VFA, µmol/g feces	48.9 ± 11.9	56.0 ± 17.9	0.334	76.3 ± 15.9	84.5 ± 34.2	0.525
Acetate, mol/100 mol	55.5 ± 5.0	52.9 ± 5.3	0.305	60.0 ± 5.4	58.4 ± 2.7	0.431
Propionate, mol/100 mol	27.6 ± 3.0	28.0 ± 3.5	0.814	25.9 ± 2.4	27.8 ± 3.5	0.207
Butyrate, mol/100 mol	11.9 ± 2.7	14.5 ± 3.3	0.080	8.8 ± 1.6	9.4 ± 1.8	0.516
Isobutyrate, mol/100 mol	1.2 ± 0.7	1.6 ± 1.2	0.425	1.4 ± 0.8	1.9 ± 1.0	0.219
Isovalerate, mol/100 mol	1.5 ± 0.9	1.9 ± 1.7	0.640	1.5 ± 1.0	1.4 ± 1.1	0.762
Valerate, mol/100 mol	2.3 ± 1.8	1.2 ± 1.2	0.131	2.3 ± 1.5	1.2 ± 0.6	0.047
Ammonia, µgN/g feces	0.27 ± 0.12	0.30 ± 0.34	0.831	0.21 ± 0.12	0.23 ± 0.14	0.782
Total lactate, µmol/g feces	5.51 ± 3.14	6.31 ± 7.71	0.779	14.59 ± 5.67	13.74 ± 8.94	0.815
Indole, µg/g feces	12.67 ± 8.52	14.02 ± 18.54	0.846	6.62 ± 3.33	7.77 ± 3.32	0.474
Skatole, µg/g feces	104.8 ± 46.4	119.5 ± 101.3	0.700	77.4 ± 30.5	87.9 ± 30.4	0.474

Values are wet weight basis and shown as mean ± SD.

[1] Each parameter was compared between control and trehalose groups at respective sampling points. *P*-values were calculated by Welch's t-test.

Table 3. Effect of trehalose feeding on fecal microbial abundances in the calves at Day 22 and Day 55 (Experiment 2)

Taxa [1]	Control	Trehalose	*P*-value [2]
Day 22			
Clostridium spp.	1.575 ± 1.347	0.448 ± 0.390	0.036
Dialister spp.	0.379 ± 0.693	3.680 ± 4.577	0.060
[Eubacterium]	0.128 ± 0.119	0.352 ± 0.332	0.083
Day 55			
Unclassified S24-7	10.268 ± 3.309	15.511 ± 5.709	0.031
[Prevotella]	2.894 ± 2.758	5.930 ± 2.765	0.031
Unclassified Coriobacteriaceae	0.773 ± 0.349	0.435 ± 0.209	0.025
Coprococcus spp.	0.506 ± 0.305	0.850 ± 0.493	0.095
Odoribacter spp.	0.151 ± 0.072	0.314 ± 0.229	0.068
Dialister spp.	0.036 ± 0.038	0.182 ± 0.214	0.073
Lachnospira spp.	0.025 ± 0.027	0.110 ± 0.095	0.027

Abundance of bacterial taxa with a relative abundance of >0.1% in at least 5 individuals in either control and trehalose groups on Day 22 or Day 55 were compared between groups at respective sampling points.

Name of taxa with square brackets indicates putative assignment.

Values mean relative abundance (% of total reads) and are shown as mean ± SD.

[1] Bacterial taxa showing statistical significance or trend in the relative abundance are listed.

[2] *P*-values were calculated by Welch’s t-test.

Figure legends

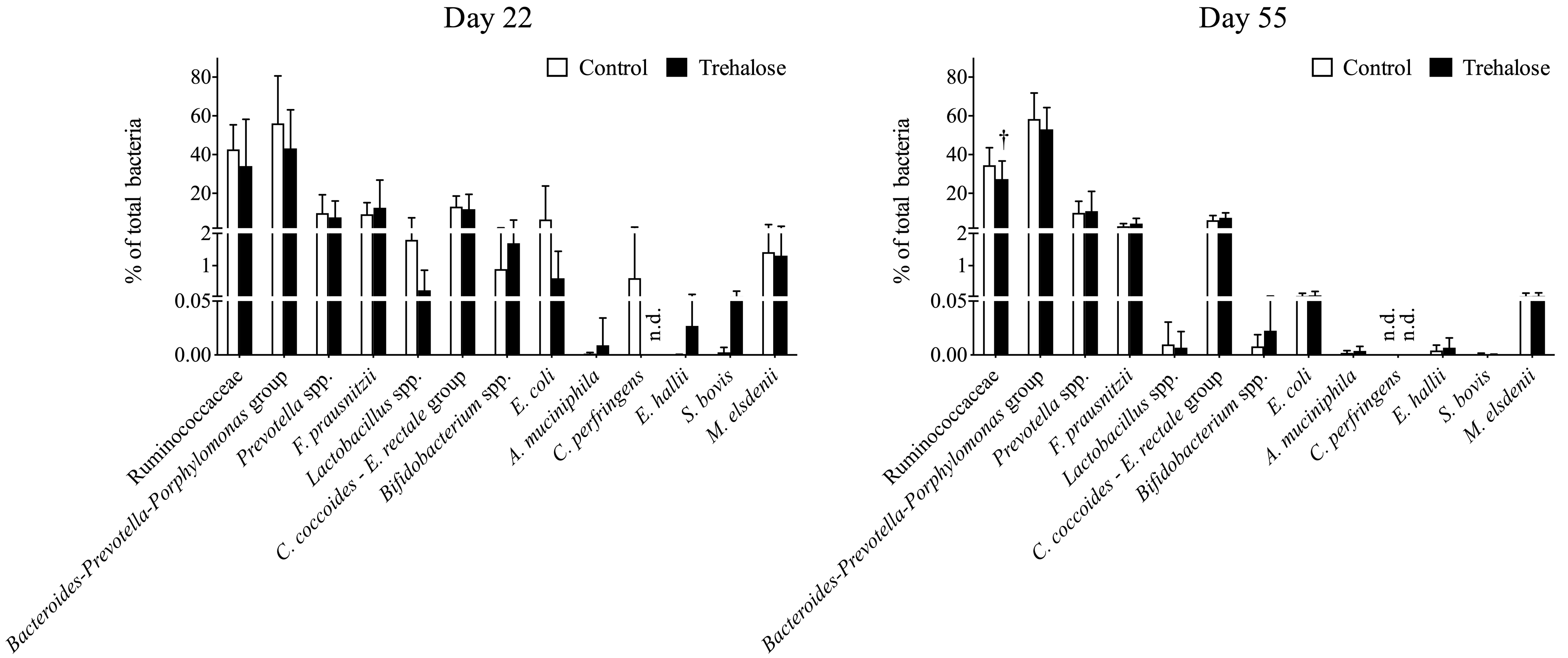
Figure 1. Effect of trehalose feeding on the average fecal score of calves during the suckling period in Experiment 2. Fecal score was recorded daily by the experienced professional farmers as a part of routine work on the farm according to the following criteria: 1 = normal feces, 2 = slightly liquid feces, 3 = moderately liquid feces, and 4 = primarily liquid feces. Average ages at Experimental Day 1 were 11.9 ± 2.0 days for the control group and 11.4 ± 2.0 days for the trehalose group (Table S1).
*$P < 0.05$ and †$P < 0.10$ (compared between control and trehalose group at respective days by Welch's t-test).

Figure 2. Pearson correlation between proportion of VFA and bacterial abundance in the feces of calves at Day 22 (Experiment 2). The values for respective VFA represents proportion against total VFA (mol/100 mol). Bacterial abundance represents relative abundance (% of total reads). Taxa with a relative abundance of >0.1% in at least 5 individuals in either control and trehalose groups on Day 22 or Day 55 were included in the matrix. Name of taxa with square brackets indicates putative assignment. Only the plots showing significance or trend are indicated in the panel. The scale colors indicate the correlation; positive (closer to 1, red circle) or negative (closer to -1, blue circle).

Figure 3. Effect of trehalose feeding on population sizes of major gut bacterial groups in the feces of calves at Day 22 and Day 55 determined by real-time PCR (Experiment 2). The population size of each bacterial target was expressed as the proportion (%) of the abundance of 16S rRNA genes of each bacterial target against that of the total bacteria. Error bars indicate SD.
*$P < 0.05$ and †$P < 0.10$ (compared between control and trehalose group at respective days by Welch's t-test).
n.d.: not detected


Control
Trehalose
Fecal score
4
3
2
1
*
†
†
*
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
Experimental Day


(% of total reads)
Prevotella
Bacteroides
Unclassified Clostridiales
Faecalibacterium
Unclassified Ruminococcaceae
Unclassified S24-7
[Prevotella]
Unclassified Enterobacteriaceae
Phascolarctobacterium
Ruminococcus
Blautia
Unclassified Lachnospiraceae
Oscillospira
Unclassified Bacteroidales
Parabacteroides
Clostridium
[Ruminococcus]
Butyricicoccus
Megasphaera
Succinivibrio
Dorea
Sutterella
Collinsella
Unclassified Coriobacteriaceae
Unclassified Clostridiaceae
Anaerovibrio
Lachnobacterium
Unclassified Erysipelotrichaceae
Bifidobacterium
Dialister
Megamonas
Coprococcus
CF231
Mitsuokella
Odoribacter
p-75-a5
[Eubacterium]
Unclassified YS2
Lachnospira
Unclassified Rikenellaceae
Unclassified RF39
Desulfovibrio
Unclassified Christensenellaceae
Acetate
Propionate
Butyrate
Isobutyrate
Isovalerate
Valerate
(mol/100mol)
1.0
0
-1.0



Day 22
Day 55
Control
Trehalose
% of total bacteria
80
60
40
20
2
1
0.05
0.00
†
n.d.
Ruminococcaceae
Bacteroides-Prevotella-Porphylomonas group
Prevotella spp.
F. prausnitzii
Lactobacillus spp.
C. coccoides - E. rectale group
Bifidobacterium spp.
E. coli
A. muciniphila
C. perfringens
E. hallii
S. bovis
M. elsdenii