Direct evidence of preventive effect of severe diarrhea in calf by milk replacer-based probiotic feeding

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Abstract

Background Diarrhea is one of the major causes of death in calves directly linked to economic loss in cattle industry. Fermented milk has been widely used for calf feeding to reduce the diarrhea in clinical settings. However, the fermentation of raw milk has many difficulties, such as unstable fermentation due to large variances of nutrient composition in raw milk and contamination with coliform. Practical use of fermented milk replacer (FMR) has a strong potential to overcome these difficulties. However, the clinical efficacy of FMR on calf diarrhea is still unclear.

Result In this study, we developed a stable condition of fermentation for FMR and verified the preventive effects of FMR feeding on calf diarrhea by an experimental infection model of bovine rotavirus (BRV) in newborn calf and a field study in dairy farms with calf diarrhea. Additionally, the clinical efficacy of lactic acid bacteria (LAB)-supplemented milk replacer (LAB-MR) was also evaluated in the experimental infection model. In the experimental infection model, control calves fed a milk replacer showed severe watery diarrhea after BRV challenge. Two out of three control calves were died after rapid decline of milk intake. In contrast, calves fed FMR or high-concentrated LAB-MR showed diarrhea, but the water content of feces was low and stable. In addition, the amount of milk intake decreased temporarily, but recovered immediately in the FMR- or LAB-MR-fed calves. Histopathological analysis of intestinal tracts revealed mucosal lesions were slighter in the FMR- or LAB-MR-fed calves than in the control calves. In a field study of FMR feeding in dairy farms with mixed infection with BRV and *Cryptosporidium parvum*, the incidence of enteritis and mortality of calf by enteritis were significantly reduced by the allocation of FMR. Additionally, the FMR-fed calves exhibited shorter duration of treatment, fewer consultations, and lower cost of medical care for enteritis compared with control calves.

Conclusion These results suggest that feeding of milk replacer-based probiotics to calves reduces severity of diarrhea and tissue damage to intestinal tracts caused by BRV infection and provides significant clinical benefits for the prevention and treatment of calf diarrhea.

Introduction

Calf diarrhea is one of the common diseases in calf, caused by infectious factors such as viral, bacterial and protozoan, and non-infectious factors such as dietary and nervous, and causes severe economic losses in cattle industry. Among those factors, bovine rotavirus (BRV) infection and bovine cryptosporidiosis are prevalent among cattle in Japan, and cause severe diarrhea in calf [1]. Bovine rotavirus infects cattle of all ages, but calf has a higher incidence and more severe symptoms [2]. Bovine cryptosporidiosis with diarrhea caused by *Cryptosporidium parvum* results in decreased appetite, weakness, and dehydration in calf [3]. Although mortality from single infections is low, it is a serious problem in the dairy farm because mixed infections including rotavirus have increased the mortality. Unfortunately, no effective vaccines have been developed against *C. parvum* infection. The infected calves exhibit diarrhea during the developmental period, causing growth retardation, and the resulting economic attrition has been a problem [4]. Antibiotics are useful chemotherapeutic agents for the treatment of bovine bacterial diarrhea [5]. However, they are not expected to be effective for viral or protozoan diarrhea in addition to the problem of the emergence of antibiotic-resistant bacteria [6]. Therefore, a novel alternative preventive strategy for bovine diarrhea is required.

Probiotics are defined as living microorganisms that have a health effect on the host, or foods containing the same. Various clinical effects have been found in humans, including not only improved stool and improved balance of intestinal resident bacteria, but also allergy-reducing, influenza infection-preventing, and *H. pylori*-suppressing effects [7–9]. A previous report indicated the use of probiotics (lactic acid bacteria) to prevent infectious diseases in newborns born in rural India, where many children are still dying from infectious diseases [10]. The results were better than expected, and the combined index of sepsis and death showed a significant drop from 9.4–5.4%, with a 40% reduction. In addition, the effect was high when only sepsis, in which bacteria were detected in the blood, was clearly high, with a reduction effect of 70% or more. It is also reported that it is effective in preventing other infectious diseases. This strongly suggests that probiotics can also be applied to disease control in calves that often die from infectious diseases. However, the use of probiotics as a supportive therapy in veterinary field is still limited and incredible because the efficacies of probiotics vary and are inconsistent. Indeed, the immunostimulation method using probiotics such as lactic acid bacteria (LAB) has been applied for a long time in the field of animal husbandry [11, 12], but its effect up to the prevention of infectious diseases is inevitable. In addition, although a test of administration of fermented milk to calves using fresh milk has been reported, in this case, coliform also grows at the same time as useful lactic acid bacteria, and possibly causes severe diarrhea. Therefore, we modified the previous method as novel fermented milk production technology using milk replacer with high nutritional value without the risk of coliform contamination. And then, we verified the anti-diarrheal effects of the two MR-based probiotics, fermented milk replacer (FMR) and Lactic acid bacteria (LAB)-supplied milk replacer (LAB-MR), using experimental infection model of bovine rotavirus in calves.

Materials And Methods

Preparation and component analysis of fermented milk replacer

A probiotics mixed feed BIO-THREE Ace (Toa Biopharma, Tokyo, Japan) including *Streptococcus faecalis* T-110 strain (1×10^8 CFU/g), *Clostridium butyricum* TO-A strain (1×10^6 CFU/g), *Bacillus mesentericus* TO-A strain (1×10^6 CFU/g) was added to milk replacer (GREATBABY; Nosan Corporation, Yokohama, Japan) prepared at 3.5- and 7-fold concentrations. The 1% mixtures were incubated at room temperature (22–25 °C) until 5 days, and the pH, glucose and the number of bacteria were chronologically examined. The pH of the mixture was measured with a pH meter (AS-600; AS ONE, Osaka, Japan). The glucose in the mixture was evaluated by Uro Paper III (Eiken Chemical, Tokyo, Japan). The bacteria in the mixture were isolated by using 5% sheep blood agar plate (Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C overnight and then morphologically identified, and numbers of bacteria were counted.

Experimental trial of probiotic treatment in BRV-challenged calves

The animal experiments were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Hokkaido University (No. 18–0147). Newborn calves (0–3 days old) without history of colostrum feeding were launched for this experiment (Table 1) and kept separately at the isolated rooms in a biosafety level II animal facility at the Faculty of Veterinary Medicine, Hokkaido University (Sapporo, Japan). In this experiment, animals were divided into three feeding groups: 1) animals fed milk replacer, 2) FMR, and 3) LAB-MR. The first group was fed milk replacer (GREATBABY; Nosan Corporation) at 7-fold dilution. The second group was fed FMR at 7-fold dilution. FMR was prepared by the fermentation of a milk replacer (3.5-fold dilution, GREATBABY; Nosan Corporation) supplemented with 1% of BIO-THREE Ace (Toa Biopharma) at 22–25 °C for 2 days. For the third group, another milk replacer (YUKIMIRUKU, Snow Brand Seed, Sapporo, Japan) including 1.3×10^7 CFU/kg/day of *Lactobacillus plantarum* (HOKKAIDO strain; Food Processing Research Center, Hokkaido Research Organization, Ebetsu, Japan; Japanese Patent number 3925502) was fed to an animal as low-concentrated LAB-MR. The other two animals were fed high-concentrated LAB-MR prepared from the milk replacer (YUKIMIRUKU) additionally supplemented with *L. plantarum* HOKKAIDO strain (2.5×10^9 CFU/kg/day). LAB-MR was immediately fed to calves after the preparation without fermentation. Amount of the feeding was determined by their body weight. All of the animals were fed 7-fold diluted MR, FMR, or LAB-MR twice per day.

On the fifth day of the experiment, the animals were inoculated orally with fecal samples from an infected calf containing bovine rotavirus (G6P[11], 3.2×10^6 $TCID_{50}$/dose) on the fifth day after the start of feeding. The titer of BRV in the fecal samples was determined by CPE assay using MA104 cell line as described previously (XX et al., 19xx). The feedings of them were continued until 10 days after the virus inoculation. The tested calves were monitored vital signs, milk intake, and clinical score of diarrheas. In addition, fresh feces were collected on each day and measured the water content in the feces by dry weight method using microwave. Furthermore, on day 10, the intestinal tract of the tested calves was collected, and the presence or absence and degree of histological damage were histopathologically evaluated.

Field investigation

To evaluate the efficacy of FMR feeding in the clinical setting, a field trial of FMR treatment to newborn calves was conducted in two dairy farms with history of high prevalence of BRV and bovine cryptosporidiosis over three years. BRV infection was diagnosed by the detection of viral antigen in feces using a commercial immunochromatography kit for group A rotavirus (DIPSTICK ROTA; Eiken Chemical, Tokyo, Japan) [13]. Bovine cryptosporidiosis was diagnosed by the microscopic identification of oocysts in feces collected by sucrose density gradient centrifugation. In Farm A, male and female newborn calves of Holstein and the crossbred (Japanese Black × Holstein cattle) were enrolled for this experiment. In Farm B, male and female newborn Holstein calves were enrolled for this experiment. FMR were prepared by the fermentation of a milk replacer (GREATBABY; Nosan Corporation) supplemented with 1% of BIO-THREE Ace (Toa Biopharma) as shown above in each farm. The enrolled calves were fed colostrum just after birth and the milk replacer (control) or FMR twice per day from birth to weaning. The incidence of enteritis and mortality by enteritis was monitored in the calves until weaning. Additionally, duration of a series of treatments for enteritis, the number of consultations during a single series of treatments, total medical consultation fee required for the treatment of enteritis and, the age of onset of enteritis were retrospectively investigated after the field study completed.

Statistics

Significant associations between treatments and clinical outcomes were determined by Fisher's exact test. Statistical significances between two treatment groups was determined by Mann-Whitney U test. All statistical tests were performed with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). A *p*-value < 0.05 was considered statistically significant.

Results

Component analysis of fermented milk replacer

According to the Ministerial Ordinance on Milk and Milk Products Concerning Compositional Standards, etc. (https://elaws.e-gov.go.jp/search/elawsSearch/elaws_search/lsg0500/detail?lawId=326M50000100052), fermented milk must meet the following criteria: 1) Number of lactic acid bacteria is more than 10^7 CFU/mL, 2) The pH is less than 5.3, 3) Coliform should be negative. In this study, we evaluated FMR based on the criteria. 7-fold FMR supplemented with BIO-THREE Ace was quickly fermented with pH 4.86 on day 1, and pH 4.78 on day 2 (Fig. 1A). On day 3, pH of the mixture was 3.75, and the acidity increased rapidly indicating not suitable for feeding due to its too sour taste (Fig. 1A). 3.5-fold FMR supplemented with BIO-THREE Ace was fermented with pH 5.95 on day 1, pH 5.0 on day 2, pH 4.80 on day 3 and pH 4.6 on day 5 (Fig. 1B). The number of LAB (*Streptococcus* spp.) increased to 10^7 CFU/mL in 3.5-fold FMR on day 1 (Fig. 1A, B). *Bacillus* spp. was stably detected during the fermentation in both of the FMRs (Fig. 1A, B). Most importantly, coliform was not detected in the FMRs. Thus, we used the FMR prepared at 3.5-fold concentration on day 2 for the following clinical trial. In addition, we also tested milk replacer supplemented with LAB without fermentation in the clinical trial in order to establish alternative fermented milk without risk of coliform contamination.

Clinical efficacies of FMR and LAB-MR in BRV-infected calves

Experimental infection of BRV was performed in three milk replacer-fed control calves (C-1, C-2, C-3), three FMR-fed calves (F-1, F-2, F-3), and three LAB-MR-fed calves (Y-1, Y-2, Y-3) (Table 1). In the LAB-MR-fed group, one calf was fed low-concentrated LAB-MR and the other two were fed high-concentrated LAB-MR (Table 1). After the virus administration, two control calves (C-1, C-3) and a LAB-MR fed calf (Y-3) showed high fiver (> 41 °C), and two control calves (C-1, C-2), a FMR-fed calf (Y-2), and a low-concentrated LAB-MR fed calf (Y-1) died of acute enteritis (Fig. 2A). On the other hand, the FMR- (F-1, F-3) and high-concentrated LAB-MR-fed (Y-2, Y-3) calves survived until the end of the study (Fig. 2A). Although one of FMR-fed calves died on eighth day, the calf (F-2) did not show severe diarrhea (Fig. 2B). Meanwhile the other dead calves (C-1, C-2, Y-1) quickly showed severe water-soluble diarrhea after the virus inoculation, FMR-fed calves did not become severe (F-2) or recovered after the severe diarrhea (F-1, F-3) (Fig. 2B). The two high-concentrated LAB-MR-fed calves (Y-2, Y-3) also recovered from diarrhea, although they temporarily became severe (Fig. 2B). Fecal water content was consistently low and did not show severe water-soluble diarrhea in FMR and high-concentrated LAB-MR fed calves (F-1, F-2, F-3, Y-2, Y-3). All of the dead control calves (C-1, C-2) clearly decreased amount of feed with worsening clinical symptoms, and died (Fig. 2C). On the other hand, all of the FMR-fed calves (F-1, F-2, F-3) and a LAB-MR-fed calf (Y-2) recovered amount of milk intake even during diarrhea (Fig. 2C). The other high-concentrated LAB-MR-fed calf (Y-3) did not decrease the amount of milk intake (Fig. 2C).

Table 1. Calves used in the clinical study of the probiotic treatments.

Group	Control			FMR			LAB-MR		
Animal number	C-1	C-2	C-3	F-1	F-2	F-3	Y-1	Y-2	Y-3
Breed*	H	H	H	H	H	H	F1	H	F1
Sex	M	M	M	M	F	M	F	F	F
Age (days old) (at day -4)	2	2	3	2	2	2	0	3	0
Body weight (kg) (at day -4)	46	38	50	42	40	50	46	40	48
LAB-MR	-	-	-	-	-	-	Low	High	High
Result of BRV challenge (day post-inoculation)	Dead (day 3)	Euthanized** (day 8)	Alive (day 10)	Alive (day 10)	Dead (day 8)	Alive (day 10)	Dead (day 1)	Alive (day 10)	Alive (day 10)

* H: Holstein, F1: crossbred (Japanese Black × Holstein)
** The calf was euthanized due to the decline of its clinical condition based on an ethical guideline of the animal experiment.

Intestinal tracts in the control calves showed shortened intestinal villi and strong inflammation (Fig. 3, Tables 2–4). On the other hand, intestinal tracts in the FMR-fed group showed no or mild intestinal villus shortening and inflammation (Fig. 3, Tables 2–4). Intestinal villi were not shortened or inflamed in the intestinal tract of the high-concentrated lactobacillus-supplied MR fed calves (Fig. 3, Tables 2–4). Taken together, the treatment of milk replacer-based probiotics is effective to prevent severe diarrhea and clinical symptoms by BRV infection in calves.

Table 2. Shortened length of intestinal villi in BRV-challenged calves.

Group	Control			FMR			LAB-MR		
Animal number	C-1	C-2	C-3	F-1	F-2	F-3	Y-1	Y-2	Y-3
Duodenum	+	−	−	−	ND	−	−	−	−
Anterior jejunum	+	−	−	−	ND	−	−	−	−
Posterior jejunum	ND	−	ND	+	ND	−	−	−	−
Anterior ileum	ND	−	+	+	ND	−	−	−	+
Posterior ileum	ND	+	−	−	ND	−	−	−	+
Cecus	ND	−	ND	−	ND	−	−	ND	−
Colon	ND	−	−	−	ND	−	−	−	ND

+: short, −: normal, ND: not determined due to artifact

Table 3. Histopathological score of crypt abscess in BRV-challenged calves.

Group	Control			FMR			LAB-MR		
Animal number	C-1	C-2	C-3	F-1	F-2	F-3	Y-1	Y-2	Y-3
Duodenum	++	+	−	−	++	−	−	+	+
Anterior jejunum	+++	+++	++	−	++	−	ND	−	+
Posterior jejunum	++	++	+	+++	++	+	−	++	+
Anterior ileum	++	−	−	++	++	++	−	+	+
Posterior ileum	++	++	−	++	ND	++	−	++	++
Cecus	++	++	+	++	++	+	−	−	+
Colon	++	++	++	++	ND	+	−	+	+

−: none (normal), +: mild, ++: moderate, +++: severe, ND: not determined due to artifact

Table 4. Histopathological score of necrotic lymphoid follicle in BRV-challenged calves.

Group	Control			FMR			LAB-MR		
Animal number	C-1	C-2	C-3	F-1	F-2	F-3	Y-1	Y-2	Y-3
Anterior ileum	ND	++	−	++	ND	+	++	++	−
Posterior ileum	+	++	−	+	ND	+	++	++	+

−: none (normal), +: mild, ++: moderate, +++: severe, ND: not determined due to artifact

Clinical efficacy of FMR in model dairy farms

Clinical efficacy of FMR treatment was evaluated using newborn calves in two model dairy farms which had a high incidence of enteritis due to mixed infection with BRV and *C. parvum*. Interestingly, the incidence of enteritis in calves decreased significantly in the FMR-fed group of the both farms, compared with the group fed with control milk replacer (Fig. 4A, Table 5). The mortality of calves also decreased significantly by the FMR treatment in Farm A (Fig. 4B, Table 5). In addition, outcome and several parameters of clinical consultations were monitored in calves with enteritis of Farm A. In Farm B, it was difficult to evaluate these issues due to the small number of FMR-fed calves with enteritis. The treatment of FMR significantly reduced the duration of treatment, the number of consultations, and total medial consultation costs related to enteritis (Figs. 4C-E). The age of the onset of enteritis was similar between control and FMR-fed groups (Fig. 4F). This field study indicates that feeding of FMR has significant clinical benefits in cattle industry by the prevention and treatment of calf diarrhea.

Table 5. The incidence of enteritis and mortality by enteritis in the field study.

		Control	FMR	*p*-value*
Farm A - incidence of enteritis				
	No colitis	10 (10.1%)	59 (61.5%)	< 0.0001
	Colitis	89 (89.9%)	37 (38.5%)	
Farm A - mortality by enteritis				
	Alive	83 (83.8%)	93 (96.9%)	0.0029
	Dead	16 (16.2%)	3 (3.1%)	
Farm A	Total	99 (100%)	96 (100%)	
Farm B - incidence of enteritis				
	No colitis	150 (69.1%)	50 (89.3%)	0.0021
	Colitis	67 (30.9%)	6 (10.7%)	
Farm B - mortality by enteritis				
	Alive	202 (93.1%)	55 (98.2%)	0.2067
	Dead	15 (6.9%)	1 (1.8%)	
Farm B	Total	217 (100%)	56 (100%)	

*Fisher's exact test was used for statistic analysis.

Discussion

In 2017, the agricultural output of Hokkaido was 1,276,2 billion yen corresponding 13% of the whole value in Japan. Of which, the livestock production was 727.9 billion yen corresponding 57% of the total agricultural output of Hokkaido. In Hokkaido, production rates of livestock and milk account for 21% and 51% of the total productions in Japan, respectively, which is reason why it is called the livestock kingdom.

In dairy industry, calf diarrhea has been causing considerable economic losses because of calf mortality, weight loss in surviving calves all over the world, including Hokkaido. Several probiotics have been used as preventive or supportive therapy for dairy cattle and neonatal calf for a long time, and previous reports indicated the benefit calf performance and health [14–25]. Fermented milk, an old probiotics for dairy cattle, provides several clinical benefits for calf breeding, and has been widely used as economical and safe probiotics [26–28]. However, the efficacy of fermented milk is often inconsistent, and vary in the fed-calf performance because lack of the unifying protocol for the preparation. In addition, there is no direct evidence of the efficacy of fermented milk against infectious diseases. In this study, in order to examine the efficacy of probiotics, we conducted the experimental infection of BRV for clinical evaluation of the probiotics-fed calves, and showed milk replacer-based probiotics reduces severity and tissue damage of the intestinal tract in calf diarrhea by rotavirus. This is first direct evidence of efficacy of milk replacer-based probiotics against infectious disease in calf.

Fermented milk includes the rich amino acids required for calf growth, and has been used for many years in the dairy industry [26–28]. A recent report indicates that the fermented milk enhanced nutritional parameters such as height, weight, and body performance [24]. In addition, the feeding of fermented milk reduced in diarrhea mobility and mortality [24]. However, fermentation of whole fresh milk is easily influenced temperature condition, and the preparation is needed to take long time for the fermentation. Furthermore, the worst drawback of fermented milk is to be easy to contaminate coliform during the fermentation. Indeed, the poor quality of fermented milk often causes diarrhea by contaminated coliform in neonatal calves. Thus, in this study, we firstly tried to establish the protocol of milk replacer-based fermented milk without a risk contamination of coliform. The coliform was not detected in the FMR produced by our protocol. The number of LAB in the FMR efficiently increased compare to fermented whole fresh milk, and the maximum bacteria load reached at 2 days in the cultivation. By the comparative component analysis, quality of the FMR derived 3.5-fold concentrated FMR was better than those of 7-fold concentrated FMR. Thus, we used 3.5-fold concentrated FMR for the evaluation of efficacy against diarrhea caused by BRV infection.

In the clinical experiment, meanwhile control calves consistently showed severe watery diarrhea during the trial, the FMR-fed calves showed diarrhea, but the water content of feces was low and stable, and the diarrhea score kept low or recovered. In addition, the amount of milk intake decreased temporarily, but recovered immediately in the FMR-fed calves. The pathological score for enteritis tended to be lower in the FMR-fed calves than that of the control calves. Interestingly, although the protocols and used LAB for fermentation were different, similar efficacy of fermented milk in rotaviral diarrhea have been reported in children [29–31] and sucking rats [32, 33]. In case of fermented milk made by using human *Lactobacillus* sp strain GG, the fermented milk promoted recovery from rotavirus diarrhea via augmentation of the local immune defense by specific IgA response to virus [30]. Although the mechanism of the efficacy of FMR is still unknown, FMR might induce similar local immune defense. Further studies on the mechanism of the efficacy of FMR might help to improve the disease control in calf by probiotics.

In the present study, three lactic acid bacteria mixture including *Streptococcus faecalis* T-110 strain, *Clostridium butyricum* TO-A strain, *Bacillus mesentericus* TO-A strain, a probiotics used widely for dairy cattle in Japan, was used to prepare the FMR. The beneficial effects of probiotics have been reported in inflammatory bowel diseases, including the suppression of pathogenic enteric bacteria. In human, the probiotics have been clinically used for the treatment of patients with symptoms owing to imbalanced intestinal flora, such as refractory diarrhea, constipation and abdominal distension. Furthermore, several previous studies showed the effectiveness of probiotic therapy using the three LAB mixture, such as in both clinical and bacteriological responses in the patients with vaginosis and vaginitis [34], the reduction of postoperative infectious complications after pancreaticoduodenectomy [35], the prevention of relapse in patients with inactive ulcerative colitis [36], the improvement of the clinical symptoms and endoscopic findings in patients with ulcerative colitis refractory to conventional therapy [37]. Interestingly, Huang et al. reported seven-days administration of the LAB mixture demonstrated high efficacy in infants and children with severe gastroenteritis by salmonella and rotavirus [38]. Indeed, the incidence of severe gastroenteritis was significantly reduced in the lactic acid bacteria mixture-treated groups. As result, the total diarrhea duration was significantly shorter for children treated with the lactic acid bacteria mixture. After lactic acid bacteria mixture administration, there were significantly less intervention group patients with severe diarrhea at intervention day 3. In addition to this report, Chen et al. also reported that the probiotics mixture reduced the severity of diarrhea and length of hospital stay in children with acute diarrhea [39]. Interestingly, Hayakawa et al. reported that administration of the probiotics containing *Streptococcus faecalis* T-110 strain, *Clostridium butyricum* TO-A strain, *Bacillus mesentericus* TO-A strain to sows and/or their neonates improves the reproductive performance, incidence of post-weaning diarrhea and histopathological parameters in the intestine of weaned piglets [40]. In addition, Inatomi et al. reported that administration of the probiotics to porcine epidemic diarrhea (PED)-vaccinated sows were healthier, transferred PED-specific antibodies via colostrum to piglets, had greater litter weight at birth, and reduced mortality during suckling [41]. Furthermore, more recently, Tsukahara et al. reported that the probiotics also improved reproductive performance of unvaccinated farmed sows infected with porcine epidemic diarrhea virus [42]. However, there is no information concerning efficacy of the FMD richly containing *Streptococcus faecalis* T-110 strain, *Clostridium butyricum* TO-A strain, *Bacillus mesentericus* TO-A strain in dairy cattle or calf. Further research is necessary to evaluate the potential of FMR to improve health in calf production.

Although the immunological mechanism of the clinical efficacy of probiotics is still unclear, Isono et al. examined whether the probiotics affect Toll-like receptor 4 (TLR4), which play a role in continuous inflammation of the intestinal mucosa in inflammatory bowel diseases, expression in human colonic epithelial cells *in vitro*, and showed that butyrate produced by *C. butyricum* TO-A specifically downregulated TLR4 mRNA level in human colonic epithelial cells [43]. TLR4-mediated recognition of microbial components triggers aberrant IL-12p40 production by myeloid cells, leading to the development of enterocolitis. The report suggested that the development and persistence of colitis could be prevented by suppression of TLR4 expression in intestinal epithelial cells. On the other hand, Hua et al. reported the several immunological functions of three lactic acid bacteria mixture such as induction of Th1 immune response, downregulation of pro-inflammatory cytokines (TNF-α), upregulation of anti-inflammatory cytokine (IL-10), enhancement of CD11b, HLA-DR, CD4, CD45RA, CD25, CD44 and CD69 expression [44]. Although the detailed mechanism of the reduction severity of diarrhea in fed calves is still unknown, FMR may restore the beneficial intestinal flora and enhance host protective immunity such as down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines. Further investigation is needed.

In this study, as an alternative way, we evaluated the possibility of LAB-MR for calf breeding. However, LAB-MR was not fermented under the tested several conditions (data not shown). *L. plantarum* HOKKAIDO strain was isolated from well-pickled vegetables in Hokkaido, and used for the fermentation of soymilk [45]. *L. plantarum* HOKKAIDO strain, a plant lactobacillus, might be difficult to metabolize the animal carbohydrate in the milk, resulted in not growth in the cultivated milk. However, interestingly, direct use of the high-concentrated LAB-MR-fed calves reduced the severity of diarrhea as well as FMR-fed calves, meanwhile a low concentrated LAB-MR-fed calf was not effective to rotaviral diarrhea. As immunological function of *L. plantarum* HOKKAIDO strain, it is seemed that the lactobacillus induced IL-8, IL-12, IP-10, important cytokines for activation of cell mediated immunity, form human dendritic cell lines [46, 47]. Although it was other *L. plantarum* strain, orally administered *L. plantarum* No.14 reduce inflammatory cytokine production by circulating exosomes in the mice model [48]. In addition, the *L. plantarum* strain strongly induced the gene expression of Th1-type cytokines by suing pig model [49]. The mechanism of the preventive effects of *L. plantarum* HOKKAIDO strain was also still unknown, these finding might suggest that the HOKKAIDO strain improves immune function including the activation of cell mediated immunity. Further research is necessary to identify underlying mechanisms and to evaluate the potential of direct use of the unfermented milk replacer.

In conclusion, the feeding of two milk replacer-based probiotics, FMR and high-concentrated LAB-MR, reduce BRV-induced diarrhea and tissue damage to the intestinal tract, and suppress the clinical symptoms of acute enteritis by maintaining milk intake. This direct evidence could be helpful to the development of potential novel methods for the control of diarrhea in neonatorum calf. Indeed, our retrospective investigation indicated the FMR feeding reduced the incidence of diarrhea in a dairy farm with a high incidence of enteritis due to mixed infection with rotavirus and *C. parvum*. Although there is no direct evidence of efficacy of FMR against *C. parvum* infection, the feeding with fermented milk was reported as a protective factor against the shedding of *C. parvum* under field conditions [50]. In addition, the feedings with FMR reduced the percentage of calves that required therapy and the amount of treatments needed against digestive diseases.

Conclusion

This study indicates that the milk replacer-based probiotic feeding has a potential for the disease control including diarrhea in neonatorum calf. Further experiments using a large number of infected calves are required to confirm the efficacy of this novel probiotic approach for clinical application to reduce the incidence of calf scours, severity, and duration of infectious diseases.

Declarations

Ethics approval and consent to participate

The animal experiments in this study were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Hokkaido University (No. 18-0147). Informed consent was obtained from all owners of cattle participated in the field study.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

H.N. and M.H. are employed by Snow Brand Seed Co., Ltd.

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Authors' contributions

FK, SK, MK, and KO were responsible for the conception and design of the study. FK, TO, SK, JK, YS, KW, GO, SG, H Nakamura, HS, EM, AK, MK, H Noda, and MH performed the experiments. FK, TO, SK, JK, EM, AK, MK, NT, AT, MH, NM, SM, and KO analyzed the data. JK, MK, NT, AT, H Noda, MH, NM, SM, and KO provided intellectual input, laboratory materials, reagents and/or analytic tools. FK, TO, SK wrote the manuscript. FK, TO, SK, JK, YS, AK, MK, MH, NM, SM, and KO contributed to the revision of the manuscript. All authors reviewed and approved the final manuscript.

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Figures

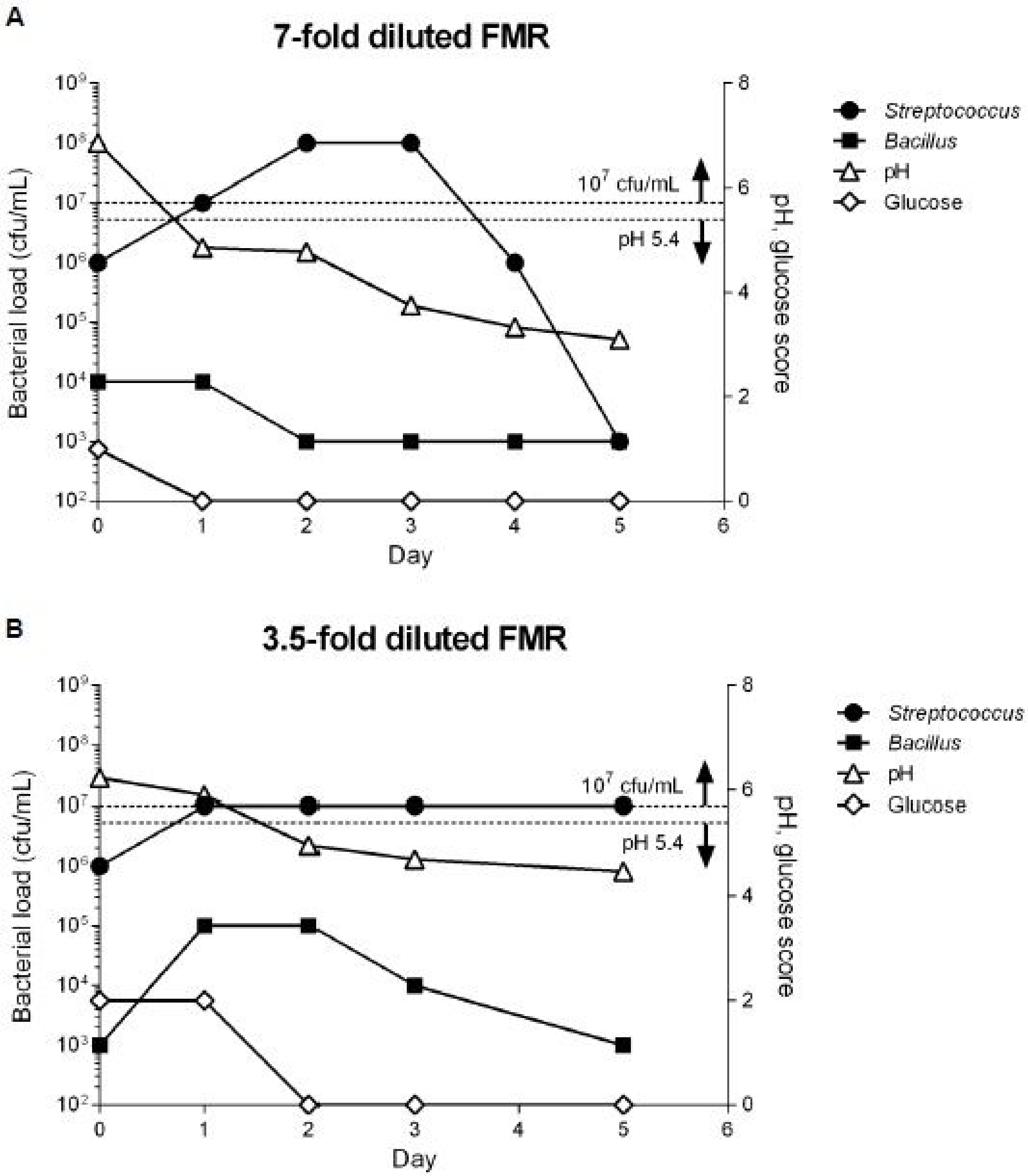

Figure 1

Fermentation of a milk replacer supplemented with BIO-THREE Ace. A milk replacer suspended by hot water at 3.5-fold (A) and 7-fold dilution (B) was supplemented with 1% BIO-THREE Ace and fermented at 22–25°C for 5 days. They were mixed once per day and sampled for bacterial colonization on 5% sheep blood agar plate and the measurement of pH (white triangle, right axis) and glucose score (white diamond, right axis). The bacterial loads (left axis) were calculated from the numbers of Streptococcus-like (black circle) and Bacillus-like colonies (black square) were counted after the overnight cultivation of the plate at 37°C. The dashed lines indicate the criterion values of fermented milk, “> 107 cfu/mL of Streptococcus spp. (LAB)” and “< pH 5.4”.

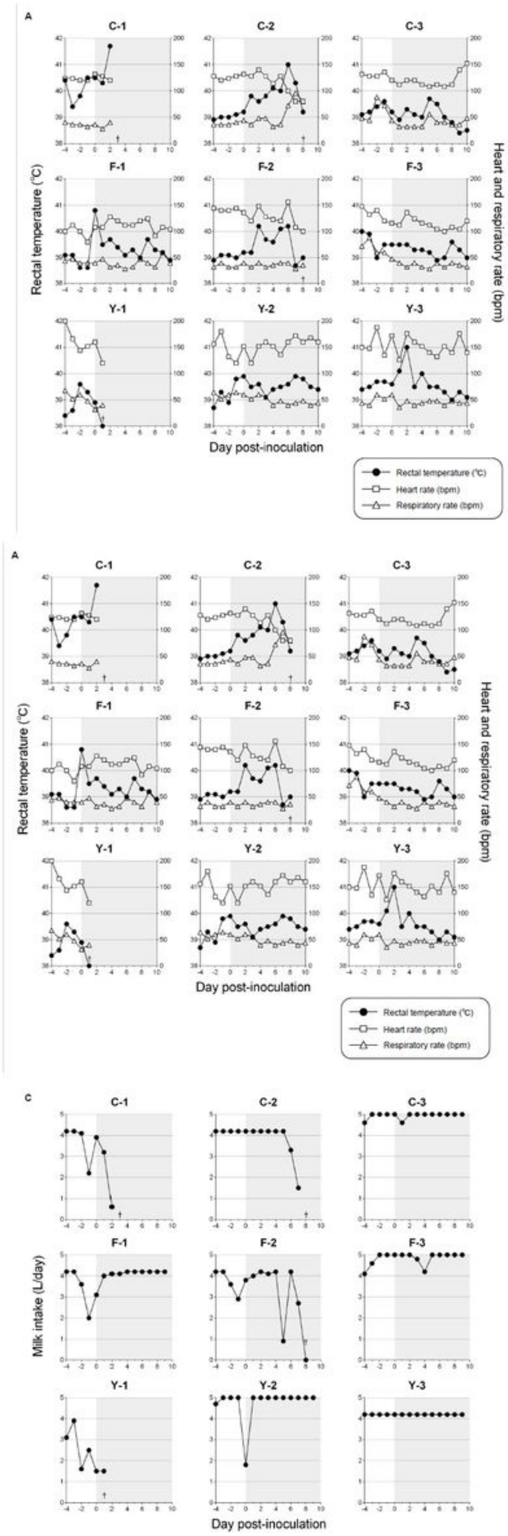

Figure 2

Clinical symptoms of calves treated with milk replacer, FMR, and LAB-MR during BRV challenge. Newborn calves fed with control milk replacer (C-1, C-2, and C-3), FMR (F-1, F-2, and F-3), and LAB-MR (Y-1, Y-2, and Y-3) were orally inoculated with BRV at day 0 and observed by 10 days post-inoculation. (A) Rectal temperature (left axis, black circle), heart (right axis, white square), and respiratory rate (right axis, white triangle) were measured. (B) Diarrhea score (left axis, black circle) was determined by visual observation of fresh feces as follows: normal (0), soft (1), mushy (2), watery (3), very watery (4). Water content in fresh feces (right axis, white square) was calculated from weight change of fresh feces by microwaving. (C) The calves were fed control milk replacer, FM, or LAB-MR twice per day. Total intake of them was monitored. (A–C) Gray background indicate the period of BRV challenge. Cross indicate the death or euthanization of animals.

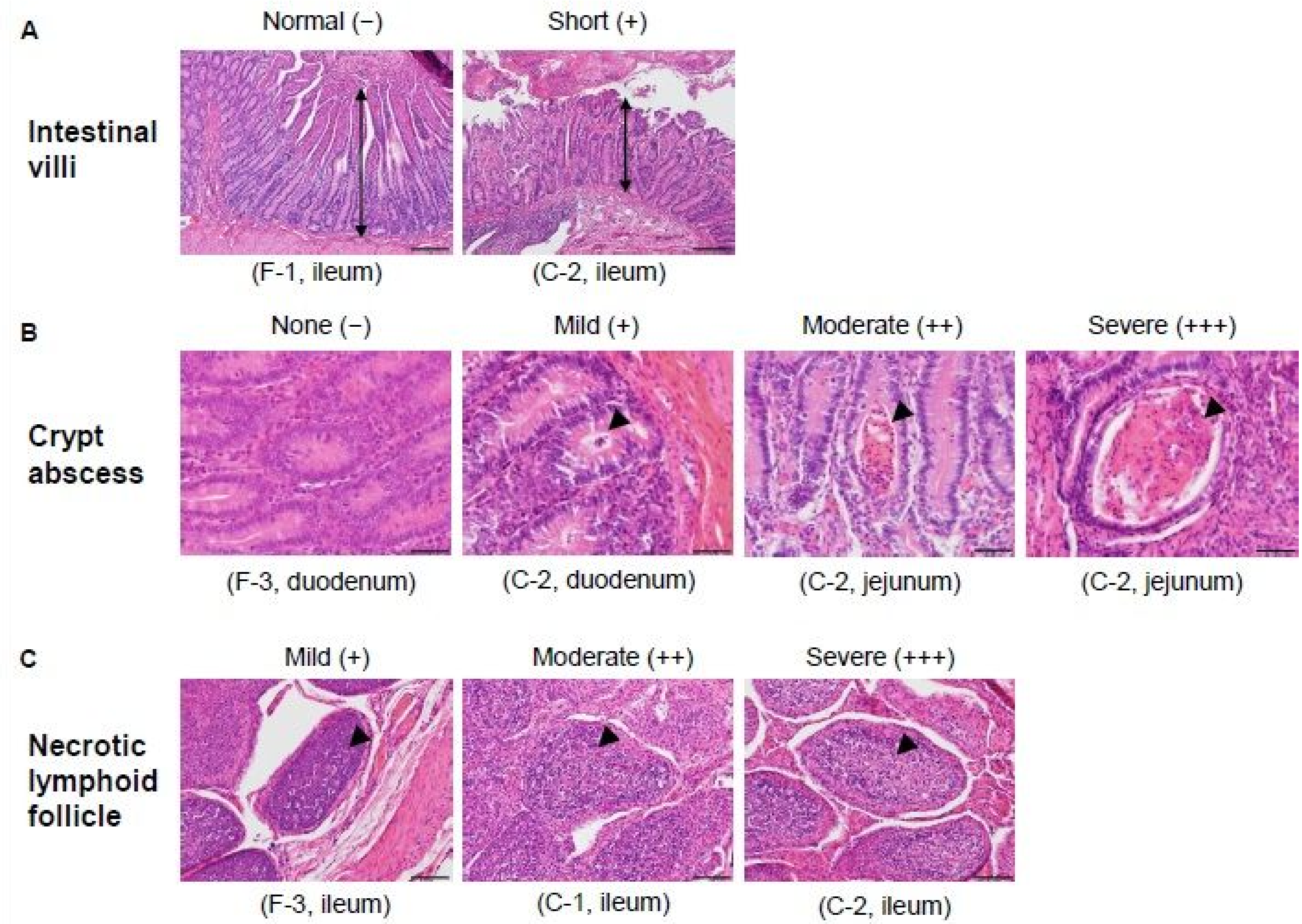

Figure 3

Histopathological evaluation of the intestinal tract in BRV-challenged calves. All of the BRV-challenged calves in the clinical study was dissected and their intestinal tracts were collected at the day of death or at day 10. Intestinal lesions of the specimens were evaluated histopathologically. (A) Length of intestinal villi (arrow) was observed and scored as normal (–) or short (+). All of the scores were shown in Table 2. (B and C) Crypt abscess in intestinal tracts (B) and necrotic lymphoid follicle in ileum (C) were confirmed and scored as none (normal) (–), mild (+), moderate (++), or severe (+++). Arrowheads indicate crypt abscess or necrotic lymphoid follicle. All of the scores were shown in Tables 3 and 4.

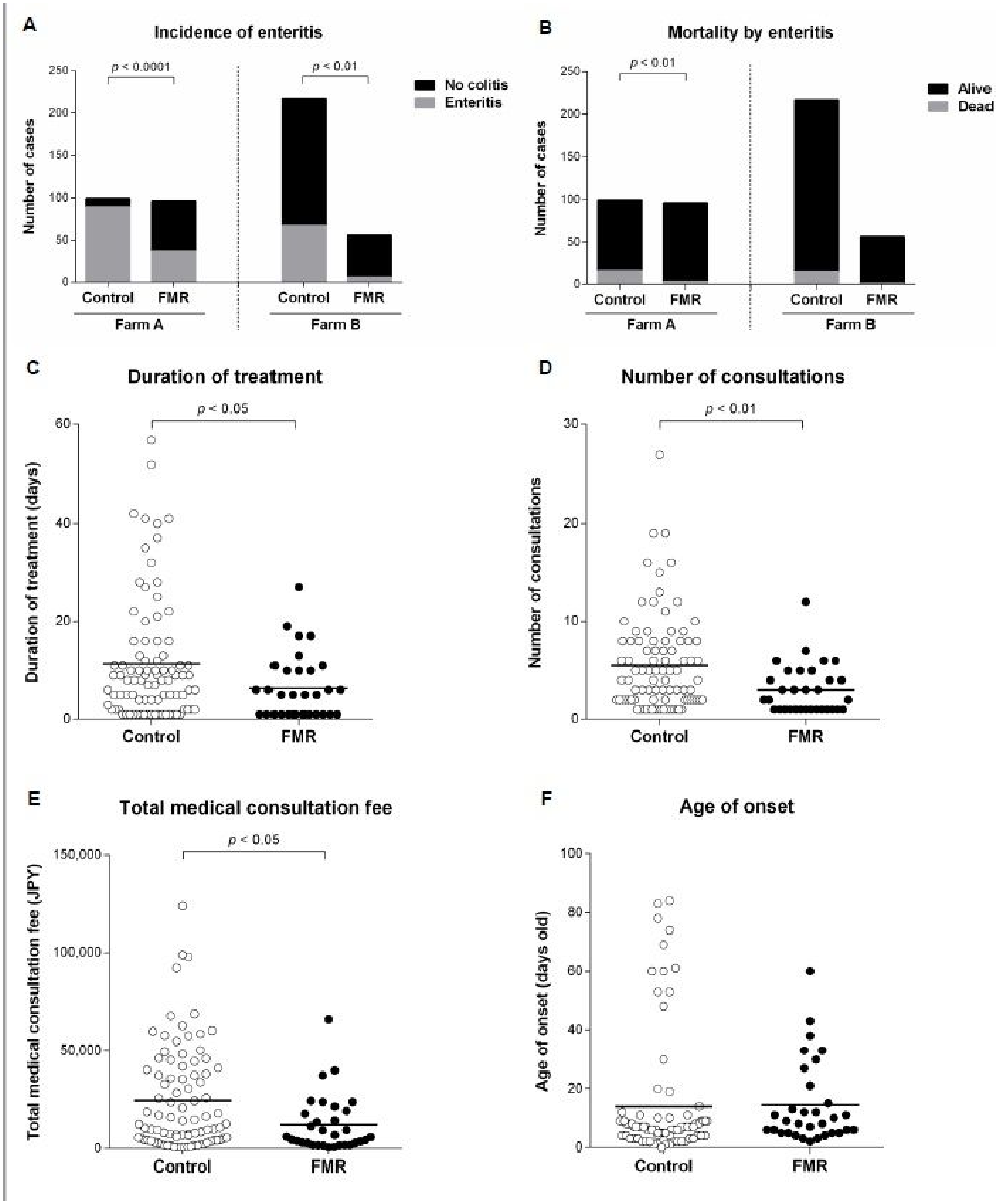

Figure 4

Field evaluation of the effect of FMR treatment against enteritis in calves. Newborn calves in two independent farms (farms A and B) were fed with milk replacer (control) or FMR from birth to weaning. (A and B) The incidence of enteritis (A) and mortality by enteritis (B) were shown among all of the newborn calves in each treatment group of both farms. Significant associations between treatments and values were determined by Fisher's exact test. The numbers of the enteritis cases and deaths were summarized in Table 5. (C–F) Duration of a series of treatments (C), the number of consultations during a single series of treatments (D), total medical consultation fee (JPY/head) required for the treatment of enteritis (E), the age of onset of enteritis (F) were shown for control calves (n = 89) and FMR-fed calves (n = 32) in farm A. Significant difference between each two groups was determined by Mann-Whitney U test.