



Title	Direct evidence of the preventive effect of milk replacer-based probiotic feeding in calves against severe diarrhea
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1 **Research Paper**

2 **Direct evidence of the preventive effect of milk**
3 **replacer–based probiotic feeding in calves against**
4 **severe diarrhea**

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2

3 **Running title: Fermented milk replacer against calf diarrhea**

4

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10

1 **Abstract**

2 Diarrhea is a major cause of death in calves and this is linked directly to
3 economic loss in the cattle industry. Fermented milk replacer (FMR) has been used widely
4 in clinical settings for calf feeding to improve its health and growth. However, the
5 protective efficacy of FMR on calf diarrhea remains unclear. In this study, we verified the
6 preventive effects of FMR feeding on calf diarrhea using an experimental infection model
7 of bovine rotavirus (BRV) in newborn calves and a field study in dairy farms with calf
8 diarrhea. In addition, we evaluated the protective efficacy of lactic acid bacteria-
9 supplemented milk replacer (LAB-MR) in an experimental infection model. In the
10 experimental infection, calves fed FMR or high-concentrated LAB-MR had diarrhea, but
11 the water content of feces was lower and more stable than that of calves fed normal milk
12 replacer. The amount of milk intake also decreased temporarily, but recovered
13 immediately in the FMR- and LAB-MR-fed calves. As compared with the control calves,
14 FMR- or LAB-MR-fed calves showed less severe or reduced histopathological lesions
15 of enteritis in the intestinal mucosa. In a field study using dairy calves, FMR feeding
16 significantly reduced the incidence of enteritis, mortality from enteritis, duration of a
17 series of treatment for enteritis, number of consultations, and cost of medical care for the
18 disease. These results suggest that feeding milk replacer-based probiotics to calves
19 reduces the severity of diarrhea and tissue damage to the intestinal tract caused by BRV
20 infection and provides significant clinical benefits to the prevention and treatment of calf
21 diarrhea.

22 **Key words:** calf diarrhea, fermented milk replacer, lactic acid bacteria-supplemented

1 milk replacer, rotavirus, enteritis

2 **Highlights**

3 ● Fermented milk replacer (FMR) feeding prevented severe diarrhea in newborn calves
4 challenged with rotavirus.

5 ● Probiotic treatment reduced histopathological lesions of enteritis in the intestinal
6 mucosa of the challenged calves.

7 ● FMR feeding reduced the incidence of enteritis and the associated mortality in dairy
8 calves.

9

1 **1. Introduction**

2 Diarrhea is a common disease observed in calves, caused by viral, bacterial,
3 and protozoan infections as well as non-infectious factors, such as dietary and nervous
4 factors. Moreover, calf diarrhea causes severe economic losses in the cattle industry (Cho
5 and Yoon, 2014). Among these factors, bovine rotavirus (BRV) infection and bovine
6 cryptosporidiosis are prevalent among cattle in Japan and cause severe diarrhea in calves
7 (Ichikawa-Seki et al., 2014). BRV infects cattle of all ages, but a higher incidence and
8 more severe clinical signs are observed in calves (Dhama et al., 2009). Bovine
9 cryptosporidiosis with diarrhea caused by *Cryptosporidium parvum* results in decreased
10 appetite, weakness, and dehydration in calves (Thomson et al., 2017). Although the
11 mortality rate from single infections is low, it remains a serious problem in dairy farms,
12 as mixed infections, including BRV, have increased the mortality rate. Unfortunately, no
13 effective vaccines have been developed against *C. parvum* infection. Infected calves
14 exhibit diarrhea during the developmental period, which causes growth retardation, and
15 the resulting economic attrition has presented a problem in the industry (Cho and Yoon,
16 2014). Antibiotics are useful chemotherapeutic agents in the treatment of bovine bacterial
17 diarrhea (Constable, 2009). However, they are not effective for viral or protozoan diarrhea
18 or for antibiotic-resistant bacteria (Smulski et al., 2020). Therefore, a novel alternative
19 preventive strategy for bovine diarrhea is required.

20 Probiotics are defined as living microorganisms that have a health effect on the
21 host. Various clinical effects have been found in humans, including not only improved
22 stool consistency and balance of intestinal resident bacteria, but also allergy-reducing,

1 influenza infection–preventing, and *Helicobacter pylori*–suppressing effects (Kechagia
2 et al., 2012; Lei et al., 2017; Hamilton-Miller, 2003). A previous report indicated the use
3 of probiotics (lactic acid bacteria [LAB]) to prevent infectious diseases in newborns
4 babies in rural India, where many children are still dying from these diseases (Panigrahi
5 et al., 2017). The results were better than expected, and the combined index of sepsis and
6 death showed a significant drop from 9.4% to 5.4%, with a 40% reduction. In addition,
7 when only sepsis was considered, the reduction was 70% or greater. Numerous studies
8 have reported that probiotics are effective in preventing other infectious diseases,
9 suggesting that they can also be used for disease control in calves that often die from
10 infectious diseases. However, as the efficacies of probiotics vary and are inconsistent, the
11 use of probiotics as a supportive therapy in veterinary field is still limited. Indeed,
12 fermented milk replacer (FMR) made from fresh milk or colostrum has been used widely
13 in clinical settings in calf feeding to increase their growth rate and reduce diarrhea
14 (Otterby et al., 1976; Maldonado et al., 2018). However, there are many difficulties in the
15 fermenting raw milk, such as the occurrence of unstable fermentation because of the large
16 variances in the nutrient composition of raw milk and coliform contamination, which may
17 cause severe diarrhea in calves. The practical use of FMR has the strong potential to
18 overcome these difficulties. However, the protective efficacy of FMR on calf diarrhea
19 remains unclear. Therefore, we modified the previous method to obtain a novel fermented
20 milk production technology using milk replacer with high nutritional value without the
21 risk of coliform contamination. We subsequently verified the antidiarrheal effects of the

- 1 two milk replacer-based probiotics, FMR and lactic acid bacteria-supplemented milk
- 2 replacer (LAB-MR), using an experimental infection model of BRV in calves.

1 **2. Materials and Methods**

2 *2.1. Preparation and component analysis of FMR*

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

14

15 *2.2. Experimental trial of probiotic treatment in BRV-challenged calves*

16 All animal experiments were approved by the Ethics Committee of the Faculty
17 of Veterinary Medicine, Hokkaido University (No. 18-0147). Newborn calves (0–3 days
18 old) with no history of colostrum feeding were included from [dairy farms without the](#)
19 [recent history of calf diarrhea](#) in this experiment (Table 1) and housed in isolated rooms
20 in a biosafety level II animal facility at the Faculty of Veterinary Medicine, Hokkaido
21 University. In this experiment, animals were divided into the following three feeding
22 groups: (1) milk replacer; (2) FMR; and (3) LAB-MR. The first group was fed milk

1 replacer (GREATBABY; Nosan Corporation) at a 7-fold dilution and the second group
2 was fed FMR at a 7-fold dilution. FMR was prepared by fermenting milk replacer (3.5-
3 fold dilution, GREATBABY; Nosan Corporation) supplemented with 1% BIO-THREE
4 Ace (Toa Biopharma) at 22–25°C for 2 days. For the third group, one animal was fed
5 another milk replacer (YUKIMIRUKU, Snow Brand Seed, Sapporo, Japan) including 1.3
6 $\times 10^7$ cfu/kg/day of *Lactobacillus plantarum* (HOKKAIDO strain; Food Processing
7 Research Center, Hokkaido Research Organization, Ebetsu, Japan; Japanese patent No.
8 3925502) as low-concentrated LAB-MR. The other two animals were fed high-
9 concentrated LAB-MR prepared from the milk replacer (YUKIMIRUKU) which was
10 additionally supplemented with *L. plantarum* HOKKAIDO strain (2.5×10^9 cfu/kg/day).
11 LAB-MR was fed immediately to calves after the preparation without fermentation. The
12 feeding amount was determined by body weight of each calf. All animals were fed 7-fold
13 diluted MR, FMR, or LAB-MR twice per day.

14 On day 5, the animals were inoculated orally with fecal samples from an
15 infected calf containing BRV (G6P[11], 3.2×10^6 TCID₅₀/dose). The BRV titer in the
16 fecal samples was determined by cytopathogenic effect assay using the MA104 cell line
17 as described previously (Ojeh, 1984). The inoculum samples also tested for BRV, bovine
18 coronavirus (BCoV), pathogenic *Escherichia coli*, *Salmonella* spp., *Cryptosporidium*
19 spp., and *Eimeria* spp. To detect BRV and BCoV, viral RNA (vRNA) was extracted from
20 fecal samples using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according
21 to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-
22 PCR) assays were performed to test the fecal vRNA samples using SuperScript IV One-

1 Step RT-PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and specific
2 primers 5'- ACCACCAAATATGACACCAGC-3' and 5'-
3 CATGCTTCTAATGGAAGCCAC-3' targeting the *VP6* gene of rotavirus group A or 5'-
4 CCGATCAGTCCGACCAATC-3' and 5'-AGAATGTCAGCCGGGGTAT-3' targeting
5 the *N* gene of BCoV (Cho et al., 2010). To detect *E. coli* and *Salmonella* spp., fecal DNA
6 was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen). DNA samples were
7 tested for PCR assays using Ex Taq DNA polymerase (Takara Bio, Otsu, Japan) and
8 specific primers 5'- GCGACTACCAATGCTTCTGCGAATAC-3' and 5'-
9 GAACCAGACCAGTCAATACGAGCA-3' targeting *K99* gene of *E. coli* or 5'-
10 TGTTGTGGTTAATAACCGCA-3' and 5'-CACAAATCCATCTCTGGA-3' targeting
11 *16S rDNA* gene of *Salmonella* spp. (Cho et al., 2010). To detect *Cryptosporidium* and
12 *Eimeria* spp., fecal samples were centrifuged through a sucrose density gradient and
13 examined microscopically for oocysts.

14 The experiment continued until 10 days after the viral inoculation. Calves were
15 monitored for vital signs, milk intake, and diarrhea clinical score. In addition, fresh feces
16 were collected, and the water content in the feces was measured by the dry weight method
17 using a microwave. Furthermore, the intestinal tract of the calves was collected at the end
18 of experimental period or earlier, and the presence or absence and degree of histological
19 damage were evaluated.

20

21 *2.3. Field investigations*

1 To evaluate the efficacy of FMR feeding in the clinical setting, a field trial was
2 conducted in newborn calves at two dairy farms with a history of high prevalence of BRV
3 and bovine cryptosporidiosis over the past 3 years. Informed consent was obtained from
4 all owners of cattle participating in the field study. Enteritis in the calves were diagnosed
5 based on clinical signs, including diarrhea. Some of the calves with clinical diarrhea were
6 selected for further confirmation by pathogen detection in feces. BRV infection was
7 diagnosed by the detecting the viral antigen in feces using a commercial
8 immunochromatography kit for group A rotavirus (DIPSTICK ROTA; Eiken Chemical,
9 Tokyo, Japan) (Minami-Fukuda et al., 2013). Bovine cryptosporidiosis was diagnosed by
10 the microscopic identification of oocysts in feces collected by sucrose density gradient
11 centrifugation or a commercial immunochromatography kit for *Cryptosporidium* spp.
12 ([DipFit *Cryptosporidium* sp](#); Bio-X Diagnostics, Rochefort, Belgium). For Farm A, male
13 and female newborn calves of Holstein and crossbred (Japanese Black × Holstein cattle)
14 were enrolled for this experiment. For Farm B, male and female newborn Holstein calves
15 were enrolled. FMR was prepared by fermenting a milk replacer (GREATBABY; Nosan
16 Corporation) supplemented with 1% of BIO-THREE Ace (Toa Biopharma), as shown
17 above in each farm. The enrolled calves were fed colostrum just after birth and the milk
18 replacer (control) or FMR twice per day from birth to weaning. The incidence of enteritis
19 and mortality from enteritis in the calves was monitored until weaning. In addition, the
20 duration of a series of treatments for enteritis, number of consultations during a single
21 series of treatments, total medical consultation fee required for the treatment of enteritis,
22 and age at enteritis onset were investigated retrospectively after the study was completed.

1

2 *2.4. Statistics*

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

8

1 **3. Results**

2 *3.1. Component analysis of FMR*

3 According to the Ministerial Ordinance on Milk and Milk Products Concerning
4 Compositional Standards, etc. ([https://elaws.e-](https://elaws.e-gov.go.jp/search/elawsSearch/elaws_search/lsg0500/detail?lawId=326M50000100052)
5 gov.go.jp/search/elawsSearch/elaws_search/lsg0500/detail?lawId=326M50000100052),
6 fermented milk must meet the following criteria: (1) number of LAB $>10^7$ cfu/mL; (2)
7 pH <5.3 ; and (3) coliform negative. In this study, we evaluated FMR-based on these
8 criteria. A 3.5-fold FMR supplemented with BIO-THREE Ace was fermented with pH
9 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. 1A). A 7-
10 fold FMR supplemented with BIO-THREE Ace was fermented too quickly with pH 4.86
11 on day 1 and pH 4.78 on day 2 (Fig. 1A). On day 3, the pH of the mixture was 3.75, and
12 the acidity increased rapidly, indicating that it was not suitable for feeding because of its
13 strong sour taste (Fig. 1A). The number of LAB (*Streptococcus* spp.) increased to 10^7
14 cfu/mL in 3.5-fold FMR on day 1 (Fig. 1B). *Bacillus* spp. was stably detected during the
15 fermentation in both of the FMRs (Fig. 1C). Most importantly, coliforms were not
16 detected in the FMRs. The glucose score was reduced along with the growth of
17 *Streptococcus* spp. and *Bacillus* spp. in both of the FMRs (Fig. 1D). Thus, we used the
18 FMR prepared at a 3.5-fold concentration on day 2 for the following clinical trial. In
19 addition, to establish alternative fermented milk without a risk of coliform contamination,
20 we tested milk replacer supplemented with LAB without fermentation in the clinical trial.

21

22 *3.2. Protective efficacy of FMR and LAB-MR in BRV-infected calves*

1 The experimental infection of BRV was performed in three milk replacer-fed
2 control calves (C-1, C-2, C-3), three FMR-fed calves (F-1, F-2, F-3), and three LAB-
3 MR-fed calves (Y-1, Y-2, Y-3; Table 1). The inoculum samples were tested positive for
4 BRV, and negative for bovine coronavirus (BCoV), pathogenic *Escherichia coli*,
5 *Salmonella* spp., *Cryptosporidium* spp., and *Eimeria* spp. In the LAB-MR-fed group, one
6 calf was fed low-concentrated LAB-MR and two were fed high-concentrated LAB-MR
7 (Table 1). After administration of the virus, two control calves (C-1, C-2) and a LAB-
8 MR-fed calf (Y-3) showed high fever ($>41^{\circ}\text{C}$), and two control calves (C-1, C-2), an
9 FMR-fed calf (F-2), and a low-concentrated LAB-MR-fed calf (Y-1) died of or
10 euthanized due to acute enteritis (Fig. 2A). On the other hand, the FMR-fed (F-1, F-3)
11 and high-concentrated LAB-MR-fed (Y-2, Y-3) calves survived until the end of the study
12 (Fig. 2A). Although one of the FMR-fed calves died on the eighth day, the calf (F-2) did
13 not show severe diarrhea (Fig. 2B). Meanwhile, the other dead calves (C-1, C-2, Y-1)
14 quickly showed severe water-soluble diarrhea after viral inoculation. The FMR-fed
15 calves did not become severely diarrheic (F-2) or recovered after severe diarrhea (F-1, F-
16 3) (Fig. 2B). The two high-concentrated LAB-MR-fed calves (Y-2, Y-3) also recovered
17 from diarrhea, although they had transient severe diarrhea (Fig. 2B). The fecal water
18 content was consistently low and the FMR-fed (F-1, F-2, F-3) and high-concentrated
19 LAB-MR-fed calves (Y-2, Y-3) did not show severe watery diarrhea. All of the dead
20 control calves (C-1, C-2) had clearly decreased the amount of milk intake alongside
21 worsening clinical signs (Fig. 2C). On the other hand, all FMR-fed calves (F-1, F-2, F-
22 3) and a LAB-MR-fed calf (Y-2) recovered their milk intake amount, even during

1 diarrhea (Fig. 2C). The other high-concentrated LAB-MR-fed calf (Y-3) did not decrease
2 its milk intake (Fig. 2C).

3 In the control calves, the intestinal tracts displayed shortened intestinal villi and
4 strong inflammation (Fig. 3, Tables 2–4). On the other hand, the intestinal tracts in the
5 FMR-fed group showed no or mild intestinal villus shortening and inflammation (Fig. 3,
6 Tables 2–4). The intestinal villi were not shortened or inflamed in the intestinal tract of
7 the high-concentrated LAB-MR-fed calves (Fig. 3, Tables 2–4). Taken together,
8 treatment with milk replacer-based probiotics is effective in preventing severe diarrhea
9 and clinical signs of BRV infection in calves.

11 3.3. Protective efficacy of FMR in model dairy farms

12 We finally evaluated the protective efficacy of FMR treatment using newborn
13 calves in two model dairy farms that had a high incidence of enteritis resulting from
14 mixed infection with BRV and *C. parvum*. Interestingly, the incidence of enteritis in
15 calves of both farms decreased significantly in the FMR-fed group as compared with the
16 group fed the control milk replacer (Fig. 4A). The mortality of the calves in farm A also
17 decreased significantly after FMR treatment (Fig. 4B). Most of the enteritis in the calves
18 of both farms were caused by BRV infection. Some of them (approximately 30%) were
19 coinfecting with *Cryptosporidium* spp. Moreover, the outcomes and several parameters of
20 the clinical consultations were monitored in calves with enteritis of farm A. However, it
21 was difficult to evaluate these issues in farm B because of the small number of FMR-fed
22 calves with enteritis. FMR treatment significantly reduced the duration of a series of

1 treatment for enteritis, number of consultations, and total medial consultation costs
2 related to enteritis (Figs. 4C–E). The age of the onset of enteritis was similar between the
3 control and FMR–fed groups (Fig. 4F). This field study indicates that FMR feeding has
4 significant clinical benefits in the cattle industry by preventing and ameliorating calf
5 diarrhea.
6

1 **4. Discussion**

2 In 2017, the agricultural output of Hokkaido was 12,762 billion yen, which
3 corresponds to 13% of the entire agricultural value in Japan. Of this, livestock production
4 comprised 727.9 billion yen, which corresponds to 57% of the total agricultural output of
5 Hokkaido. In Hokkaido, the productions of livestock and milk account for 21% and 51%
6 of the total productions in Japan, respectively, which is why it is referred to as the
7 “livestock kingdom”.

8 Calf diarrhea has been the source of considerable economic losses in the dairy
9 industry as a result of calf mortality and weight loss in surviving calves worldwide,
10 including Hokkaido. Several probiotics have been used as preventive or supportive
11 therapy for dairy cattle and neonatal calves for many years, and previous reports have
12 indicated the benefit in calf performance and health (Abe et al., 1995, Donovan et al.,
13 2002, Ewaschuk et al., 2004, Timmerman et al., 2005, Galvão et al., 2005, Mokhber-
14 Dezfouli et al., 2007, Magalhães et al., 2008, Signorini et al., 2012, Foditsch et al., 2015,
15 Fouladgar et al., 2016, Maldonado et al., 2018, Renaud et al., 2019). Fermented milk,
16 classic probiotic source for dairy cattle, provides several clinical benefits for calf breeding,
17 and has been used widely as an economical and safe source of probiotics (Foley et al.,
18 1978, Kesler, 1981, Keith et al., 1983). However, the efficacy of fermented milk is often
19 inconsistent and its performance varies in each fed calf because of a lack of a unifying
20 preparation protocol. In addition, there is no direct evidence of the efficacy of fermented
21 milk against infectious diseases. In this study, to examine the protective efficacy of
22 probiotics, we conducted an experimental infection of BRV for the clinical evaluation of

1 probiotic-fed calves, and demonstrated that milk replacer-based probiotics reduce the
2 severity and tissue damage of the intestinal tract during calf diarrhea by BRV infection.
3 This study provides the first direct evidence of the protective efficacy of milk replacer-
4 based probiotics against infectious diseases in calves.

5 Fermented milk is rich in amino acids that are required for calf growth and
6 has been used for many years in the dairy industry (Foley et al., 1978, Kesler, 1981, Keith
7 et al., 1983). A recent report indicated that fermented milk enhanced physical parameters
8 such as height, weight, and body performance (Maldonado et al., 2018). In addition,
9 feeding fermented milk reduced diarrhea mobility and mortality (Maldonado et al., 2018).
10 However, the fermentation of whole fresh milk is influenced easily by temperature, and
11 the preparation time is lengthy. Furthermore, the primary drawback is coliform
12 contamination that occurs during fermentation. Indeed, the poor quality of fermented milk
13 often causes diarrhea from contaminated coliform in neonatal calves. Thus, in this study,
14 we first tried to establish a protocol of milk replacer-based fermented milk without the
15 risk of coliform contamination. The coliforms were not detected in the FMR produced by
16 our protocol. The number of LAB in the FMR increased efficiently as compared with
17 fermented whole fresh milk, and the maximum bacterial load was reached at 2 days in
18 cultivation. Comparative component analysis indicated that the quality of the FMR
19 derived from 3.5-fold FMR was better than that of 7-fold FMR. Thus, we used the 3.5-
20 fold FMR to evaluate efficacy against diarrhea caused by BRV infection.

21 Although control calves consistently showed severe watery diarrhea during
22 the trial in the clinical experiment, the FMR-fed calves showed diarrhea, but the water

1 content of the feces was low and stable, and the diarrhea score remained low or recovered
2 to normal. In addition, in the FMR-fed calves, the amount of milk intake decreased
3 temporarily, but recovered immediately. The pathological score for enteritis tended to be
4 lower in the FMR-fed calves than in the control calves. Interestingly, although the
5 protocols and LAB used for fermentation differed, a similar efficacy of fermented milk
6 in rotaviral diarrhea has been reported in children (Isolauri et al., 1991, Kaila et al., 1992,
7 Marteau et al., 2001) and sucking rats (Guérin-Danan et al., 2001, Rigo-Adrover et al.,
8 2019). In the case of fermented milk made by using human *Lactobacillus* sp strain GG,
9 the fermented milk promoted recovery from rotaviral diarrhea through the augmentation
10 of the local immune defense by specific immunoglobulin A response to the virus (Kaila
11 et al., 1992). Although the mechanism of the efficacy of FMR remains unknown, FMR
12 might induce a similar local immune defense. Further studies on the mechanism of FMR
13 efficacy might help to improve the disease control of calves by probiotics.

14 In this study, we evaluated the possibility of LAB-MR for calf breeding as an
15 alternative source of FMR preparation. However, LAB-MR was not fermented under the
16 several conditions tested (data not shown). *L. plantarum* HOKKAIDO strain was isolated
17 from well-pickled vegetables and used for the fermentation of soymilk (Nakagawa et al.,
18 2005). This plant lactobacillus may have difficulty metabolizing animal carbohydrates in
19 the milk, which resulted in no growth in the cultivated milk. However, interestingly, the
20 use of the high-concentrated LAB-MR-fed directly to the calves reduced the severity of
21 diarrhea, as seen in the FMR-fed calves. As for the immunological function of *L.*
22 *plantarum* HOKKAIDO strain, it seems that the lactobacillus induced interleukin (IL)-8,

1 IL-12, and IP-10, which are important cytokines for activation of cell mediated immunity,
2 from human dendritic cell lines (Nakagawa et al., 2009, Nishimura et al., 2015). Although
3 it was another *L. plantarum* strain, the orally administered *L. plantarum* No. 14 reduced
4 the production of inflammatory cytokines by circulating exosomes in the mice model
5 (Yoshida-Aoki et al., 2017). In addition, the *L. plantarum* strain strongly induced the gene
6 expression of Th1-type cytokines in a pig model (Nagata et al., 2010). The mechanism of
7 the preventive effects of *L. plantarum* HOKKAIDO strain remains unknown, and these
8 findings might suggest that the HOKKAIDO strain improves immune function. Further
9 research is required to identify the underlying mechanisms and evaluate the potential of
10 using the LAB-MR directly without fermentation.

11 The results of this study indicate that feeding two milk replacer-based
12 probiotics, FMR and high-concentrated LAB-MR, reduced BRV-induced diarrhea and
13 tissue damage to the intestinal tract and suppressed the clinical signs of acute enteritis by
14 maintaining milk intake. This direct evidence could be helpful in the development of
15 potential novel methods to control diarrhea in neonatal calves. Indeed, our retrospective
16 investigation indicated that FMR feeding reduced the incidence of diarrhea in a dairy
17 farm with a high incidence of enteritis as a result of mixed infection with rotavirus and *C.*
18 *parvum*. Although there is no direct evidence of the efficacy of FMR against *C. parvum*
19 infection, feeding with fermented milk was reported to be a protective factor against the
20 shedding of *C. parvum* under field conditions (Delafosse et al., 2015). In addition,
21 feeding with FMR reduced the percentage of calves that required therapy and the number
22 of treatments needed against digestive diseases.

1 **5. Conclusion**

2 This study indicates that the milk replacer-based probiotic feeding has a
3 potential for controlling disease, including diarrhea in neonatal calves. Further
4 experiments using a larger number of infected calves are needed to confirm the efficacy
5 of this novel probiotic approach for clinical applications, to reduce the incidence of calf
6 scours, and the severity and duration of infectious diseases.

7

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7

8 **Declaration of interest**

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

10

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16

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5

1 **Figure legends**

2 **Figure 1. Fermentation of a milk replacer supplemented with BIO-THREE Ace.**

3 A milk replacer suspended in hot water at 7-fold (A) and 3.5-fold (B) dilutions was
4 supplemented with 1% BIO-THREE Ace and fermented at 22–25°C for 5 days. These
5 were mixed once per day and sampled for bacterial colonization on 5% sheep blood agar
6 plates, with measurements of pH (white triangle, right axis) and glucose scores (white
7 diamond, right axis). The bacterial loads (left axis) were calculated from the numbers of
8 *Streptococcus*-like (black circle) and *Bacillus*-like colonies (black square) counted after
9 the overnight cultivation of the plates at 37°C. The dashed lines indicate the criterion
10 values of fermented milk, “ $>10^7$ cfu/mL of *Streptococcus* spp. (LAB)” and “ $<pH$ 5.4”.

11

12 **Figure 2. Clinical signs of calves treated with milk replacer, FMR, and LAB-MR**
13 **during BRV challenge.**

14 Newborn calves fed with the control milk replacer (C-1, C-2, and C-3), FMR (F-1, F-2,
15 and F-3), and LAB-MR (Y-1, Y-2, and Y-3) were inoculated orally with BRV on day 5
16 after the start of the feeding and observed for 10 days after inoculation. (A) The rectal
17 temperature (left axis, black circle), heart rate (right axis, white square), and respiratory
18 rate (right axis, white triangle) were measured. (B) The diarrhea score (left axis, black
19 circle) was determined by visual observations of fresh feces as follows: normal (0); soft
20 (1); mushy (2); watery (3); and very watery (4). The water content in fresh feces (right
21 axis, white square) was calculated by determining the weight change of fresh feces by
22 microwaving. (C) The calves were fed control milk replacer, FMR, or LAB-MR twice

1 per day. The total feed intake was monitored. (A–C) The gray background indicates the
2 period of the BRV challenge. The cross indicates the death or euthanization of the animals.

3
4 **Figure 3. Histopathological evaluation of the intestinal tract in BRV-challenged**
5 **calves.**

6 All BRV-challenged calves in the clinical study were dissected, and their intestinal tracts
7 were collected on the day of death or at day 10. Intestinal lesions of the specimens were
8 evaluated histopathologically. (A) The length of the intestinal villi (arrow) was observed
9 and scored as normal (–) or short (+). All scores are shown in Table 2. (B and C) Crypt
10 abscesses in the intestinal tracts (B) and necrotic lymphoid follicles in the ileum (C) were
11 confirmed and scored as none (normal) (–), mild (+), moderate (++), or severe (+++).
12 Arrowheads indicate crypt abscess or necrotic lymphoid follicle. All scores are shown in
13 Tables 3 and 4.

14
15 **Figure 4. Field evaluation of the effects of FMR treatment against enteritis in calves.**

16 Newborn calves from two independent farms (farms A and B) were fed milk replacer
17 (control) or FMR from birth to weaning. (A and B) The enteritis incidence (A) and
18 mortality from enteritis (B) are shown among all the newborn calves in each treatment
19 group of both farms. Significant differences between treatments and values were
20 determined by Fisher's exact test. The number of enteritis cases and deaths are shown in
21 Table 5. (C–F) The duration of a series of treatments (C), the number of consultations
22 during a single series of treatments (D), the total medical consultation fee (JPY/head)

1 required for enteritis treatment (E), and the age of onset of enteritis (F) are shown for
2 control calves ($n = 89$) and FMR-fed calves ($n = 32$) on farm A. A significant difference
3 between the two groups was determined by Mann–Whitney U test.
4

Figure 1

● 3.5-fold FMR
○ 7-fold FMR

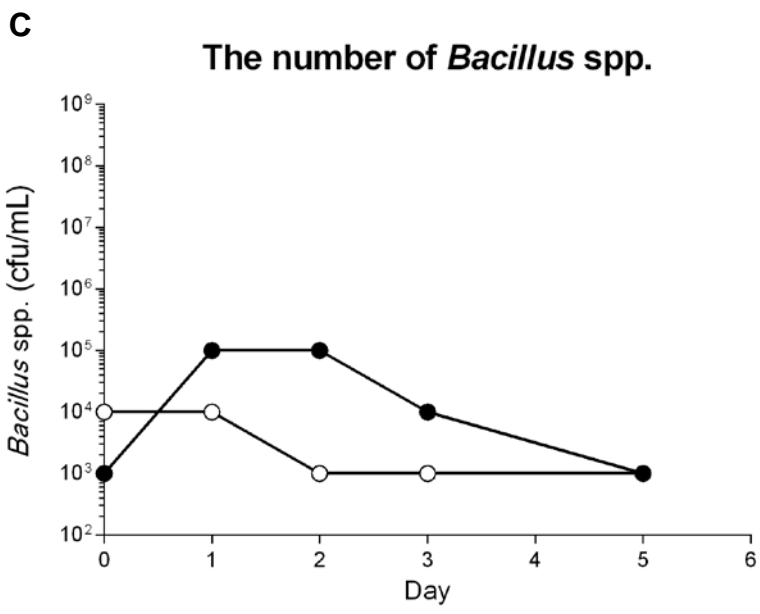
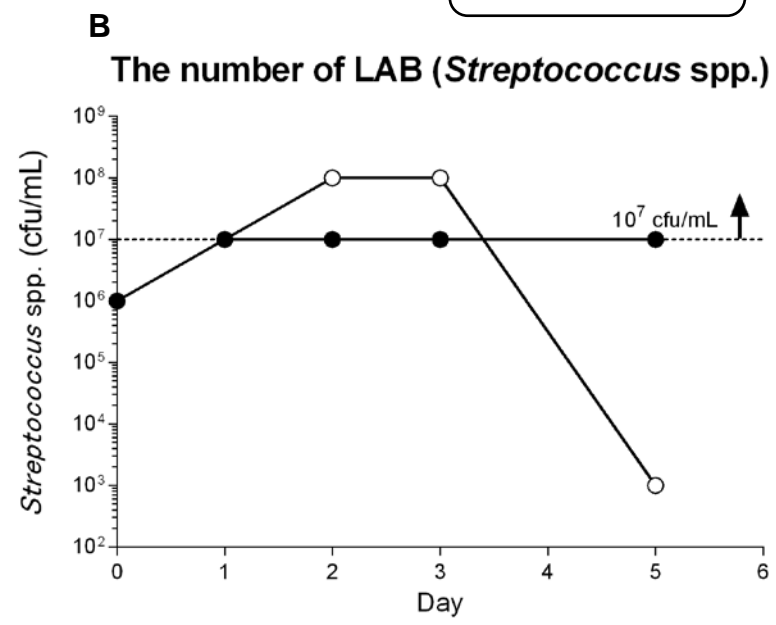
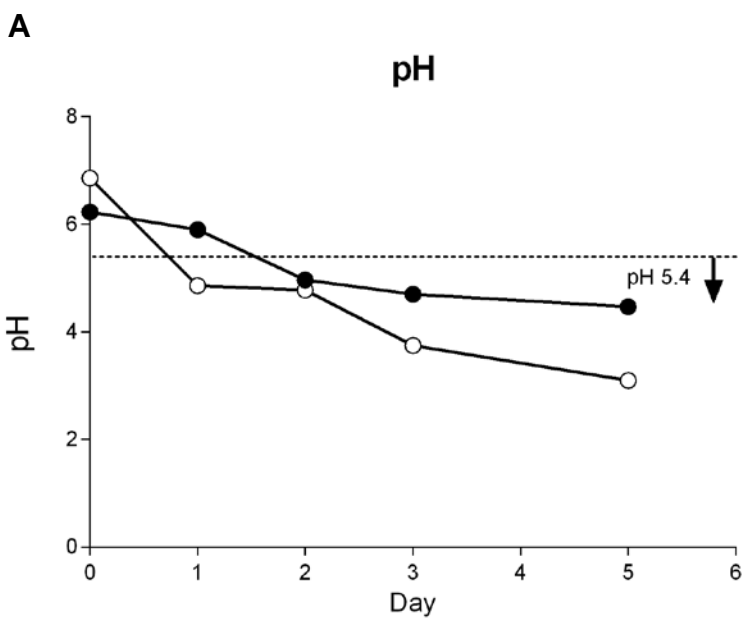


Figure 2

A

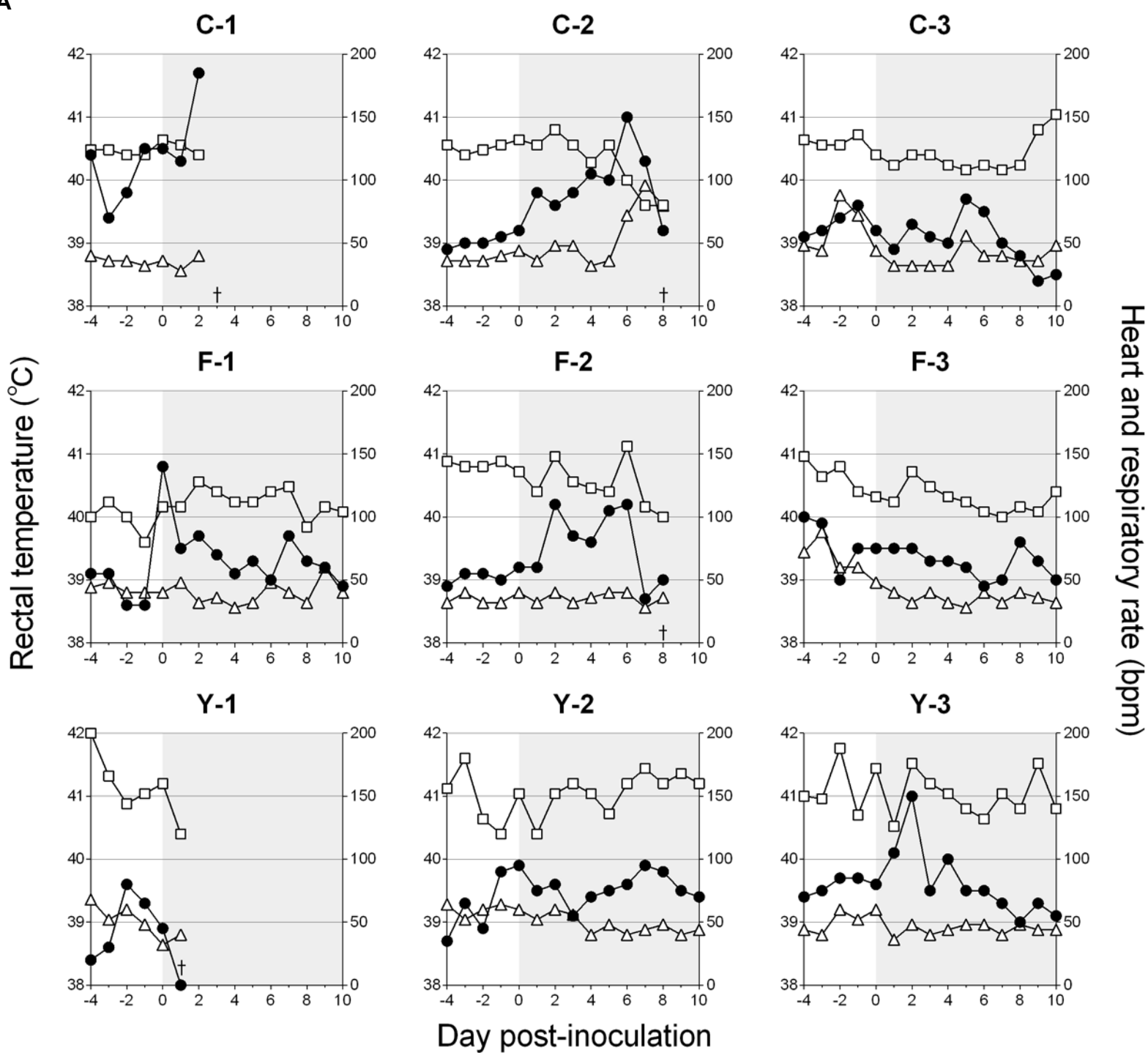


Figure 2 (continued)

B

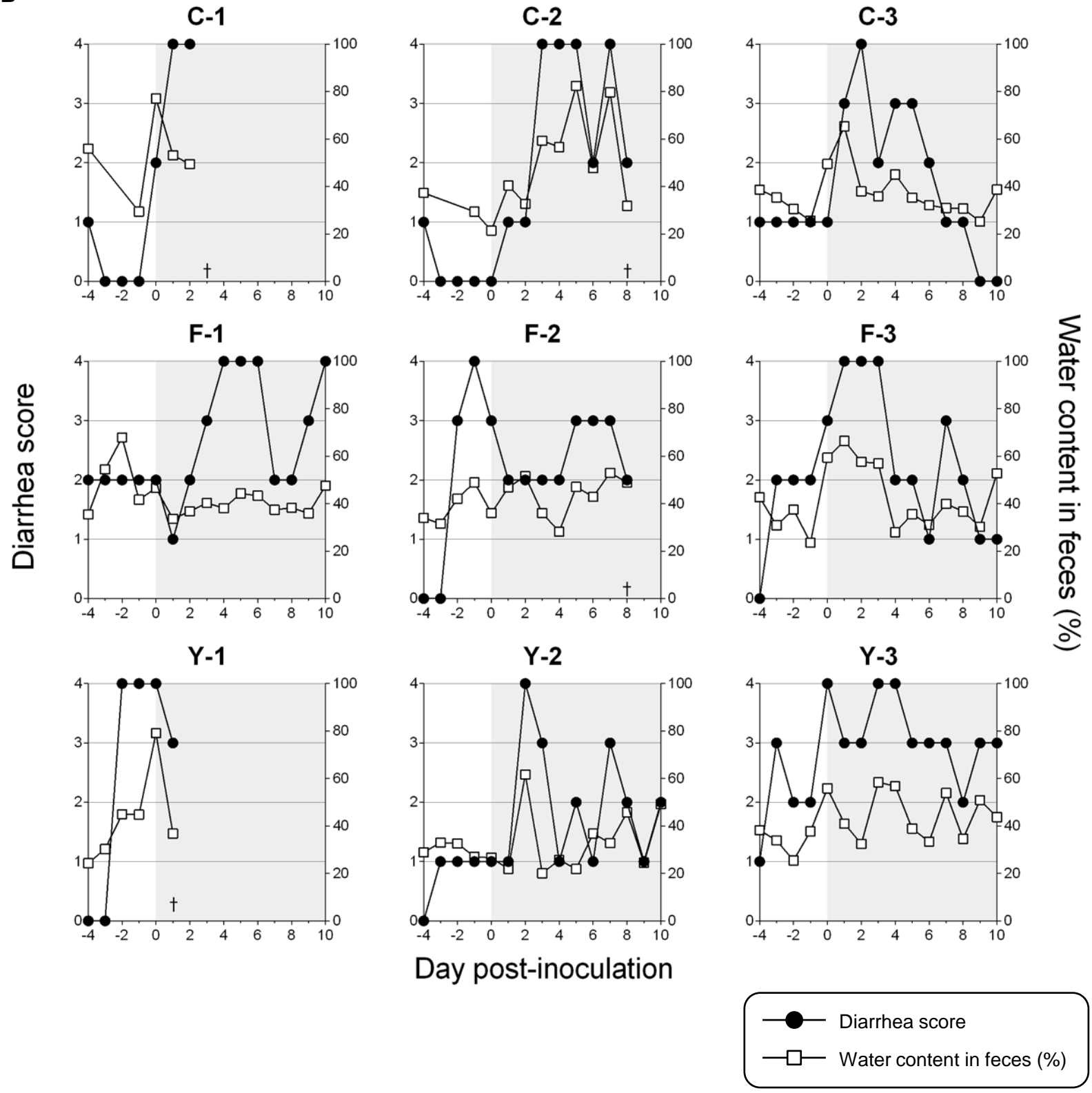


Figure 3

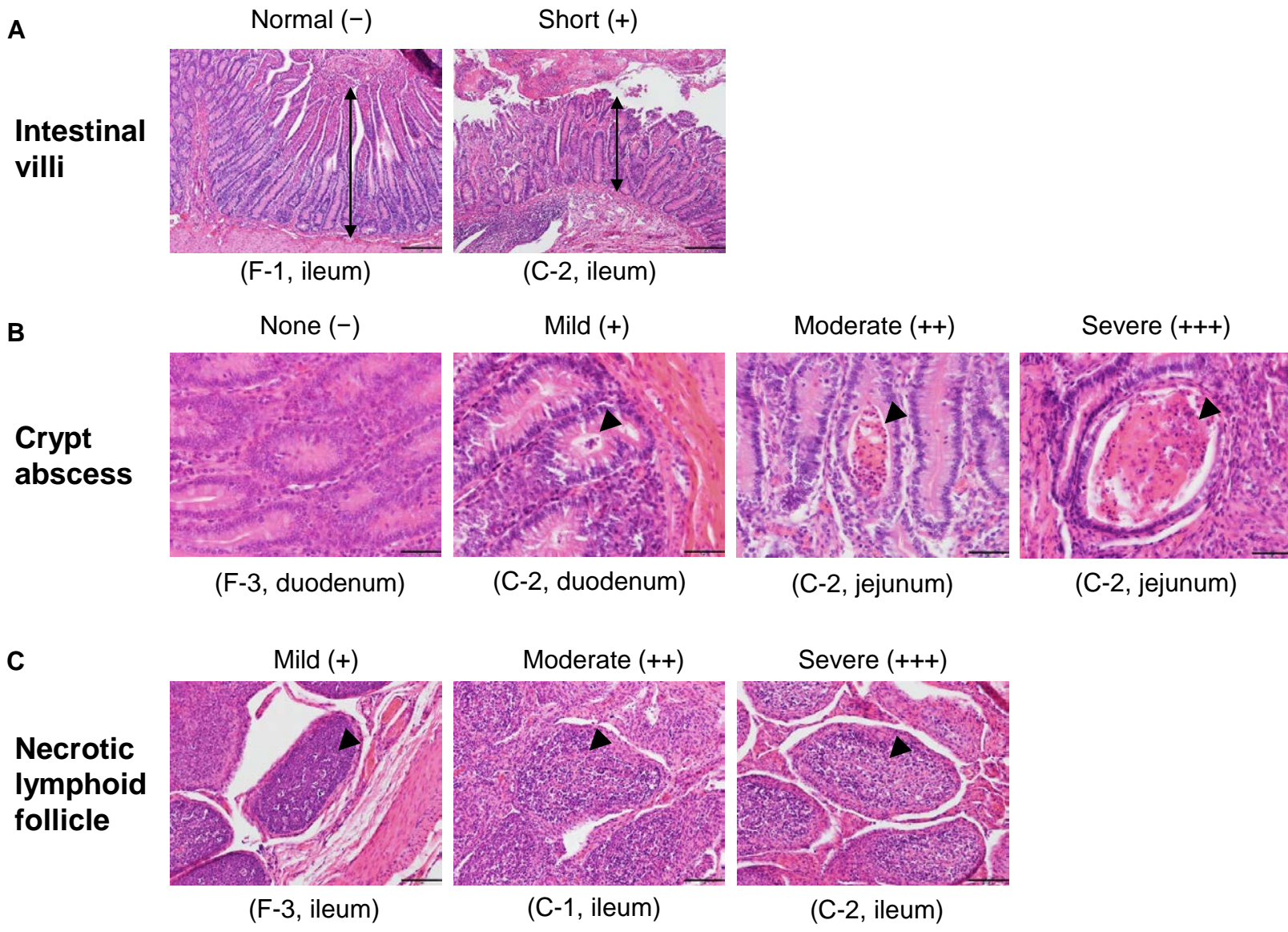
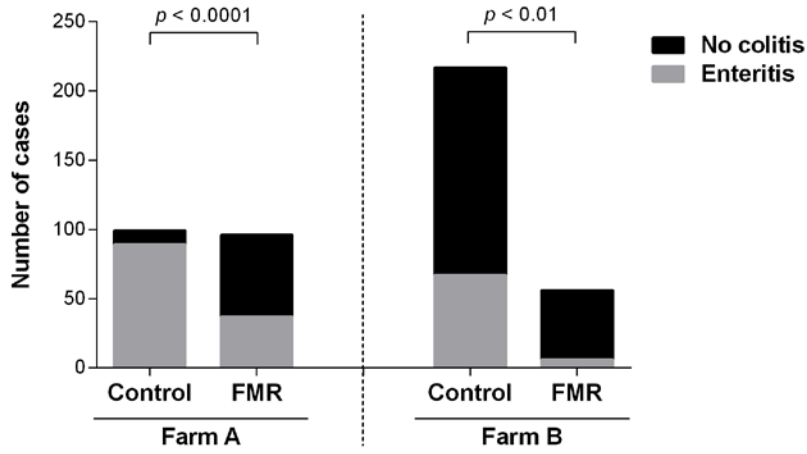


Figure 4

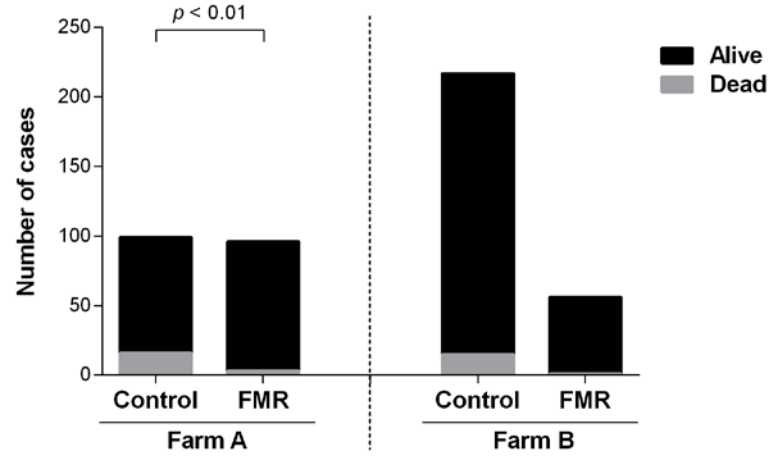
A

Incidence of enteritis



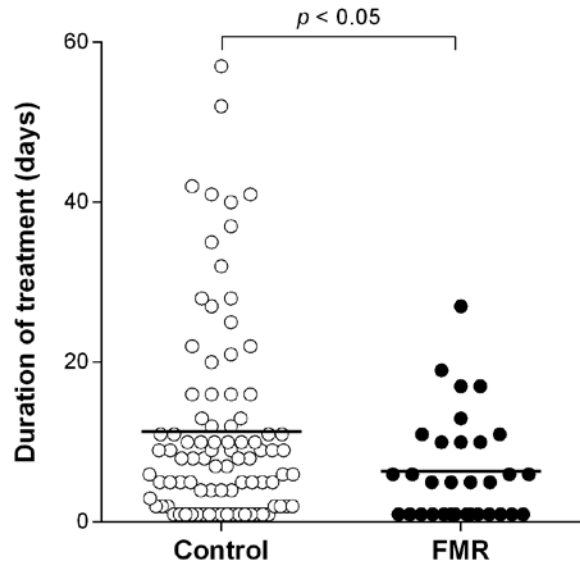
B

Mortality from enteritis



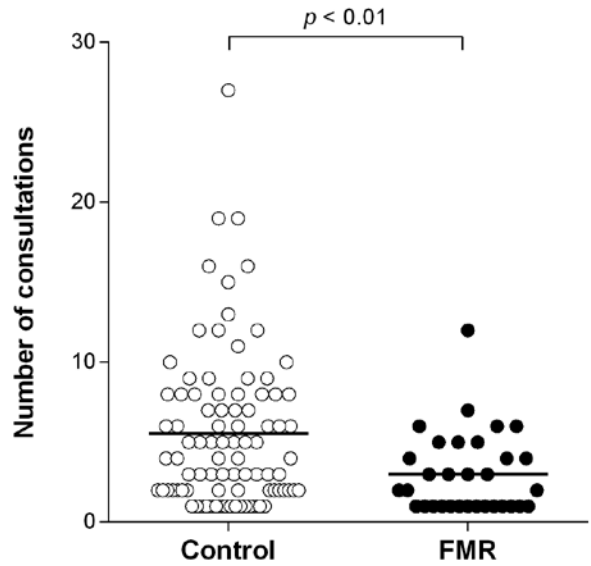
C

Duration of treatment



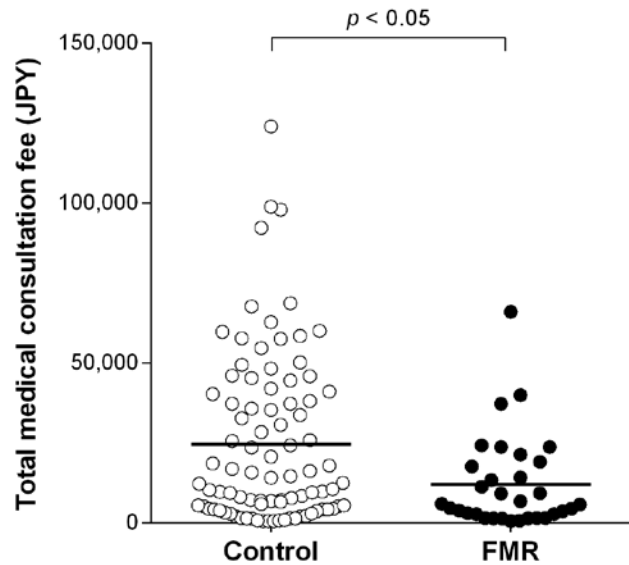
D

Number of consultations



E

Total medical consultation fee



F

Age of onset

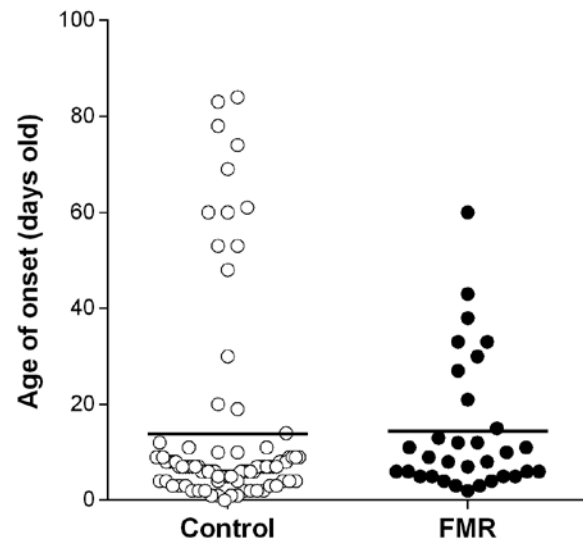


Table 1. Calves used in the clinical study of the probiotic treatments.

Group	Control			FMR			LAB-MR		
	C-1	C-2	C-3	F-1	F-2	F-3	Y-1	Y-2	Y-3
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.

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

Group	Control			FMR			LAB-MR		
	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		
	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		
	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