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# Effects of Daily Oral Administration of Difructose Anhydride III on Health Status, Blood Parameters and Faecal Shedding of Coliform Bacteria of Japanese Black Calves during the Pre-weaning Period

**M. Takagi*, H. Hasunuma[3], D. Matsumoto[3], T. Obi[1], K. Takase[1], M. Ohtani[4], T. Sato[4], U. Watanabe[5], K. Okamoto[5], T. Tanaka[2], C. Tshering and E. Deguchi**

Laboratory of Farm Animal Production Medicine
Kagoshima University, Kagoshima 890-0064, Japan

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## ABSTRACT

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The prebiotic effect of difructose anhydride III (DFA III) as a supplement in milk replacers (MRs) on the health and faecal bacteriological flora of suckling Japanese Black calves was examined. Ten calves were divided into two equal groups (5 calves per group) and fed MR supplemented with or without DFA III. The prebiotic supplement was administered orally twice daily (3 g per administration) from within 5 days after calving to 1 month of age (Day 30). Health, haematology and blood-chemistry, and population of faecal coliforms were assessed at 2-weeks intervals. The number of calves requiring medication for diarrhoea tended to be lower ($P=0.08$) in the treatment group during the treatment and 1-month follow-up periods. The mean total cholesterol concentration differed significantly ($P<0.05$) at Day 14, and the total cholesterol concentration at Day 30 and iron concentration at Day 14 tend to differ between the groups. Additionally, although the number of coliforms at each sampling point decreased significantly ($P<0.05$) in the treatment group during the DFA III treatment period, no significant change was observed in the number of coliforms of the control group between Day 14 and Day 30. The differences between the groups might reflect the clinical incidence of diarrhoea after calving. The present study revealed the potential benefit of DFA III as a prebiotic for calves during the pre-weaning period.

**Key words:** Calf, Difructose anhydride III, Prebiotics, Coliform

*Reprint request: Mitsuhiro Takagi; E-mail: mtakagi@agri.kagoshima-u.ac.jp
[1]Laboratory of Veterinary Microbiology, [2]Laboratory of Emerging Infectious Diseases, Kagoshima University, Kagoshima 890-0064, Japan; [3]Shepherd Central Livestock Clinic, Kagoshima 899-1611, Japan; [4]Research Center, Nippon Beet Sugar Manufacturing Co. Ltd., Obihiro 080-0831, Japan; and [5]Soo Veterinary Clinical Center, Soo Agriculture Mutual Aid Association, Kagoshima 899-8212, Japan

## INTRODUCTION

As the use of antibiotics in animal production may contribute to the resistance of human pathogens, alternatives such as probiotics and prebiotics have been proposed (Heinrichs *et al.*, 2003; Timmerman *et al.*, 2005; Jouany and Morgavi, 2007). There is growing interest in the health-promoting benefits of prebiotics such as mannanoligosaccharide (Heinrichs *et al.*, 2003; Franklin *et al.*, 2005), fructooligosaccharides (Donovan *et al.*, 2002), lactulose (Fleige *et al.*, 2007), and fermentation products (Heinrichs *et al.*, 2009). It has been reported that prebiotics positively influence the bacterial flora of the gastrointestinal tract (increasing the population of *Bifidobacteria* and *Lactobacillus*), thereby reducing the incidence of diseases in animals (Fleige *et al.*, 2007; Heinrichs *et al.*, 2009).

Difructose anhydride III (DFA III) is a naturally occurring nondigestible disaccharide present in commercial roasted chicory and manufactured from inulin by microbial fermentation (Yokota *et al.*, 1991; Tamura *et al.*, 2004). DFA III promotes calcium absorption in rats (Mineo *et al.*, 2002; Shiga *et al.*, 2003), humans (Shigematsu *et al.*, 2004; Tomita *et al.*, 2007), and cattle (Sato *et al.*, 2007). Furthermore, Minamida *et al.* (2005, 2006) reported that the oral administration of DFA III in laboratory animals may help maintain a healthy balance of intestinal microbiota, and suggested that DFA III is a novel prebiotic. We speculated that the etiotropic effects of DFA III could be applied to group-housed calves fed milk replacers and that DFA III would help maintain the health of calves and hence reduce the need for antibiotics. Recently, we reported the efficacy of DFA III as a supplement in a colostrum replacer for calves during the pre-weaning period (Matsumoto *et al.*, 2009) and suggested the use of DFA III as a prebiotic for cattle. However, no reports are available concerning the efficacy of DFA III after daily oral administrations on clinical health status, concomitant with parameters based on blood examinations and the faecal bacteriological flora of calves.

Therefore, in this preliminary study, we aimed to investigate, under commercial farming conditions, the prebiotic effects of the oral administration of DFA III as a daily supplement in a milk replacer (MR) to calves during the early pre-weaning period. We examined the health (frequency of fever and/or diarrhoea), haematology and blood-chemistry, and faecal bacteria (enterobacteriaceae) of calves treated with DFA III in the pre-weaning period.

## MATERIALS AND METHODS

Ten Japanese Black calves (4 males and 6 females) born between August and October on a private farm in Kagoshima Prefecture, Japan were studied. Calving occurred naturally on the stall in all cases, and the calves were fed fresh colostrum from their dam within 2h after birth. After the first feeding, the calves were orally administered a colostrum replacer (Glomlin; Toa Pharmaceutical Co. Ltd., Tokyo, Japan) and a colostrum-derived immunoglobulin preparation (First defense; ImmuCell Corporation, Portland, ME) within 6h of the calving. Additionally, they were orally administered 10g of a

probiotic preparation (*Enterococcus faecium* BIO-4R, Balantol; Kohkin Chemical Co. Ltd., Osaka, Japan) once a day before being separated from their dam within 5 days after calving and moved to a group-housing pen bedded with wood shavings. Thus, all the calves were considered to have similar levels of stress due to infection. New calves were continually introduced into the existing group (maximum number of calves was 3-4).

The calves were randomly assigned to a treatment group (n = 5; 3 males and 2 females) or a control group (n = 5; 1 males and 4 females). All were fed a MR (total digestive nutrients > 110%, crude protein>24%, fat>20%; Premium Meiluck, Meiji Co. Ltd., Tokyo, Japan) via a nursing bottle twice a day. The calves in the treatment group received a MR supplemented with 3g per administration of DFA III (Nippon Beet Sugar Manufacturing Co. Ltd., Obihiro, Japan) twice a day, the dose recommended for the prevention of hypocalcemia in dairy cows (Sato *et al.*, 2007). Calves in the control group were fed the same MR without DFA III. The volume of the MR provided was initially 4 l/d; but this was gradually increased to a maximum of 6 l/d by one week before weaning (approximately 3 month of age), and then decreased to 3 l/d until weaning, regardless of the body weight and sex of the calves. The intake of calf starter (total digestible nutrients 86.2%, crude protein 22.9%; Premium Meistarter P, Meiji Co. Ltd., Tokyo, Japan) was monitored daily. Fresh water and a calf starter supplemented with minerals and vitamins were provided *ad libitum*.

General health, including appetite and faecal consistency, was monitored daily during the experimental and 1 month follow up periods by experienced farm staff. Additionally, a veterinarian visited the herd. Enteritis, bronchitis, and pneumonia were diagnosed on the basis of previously reported clinical criteria such as diarrhoea (gruel-like or watery feces), fever (rectal temperature>39.5°C), and signs of respiratory disease involving severely increased respiratory sounds accompanied by fever and coughing or a grayish to yellowish nasal discharge (Svensson and Liberg, 2006). Treatment consisting of mainly systematic antibiotic therapy accompanied by supportive therapy was administered according to the clinical diagnosis. Treatment data were recorded for each calf.

Additionally, blood samples from the jugular vein were collected within 5 days at separation from their dam (Day 0), 14 days (Day 14), and 30 days (Day 30) after calving in order to determine the following: a complete blood count (CBC; assessed on F-820; Sysmex, Japan) and blood urea nitrogen (BUN) and serum aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), total cholesterol, glucose, free fatty acid (FFA), calcium (Ca), magnesium (Mg) and iron (Fe) (measured on a Labospect 7080 autoanalyzer; Hitachi, Japan), immunoglobulin (Ig) IgG, IgA and IgM (measured using radial immunodiffusion method; Metabolic-Eco-System Institute, Japan), and lactoferrin (LF; measured using the latex agglutination turbidimetric immune assay method; Cosmo Bio Co. Ltd., Japan) levels. The tests were performed to monitor hepatic (AST and GGT) and renal functions (BUN), nutritional status (total cholesterol, glucose and FFA), Mineral intake (Ca, Mg and Fe), inflammation (LF), and immune status (IgG, IgA and IgM) of the calves in the two groups.

A bacteriological analysis was conducted to evaluate the prebiotical efficacy of DFA III for monitoring intestinal microflora, especially for changes in enterobacteriaceae numbers. Together with blood samples, faecal samples were collected from all calves upon rectal stimulation at Day 0, 14 and 30. The faecal samples were immediately kept refrigerated on ice, transported to the laboratory, and processed within 2 4h of the sampling. The faecal samples (1 g) were then homogenized in 10 ml of PBS buffer. Dilutions of the homogenized samples were made in reduced PBS buffer, the relevant dilutions were plated (DHL agar), and the plates were incubated aerobically at 37°C for 48h. After incubation, the agar plates were assessed for growth and colonies were counted. Using the relevant calculations for the spiral plater, total cell counts of enterobacteriaceae per gram of faecal sample were calculated and transformed into $\log_{10}$ values.

The results obtained for each group are expressed as the mean±SD or SEM. The number of calves that did not receive any medical treatment was compared between the groups by using the Fisher's Exact Test. Values for blood parameters and mean duration (in days) of medical treatment were compared between the groups using the Student *t* test in order to determine the effects of DFA III on the calves. Additionally, the colony forming unit (CFU) counts within each group at Day 0, 14 and 30 were compared using a one-way analysis of variance, followed by a post-hoc test, with SPSS Statistics software (IBM, USA). $P<0.05$ were considered to indicate a statistically significant difference, while $P<0.1$ were considered to indicate a significant tendency.

## RESULTS

### *Health and medical treatments*

The health of the calves and results of the medical treatments are shown in Table 1. The mean (±SEM) duration of medical treatment per calf was 0.8±0.6 d (treatment group) and 0.2±0.2 d (control group) for fever, and 1.8±1.2 d and 2.4±0.5 d for diarrhoea, during the period that DFA III was administered. For the 1-month follow-up period, the corresponding period of treatment averaged 4.8±1.8 d and 3.4±1.7 d for fever, and 1.8±1.2 d and 2.6±0.6 d for diarrhoea. The number of calves requiring medical treatment for diarrhoea during both the treatment and follow-up periods tended ($P=0.08$) to be lower in the treatment group (40%, 2/5) than that in the control group (100%, 5/5).

### *Blood analysis*

The results of the haematological and serum biochemical analyses are shown in Figures 1 and 2 (a and b). No significant differences were observed between the groups at any of the sampling times with regard to red and white blood cell counts, haemoglobin (Hb) levels, hematocrit values and total protein concentrations. The mean serum IgG concentrations at Day 0 of both the treatment (22.9±11.0 mg/ml; range, 5.9 to 35.7 mg/ml) and control (28.4±5.3 mg/ml; 21.8 to 33.6 mg/ml) groups exceeded 10 mg/ml, except for one calf in the former group. Moreover, the difference in serum IgG

Table 1. Effect of DFA III supplementation on number of medical treatments and frequency of diseases in calves.

| | Treatment period | | Follow up period (1 month) | |
|---|---|---|---|---|
| | DFA III | Control | DFA III | Control |
| % with fever | 40 (2/5) | 20 (1/5) | 100 (5/5) | 100 (5/5) |
| No. of treatments | 0.8±0.6 | 0.2±0.2 | 4.8±1.8 | 3.4±1.7 |
| % with diarrhoea | 40 (2/5)[a] | 100 (5/5)[b] | 40 (2/5)[c] | 100 (5/5)[d] |
| No. of treatments | 1.8±1.2 | 2.4±0.5 | 1.8±1.2 | 2.6±0.6 |

[abcd]Significant difference between the groups (P=0.08).

concentrations between the groups was not significant. This might indicate that almost all of the calves in both groups possessed IgG in amounts sufficient for the prevention of infections.

No significant differences were observed between the groups with regard to AST, BUN, FFA, Ca, Mg, LF, IgG, IgA and IgM levels. The GGT concentration at the first sampling was significantly lower (P<0.05) in the treatment group (411.5±121.5 U/l vs. control: 904.8±322.1 U/l) and the glucose concentration tended to be lower (P=0.07) in the treatment group (93.7±26.0 mg/dl vs. 121.0±13.1 mg/dl). Total cholesterol concentrations were significantly higher at Day 14 (81.8±20.9 mg/dl vs. 51.8±15.6 mg/dl) and tended to be higher at Day 30 (147.6±16.6 mg/dl vs. 125.2±16.6 mg/dl) in the DFA III- treated calves. On the other hand, iron concentrations (120.1±44.1 mg/dl vs. 166.7±32.3 mg/dl) in the treatment group tended to be lower at Day 14.

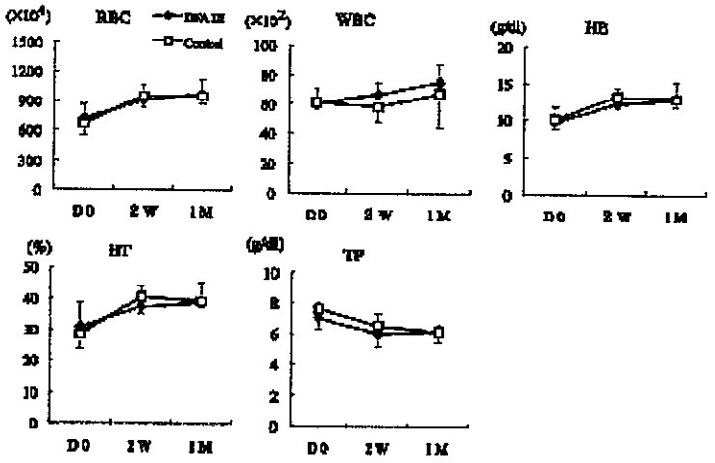


Fig. 1. Effect of DFA III supplementation on periodic changes in haematology and serum total protein of calves.

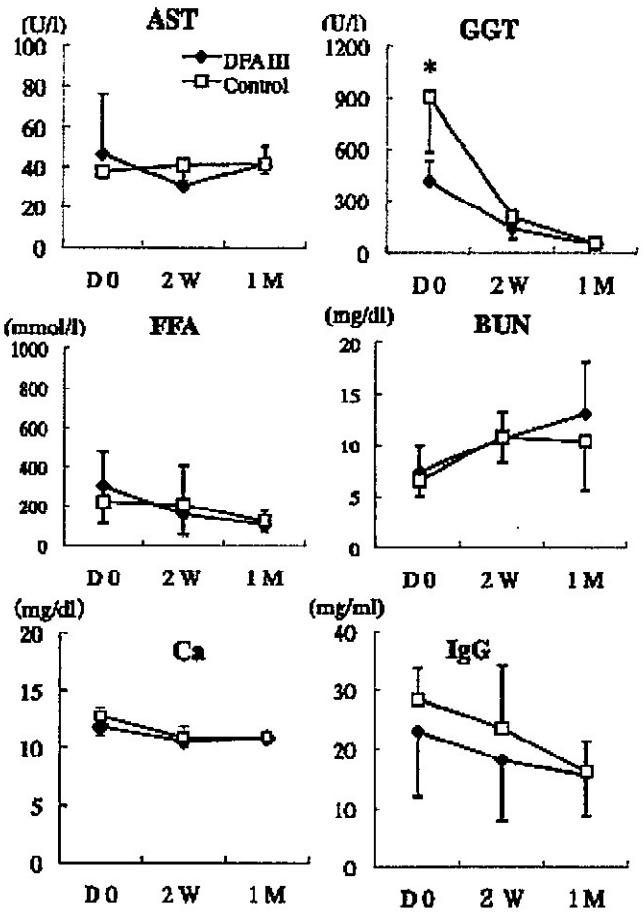


Fig. 2a. Periodic alterations serum biochemical parameters of calves supplemented with or without DFA III (Significant difference between the treatment and control groups *P<0.05).

*Faecal coliform counts in faecal samples*

Figure 3 shows the number of enterobacteriaceae in the faecal samples (CFU/g) at Day 0, 14 and 30 for the 2 animal groups. The CFU/g between the treatment and control groups at each of the 3 sampling points, Day 0 ($8.9\pm0.4$ vs. $8.5\pm0.8$), Day 14 ($7.7\pm0.5$ vs. $7.1\pm0.7$) and Day 30 ($6.9\pm0.5$ vs. $6.6\pm0.6$) did not differ significantly. On the other hand, within group comparison of CFU/g between the sampling points showed significant decrease in CFU/g with each successive sampling points ($8.9\pm0.4$ vs. $7.7\pm0.5$ vs. $6.9\pm0.5$ versus, $P<0.05$) in the treatment group, whilst the change was not significant between Day 14 and Day 30 ($7.1\pm0.7$ vs. $6.6\pm0.6$) in the control group.

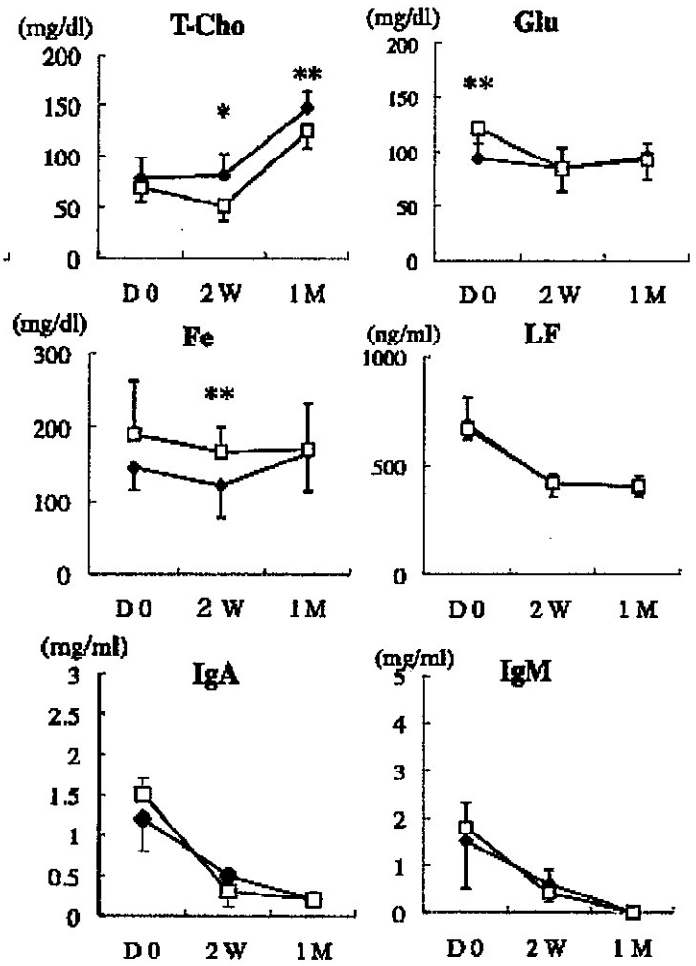


Fig. 2b. Periodic alterations serum biochemical parameters of calves supplemented with or without DFA III (Significant difference between the treatment and control groups *P<0.05; **P<0.01).

## DISCUSSION

Although the functional mechanism of prebiotics has not been fully determined, multiple mechanisms of action have been postulated, particularly, enhancement of the growth of probiotics in the intestine (Macfarlane *et al.*, 2006). The beneficial effects of prebiotics include prevention of the growth of pathogenic bacteria, production of antimicrobial agents, enhancement of the mucosal barrier function, and alteration of immunoregulation (Novak and Katz, 2006). Several effects of the oral administration of

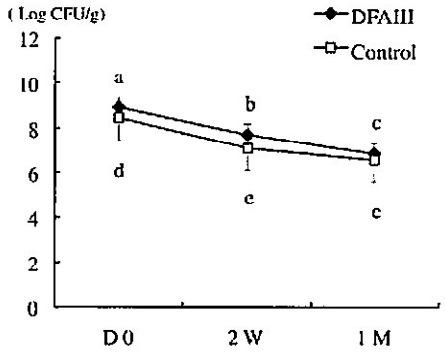


Fig. 3. Population of faecal coliforms (log CFU/g±SD) in faecal samples of calves with or without (control) DFA III at Day 0, 14 and 30 (Significant difference within the treatment group $^{abc}P<0.05$; $^{de}P<0.01$).

DFA III as a supplement have been reported in humans and rats, such as increased intestinal calcium absorption (Mineo *et al.*, 2002; Mitamura *et al.*, 2002; Shiga *et al.*, 2003; Shigematsu *et al.*, 2004) and improved balance of intestinal microbiota resulting in healthier conditions (Minamida *et al.*, 2005, 2006). Recently, we found that the oral administration of DFA III within 24 h after calving as a supplemental feed additive to the colostrum and/or a colostrum replacer reduced the total duration of medical treatment in calves; this finding suggests DFA III to have etiotropic effects on the health of calves in the pre-weaning period, and hence, DFA III was expected to function as a prebiotic in calves (Matsumoto *et al.*, 2009). Thus, the present study was conducted to confirm the effects of the daily oral administration of DFA III on the incidence of diseases and faecal counts of coliforms. The results indicated a significant decrease in CFU/g with each successive sampling points and a tendency of low number of calves not requiring medical treatment for diarrhoea both during the period of DFA III administration and follow up periods in the treatment group, suggesting the positive effects of DFA III as a prebiotic, especially on the digestive health of newborn calves.

In the present study, metabolic evaluation revealed significant differences in some serum biochemical parameters between the calves receiving DFA III and those not, for example, in levels of GGT, total cholesterol, glucose, and iron. These differences might reflect the health and nutritional status of the calves during the experimental period. The GGT concentrations of both groups decreased in a time-dependent manner, and a significant difference was observed between the groups at the first sampling point within 5 days of calving. As indicated before (Perino *et al.*, 1993; Thompson and Pauli, 1981; Wesselink *et al.*, 1999), the GGT concentration of newborn calves who have fed on the colostrum is usually extremely high (300< U/l) and decreases in a time-dependent manner. In the present study, the difference in the first sampling day between the treatment (mean; 4.2 days) and control (mean; 3.8 days) group would reflect the difference in the GGT concentration. On the other hand, the concentration of glucose tended to be lower ($P=0.07$)

in the treatment group at the first sampling, mainly due to one calf with a low glucose concentration (52.7 mg/dl). Interestingly, although there was no significant difference in the IgG concentration between the groups, the IgG concentration of the calf at the first sampling was also low (5.9 mg/ml) compared with the values for the other 9 calves (>20 mg/ml); even the GGT concentration of the calf was more than 300 U/l, which might indicate consumption of enough colostrum immediately after calving. Therefore, the ability to absorb IgG and glucose from the intestine soon after calving of the calf might be insufficient compared with the other normal calves. Daily DFA III administration until 1 month after calving had etiotropic effects on the animal's health, especially diarrhoea, and significantly reduced the number of calves diagnosed with and treated for diarrhoea. These results imply that the enteric inflammation was not as severe in the treatment group. The total cholesterol concentration was found to be significantly lower in the control group at Day 14 and Day 30. This may reflect the clinical symptoms of diarrhoea, and may be due to restriction of the enterohepatic circulation by both severe diarrhoea and an impaired intestinal mucosa and to reduced absorption of lipids from the feed.

Several effects of the oral administration of DFA III as a supplement have been reported in humans and rats, such as increased intestinal calcium absorption (Mineo *et al.*, 2002; Mitamura *et al.*, 2002; Shiga *et al.*, 2003; Shigematsu *et al.*, 2004) and improved balance of intestinal microbiota resulting in healthier conditions (Minamida *et al.*, 2005, 2006). Recently, Sato *et al.* (2007) examined the effects of oral DFA III on prepartum dairy cows and found that it prevented decreases in blood calcium concentrations at calving. Hence, they suggested that DFA III might prevent hypocalcemia in dairy cows. In the present study, we measured the concentrations of not only calcium but also other minerals such as Mg and iron, and found no significant differences between the treatment and control groups except in iron concentrations at Day 14 (DFA III; 120.1±44.1, Control; 166.7±32.3). The calcium in serum consists of two major components, ionized calcium and protein-bound calcium. Although a correlation between ionized and total calcium concentrations was indicated (Bienzle *et al.*, 1993), blood levels of ionized calcium are a better indicator of calcium status and suggested differences between the ionized and non-ionized fractions to be more important than the total level (Radostits *et al.*, 2000). Therefore, although no significant difference in calcium concentrations was found in the present study, it may be worth focusing on the effects of DFA III on ionized calcium levels.

The serum iron concentration at 2 weeks after calving tended to be significantly lower in the treatment than control group. In the present study, the calves in both groups were fed the same type and volume of milk replacer after calving. It has been reported that plasma iron concentrations are independent of the parity or sex of the calves (Kume and Tanabe, 1996). Although the normal range of serum iron concentrations in neonatal Japanese Black calves has not yet been clinically determined, we speculate that the difference between the DFA III treatment and control calves could not be attributed to the composition of the milk replacer. It has been suggested that in dairy cattle, the

plasma iron concentration decreases during acute-phase immunological responses in diseases such as mastitis. This is because of the increased secretion of binding proteins such as LF, an 80-kDa member of the transferrin family of iron-binding glycoproteins in milk; these proteins decrease the amount of available iron and thus reduce the availability of divalent iron, which is required for bacterial growth (Andrieu, 2008). Some previous studies have also suggested LF to be a marker of early-stage inflammation due to infectious diseases (Bennet and Kokocinski, 1978; Lash *et al.*, 1983; Maaks *et al.*, 1989). Although no significant differences were observed in the serum LF concentrations of the two groups, the differences in iron concentrations observed in this study may reflect differences in infection status after parturition. In the present study, a reduction in numbers of CFU/g in both groups within 30 days after calving was observed, and interestingly, the decrease was significant in the treatment group but not in control group. Thus, together with the etiotropic effects on health, these results may possibly reflect the above hypothesis concerning the difference in iron concentrations observed in the present study.

In conclusion, our findings indicated that DFA III added as a daily supplement to a milk replacer after calving has etiotropic effects on health, especially diarrhoea, and these clinical benefits were reflected by both the blood chemical analysis and the examination of coliform numbers in faeces. Therefore, our results suggest functional effects of DFA III as a prebiotic for calves during the pre-weaning period. Further research is required to elucidate the mechanism of action of DFA III as a prebiotic, which may include a change in the number of faecal anaerobic bacteria such as *Bifidobacteria* and *Lactobacilli*.

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