A Study on Efficacy of Novel Dietary Feed Supplementations Presented to Japanese Black Cattle

（黒毛和種牛における新規飼料添加物の有効性に関する研究）

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By

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We hereby recommend that the thesis prepared under supervision by Naoya Sasazaki, entitled “A Study on Efficacy of Novel Dietary Feed Supplementations Presented to Japanese Black Cattle” should be accepted as fulfilling in part for the degree of Doctor of Philosophy.

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ABSTRACT

The objective of Chapter 1 of the present study was to evaluate the effect of heat-killed *Lactobacillus sakei* HS-1 (HK-LS HS-1) on the health and fecal bacteriological change of suckling Japanese Black calves as a supplement in milk replacers. To this end, they were randomly assigned to an HK-LS HS-1 supplement or a control without HK-LS HS-1 group in milk replacers. HK-LS HS-1 was administered from separation day to 3 weeks. Blood and fecal samples were examined. The result is glucose and vitamin A levels on day 7 were significantly higher in the supplement group than in the control group. No significant differences were observed in haptoglobin or serum amyloid A between the groups. The number of *Escherichia coli* in feces was lower in the control group than in the supplement group on day 21. No difference was observed in the number of *Bifidobacteria,* but that of lactic acid bacteria was significantly higher in the supplement group on day 21. The number of medications administered was significantly lower in the supplement group than in the control group during the experimental period. The results indicated that HK-LS HS-1 is potentially beneficial for improving intestinal microbes and reducing the number of medical treatments.

In the second study, we evaluated the effects of supplementing cattle feed with difructose anhydride III (DFA III) by measuring urinary sterigmatocystin (STC) concentrations using 20 Japanese Black cattle aged 9–10 months from one herd. DFA III was supplemented for 2 weeks for 10 animals, and non-treated animals served as controls. STC concentration in the dietary feed was 0.06 mg kg−1(mixture of roughage and concentrate) at the beginning of the study (Day 0). The urine STC concentration was measured using liquid chromatography with tandem mass spectrometry 1 d prior to DFA

III administration, 9 and 14 d thereafter, and 9 d following supplementation cessation, concomitant with the measurement of serum amyloid A (SAA). The number of heifers in which STC was detected in the urine was low in the DFA III group compared to that in the control group on Day 9. After 9 d following supplementation cessation (Day 23), STC concentrations were significantly lower ($P = 0.032$) in the DFA III group than in the control group, although there was no difference in the number of heifers in which urinary STC was detected or in SAA concentrations between the two groups. Our findings demonstrate the effect of DFA III on reducing the urinary concentration of STC in Japanese Black cattle.

GENERAL INTRODUCTION

Probiotics have been defined as “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host.” Several lactic acid bacteria (LAB) strains with species belonging to the genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, are considered beneficial to the host; thus, been widely used as probiotics in cattle production (Uyeno *et al.*, 2015). A recent study with newborn calves indicated that intestinal colonization of newborn calves by *Lactobacillus* spp. takes place during the first 7 days of life (Takino *et al.*, 2017). Early colonization by LAB in the intestinal ecosystem may decrease pathogen adherence to the intestinal mucosa. Additionally, a stable microbial load of *Lactobacillus* species has been shown to improve weight gain and immunocompetence in young calves (Al-Saiady, 2010; Uyeno *et al.*, 2015). Most studies involving human or animal supplementation with LAB refer to the immunomodulatory effects of viable bacteria. However, heat-killed LAB, such as *Lactobacillus acidophilus* or *L. plantarum*, are more effective than viable LAB in immunomodulation and also stimulate phagocytic activity in macrophages (Hirose *et al.*, 2006; Lin *et al.*, 2007; Cheon *et al.*, 2011). They have a longer shelf life and are easy to store and transport (Ishikawa *et al.*, 2010). A recent study on pigs and broilers also revealed that dietary supplementation with heat-killed *Lactobacillus sakei* HS-1 (HK-LS HS-1) improves their growth performance (Shidara, 2012; Khonyoung and Yamauchi, 2019). However, the effects of heat-killed LAB on cattle, especially for JB calves, are unknown. Chapter 1 in the present study primarily aimed to investigate the effect of HK-LS HS-1 as a supplement in milk replacers on the clinical health as well as fecal bacteriological change of suckling JB calves from the early stage after birth.

Sterigmatocystin (STC) is generally considered an *Aspergillus*-derived mycotoxin; however, STC can also be produced by some fungi of the genus *Penicillium*. This mycotoxin is considered the end product of a biosynthetic pathway in some fungal species, such as *Aspergillus versicolor* and *Aspergillus nidulans*, and is also a well-known precursor for the synthesis of aflatoxin (AF) B1 (Hsieh *et al.*, 1973; Wilkinson *et al.*, 2004; Versilovskis and de Saeger, 2010). Currently, no consensus exists on the maximum tolerable limit of STC in food or feed; the European Food Safety Authority (EFSA) classified STC as a possible human carcinogen (Group 2B). This classification was based on research data indicating that STC has carcinogenic, mutagenic, neurotoxic, immunogenic, and estrogenic effects in vitro and in vivo (EFSA Panel on Contaminants in the Food Chain, 2013; Kusunoki *et al.*, 2011). Several incidences of STC contamination in food and feed (grains, grain-based products, maize, and rice) have been also reported in Japan (Kobayashi *et al.*, 2018; Nomura *et al.*, 2018; Kobayashi *et al.*, 2019; Yoshinari *et al.*, 2019). In our previous study, in which STC contamination levels in cattle feed were monitored, we measured urinary STC concentrations using liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Fushimi *et al.*, 2014b) and found that monitoring urinary STC levels helps to evaluate the contamination level in cattle herds. The benefits of difructose anhydride III (DFA III), a unique non-digestible disaccharide present in commercially roasted chicory and manufactured from inulin through microbial fermentation, have been previously reported (Sato *et al.*, 2007; Teramura *et al.*, 2015), which seems to have these properties. When DFA III was used as an anti-mycotoxin supplement, the concentration of zearalenone (ZEN) in the urine of cattle declined, as in indication of reduced systemic bioavailability in the presence of DFA III. However, the exact mechanism, which is likely related to the protective effect of DFA

III on the intestinal barrier, has to be elucidated (Toda *et al.*, 2018). Chapter 2 primarily aimed to evaluate the effects of DFA III supplementation on the concentration of STC in the urine as an indicator of the impact of DFA III on mycotoxin absorption. Additionally, serum amyloid A (SAA), one of the most reliable positive acute-phase proteins mainly produced by the liver and other tissues including the intestines (Berg *et al.*, 2011; Zhang *et al.*, 2018), was also monitored to assess inflammation status and its relationship with changes in STC concentrations.

Chapter 1

Effects of dietary feed supplementation of heat-treated *Lactobacillus sakei* HS-1 on the health status, blood parameters, and fecal microbes of Japanese Black calves

ABSTRACT

This study investigated the effect of heat-killed *Lactobacillus sakei* HS-1 (HK-LS HS-1) on the health and fecal bacteriological change of suckling Japanese Black calves as a supplement in milk replacers. Twelve calves were separated from dams to calf-hatch after calving for milk replacers feeding. They were randomly assigned to an HK-LS HS-1 supplement or a control without HK-LS HS-1 group in milk replacers. HK-LS HS-1 was administered from separation day to 3 weeks. Blood and fecal samples were examined. Two calves with a haptoglobin concentration of >500 μg/m*l* on day 0 were excluded from the experiment, and 10 calves were finally included. Glucose and vitamin A levels on day 7 were significantly higher ($P<0.05$) in the supplement group than in the control group. No significant differences were observed in haptoglobin or serum amyloid A between the groups. The number of *Escherichia coli* in feces was lower in the control group than in the supplement group on day 21 ($P=0.06$). No difference was observed in the number of *Bifidobacteria,* but that of lactic acid bacteria was significantly higher ($P<0.05$) in the supplement group on day 21. The number of medications administered was significantly lower ($P<0.05$) in the supplement group (5.2±3.9) than in the control group (10.6±5.9) during the experimental period. The results indicated that HK-LS HS-1 is potentially beneficial for improving intestinal microbes and reducing the number of medical treatments.

INTRODUCTION

The intestine of a newborn calf is sterile and colonization of the gastrointestinal (GI) tract begins immediately after birth (Uyeno *et al.*, 2015). Therefore, appropriate development of the GI tract microbiota in the early weeks of life is crucial for a functional immune system (Qadis *et al.*, 2014). The intestinal microbiota is affected several factors. These include but are not limited to: diet, antibiotic treatment, environments for as growth and feeding, and stress. The period from birth to weaning is stressful to young calves, causes decreased immunity, and reduces calf herd productivity (Salazar *et al.*, 2019; Takino *et al.*, 2017).

Japanese Black (JB) is the most popular breed of beef cattle in Japan. However, compared with other breeds, these cattle are immunologically weak. Thus, they are more prone to disease during the early postnatal period, even despite adequate passive immunity. During this period, the high risk of infections in JB calves may be ascribed to reduced lymphocyte proliferation (Inokuma *et al.*, 1995; Ohtsuka *et al.*, 2002; Ohtsuka 2011). Although progress has been made in developing vaccines and improving herd management practice and treatment protocols, diseases in calves such as diarrhea and pneumonia, during the lactating period cause considerable economic loss to the JB cattle herds. To prevent such adverse cases, instead of antibiotic use in animal production that may contribute to human pathogen resistance, alternatives such as probiotics and prebiotics have been proposed worldwide (Heinrichs *et al.*, 2003; Timmerman *et al.*, 2005; Jouany *et al.*, 2007; Matsumoto *et al.*, 2009; Uyeno *et al.*, 2015; Salazar *et al.*, 2019). Probiotics have been defined as “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host”. Several lactic acid bacteria

(LAB) strains with species belonging to the genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, are considered beneficial to the host and have, thus, been widely used as probiotics in cattle production (Uyeno *et al.*, 2015). A recent study with newborn calves indicated that intestinal colonization of newborn calves by *Lactobacillus* spp. takes place during the first 7 days of life (Takino *et al.*, 2017). Early colonization by LAB in the intestinal ecosystem may decrease pathogen adherence to the intestinal mucosa. Additionally, a stable microbial load of *Lactobacillus* species has been shown to improve weight gain and immunocompetence in young calves (Al-Saiady, 2010; Uyeno *et al.*, 2015).

Most studies involving human or animal supplementation with LAB refer to immunomodulatory effects of viable bacteria. However, heat-killed LABs, such as *Lactobacillus acidophilus* or *Lactobacillus plantarum*, are more effective than viable LABs in immunomodulation and also stimulate phagocytic activity in macrophages (Hirose *et al.*, 2006; Lin *et al.*, 2007; Cheon *et al.*, 2011). They have longer shelf life and are easy to store and transport (Ishikawa *et al.*, 2010). A recent study on pigs and broilers also revealed that dietary supplementation with heat-killed *Lactobacillus sakei* HS-1 (HK-LS HS-1) improves their growth performance (Shidara, 2012; Khonyoung and Yamauchi, 2019). However, the effects of heat-killed LAB for cattle, especially for JB calf, are unknown. We hypothesized that supplementation of HK-LS HS-1 at age 7 days will enable LABs to establish a community in the intestinal tract and increase in concentration without harmful effects on the calves. The aim of this study was to investigate the effect of HK-LS HS-1 as a supplement in milk replacers (MR) on the clinical health (frequency of diarrhea and/or fever) as well as fecal bacteriological change of suckling JB calves from the early stage after birth. Moreover, measurements of

hematology and blood chemistry were conducted to monitor and compare the states of hepatic, renal, nutritional, and mineral intake, immunoglobulins, and inflammation in calves with and without HK-LS HS-1 supplementation.

MATERIALS AND METHODS

The experiments were conducted according to the regulations concerning the protection of experimental animals and the guidelines of Yamaguchi University, Japan (No. 40, 1995; approval date: March 27, 2017) and we obtained informed consent from the farmer.

Twelve JB calves born between April and July 2019 on a private farm in Kagoshima Prefecture, Japan were studied. In this experiment, calves born to mothers who had problems during labor such as dystocia were excluded from the experiment. Calving occurred naturally in the stall in all cases, and the calves were fed fresh colostrum from their dam within 2 hr after birth. After the first feeding, the calves were orally administered a colostrum replacer including totally 60 g of IgG (Headstart; Bayer Co., Ltd., Tokyo, Japan) mixed into 1 liter warm water by feeding bottle within 6 hr of the calving. Separation of calves from dams to calf-hatch was conducted 2–12 days (mean: 6.7 ± 3.6 days) after calving for MR feeding based on the calf's health condition and willingness to feed. Thus, all the calves were considered to have similar levels of stress due to infection. The calves were randomly assigned to the HK-LS HS-1 supplement group (n=6) or control without HK-LS HS-1 group (n=6) in MR. HK-LS HS-1 (*Lactobacillus*-KDP®; Daiwa Pharmaceutical Co., Ltd., Tokyo, Japan) was administered (0.2% HK-LS HS-1 based on a preliminary trial) orally twice daily at 9:00 am and 4:00 pm from the day of separation to 3 weeks. The volume of the MR provided was initially 3 l (600 g MR)/day, but this was gradually increased to a maximum of 6 l (1,000 g MR)/day by the end of the sampling on day 21, regardless of the body weight and sex of the calves. The intake of calf starter (total digestible nutrients >76.0%, crude protein

>23.0%; Banana Calf, Nippon Agricultural Industry Co., Ltd., Yokohama, Japan) was also monitored daily. Fresh water and a calf starter supplemented with minerals and vitamins were provided ad libitum during the experimental period.

General health, including appetite and fecal consistency, was monitored daily during the experimental periods by experienced farm staff. Additionally, the veterinarians not only visited the farm at the time of sampling from the test calves, but also during the week when there was no sampling. They visited the farm once a week to observe the health of the calves and check the progress of the experiment. Enteritis, bronchitis, and pneumonia were diagnosed based on previously reported clinical criteria such as diarrhea (gruel-like or watery feces), fever (rectal temperature >39.5°C), and signs of respiratory disease (severely increased respiratory sounds accompanied by fever and coughing or a grayish to yellowish nasal discharge) (Matsumoto *et al.*, 2009; Takagi *et al.*, 2011). In this experiment, the farm staff observed the stool properties of the calf at the time of AM and PM feedings; and in cases of mild diarrhea with good appetite, an oral antidiarrheal was administered after the milking that included berberine tannate, phenyl salicylate, acacia yak powder, and torula yeast (Geritomin; Kyoritsu Seiyaku Corp., Tokyo, Japan). In cases of a calf showing severe diarrhea or fever with no appetite at feeding time, injection of antibiotics was given under the direction of a veterinarian, these included penicillin, kanamycin, or oxytetracycline was injected by the farm staff. In this study, recovery from fecal characteristics and normal body temperature was judged to be curative, and medical treatment was discontinued. All treatment data were recorded for each calf.

Blood samples from the jugular vein were collected on the day of separation (before HK-LS HS-1 supplementation; day 0), and 7 (day 7) and 21 (day 21) days after the

supplementation to determine the following: complete blood count (assessed on F-820; Sysmex, Japan) and blood urea nitrogen (BUN), serum aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), total protein (TP), total cholesterol (T-Cho), glucose (Glu), free fatty acid (FFA), 3-hydroxybutyric acid (3HB), albumin (Alb), calcium (Ca), magnesium (Mg), and inorganic phosphorus (iP) (measured on a Labospect 7080 autoanalyzer; Hitachi, Japan). Serum vitamin A (VA) and vitamin E (VE) levels were measured using a HPLC (Shimadzu, Kyoto, Japan) to evaluate the changes in the depletion of both vitamins during the experimental period. Serum haptoglobin (HG) concentration was measured using an enzymelinked solvent assay kit (Life Diagnostics, Inc., West Chester, PA, USA). Serum amyloid A (SAA) concentration was also measured using an automated biochemical analyzer (Pentra C200; HORIBA ABX SAS, Montpellier, France) with an SAA reagent special for animal serum or plasma (VET-SAA 'Eiken' reagent; Eiken Chemical Co., Ltd., Tokyo, Japan). The SAA concentration was calculated based on a standard curve made by a calibrator (VET SAA calibrator set; Eiken Chemical Co., Ltd.). Concentrations of serum immunoglobulin A (IgA) were measured using an enzyme-linked solvent assay kit (Bethyl Laboratories, Inc., Montgomery, TX, USA). The tests were performed to monitor hepatic (AST and GGT) and renal functions (BUN), nutritional status (T-Cho, glucose, FFA, 3HB, and Alb), mineral intake (Ca, Mg, and iP), inflammation (HG and SAA) and intake of immunoglobulin (GGT, TP, Glb, A/G ratio and IgA) of the calves in the two groups.

A bacteriological analysis was conducted to evaluate the effect of HK-LS HS-1 supplementation for monitoring fecal microbes, especially for the number of aerobic (Coliform group) and anaerobic bacteria (LAB, *Bifidobacteria*) in the fecal sample according to a previous method [40]. Fecal samples were collected from all calves upon

rectal stimulation on days 0, 7, and 21. The fecal samples (3 g) were immediately placed on ice in a 50 ml conical tube containing 27 ml of brain heart infusion broth (Difco; Tokyo, Japan) medium, stirred and then transported to the laboratory while refrigerated, and processed within 24 hr of the sampling. Dilutions of the fecal homogenized samples were made in modified phosphate-buffered saline (PBS) including 0.5 g of L-cysteine·HCl·H2O, 0.5 g of sodium thioglycolic acid, and 1 g of agar. The relevant dilutions were plated in de Man, Rogosa, and Sharpe agar media (Kanto Chemical; Tokyo, Japan) for *Lactobacillaceae* spp., brinated horse blood agar media (Nissui Seiyaku, Tokyo, Japan) for *Bifidobacterium* spp., and in deoxycholate hydrogen sulfide lactose agar media (Nissui Seiyaku) for the coliform group. The plates were incubated in each anaerobic and aerobic conditions at 37°C for 48 hr. Subsequently, 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 fecal sample were calculated and transformed into log10 values.

The results obtained for each group are expressed as the mean ± SD. 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 to determine the effects of HK-LS HS-1 on the calves. Additionally, the CFU within each group on day 0, 7, and 21 were compared using a one-way analysis of variance, followed by a post-hoc test, with Ekuseru-Toukei 2012 for Windows (version 1.11; Society Survey Research Information Co., Ltd., Tokyo, Japan). P values less than 0.05 were considered to indicate a statistically significant difference, whereas P values less than 0.1 were considered to indicate a significant tendency.

RESULTS

Two calves were excluded from the experiment because they showed an HG concentration higher than 500 μg/ml (SAA concentrations of these 2 calves [150.8 and 175.0 μg/ml] were also higher than the others). Finally, 5 calves (one male and four females) were assigned to the HK-LS HS-1 supplement group and 5 calves (three males and two females) to the control without HK-LS HS-1 group in MR.

Blood analysis

The results of the hematological and serum biochemical analyses are shown in Figs. 1 and 2. No significant differences were observed between the groups at any of the sampling times with regard to red and white blood cell counts, hemoglobin (Hb) levels, hematocrit values, and total protein concentrations.

No significant differences were observed between the groups with regard to AST, T-Cho, BUN, Ca, IP, Mg, VE, 3HB, TP, AG ratio, and Alb levels. The FFA at the first sampling (day 0) was significantly higher ($P<0.05$) in the HK-LS HS-1 group (355.7 ± 121.9 mmol/l versus control: 211.1 ± 43.8 mmol/l). The Glu and serum VA concentrations were significantly higher in the HK-LS HS-1 group on day 7 (98.6 ± 7.3 mg/dl versus control: 82.9 ± 14.9 mg/dl, and 91.6 ± 15.0 IU/dl versus control: 75.2 ± 5.3 IU/dl, respectively), and GGT was higher on day 21 (47.5 ± 9.8 U/l versus 29.0 ± 13.0 U/l) in the HK-LS HS-1 group.

The results of acute-phase protein (APP) in blood are shown in Fig. 3. No clear difference was found between the HK-LS HS-1 and control groups in serum HG and SAA. As for HG, the average value increased because of one calf in the HK-LS HS-1 group

(more than 500 μg/ml) on day 7. Two calves in the control group (171 and 306 μg/ml) on days 7 and 21 gave a sudden high value, whereas the others showed an average value of <5 μg/ml. On the other hand, SAA tended to be higher at 39.0 ± 12.4 μg/ml in the LS HS-1 group and 39.9 ± 11.8 μg/ml in the control group on day 0, and gradually tended to decrease the mean SAA concentrations in both groups.

Health and medical treatments

Table 1 shows the incidence of illness (diarrhea or fever) and the number of treatments during the test period, which was divided according to the second sampling time (Day 7). Totally, four of the five calves required medical treatment in the HK-LS HS-1 supplement group and all five calves in the control group. The number of calves requiring medical treatment for diarrhea with oral antidiarrheal during the experimental periods was significantly lower in the HK-LS HS-1 group (5.2 ± 3.9) than in the control group (10.8 ± 5.5) (P=0.07). The mean (± SD) duration of medical treatment per calf was 2.6 ± 1.9 day (HK-LS HS-1 group) and 5.3 ± 2.6 day (control group) for diarrhea during the experimental period. Additionally, the cost of medical treatments significantly tended to be lower in the HK-LS HS-1 group (3,619.6 yen) (P=0.07) than in the control group (7,234.2 yen).

Fecal coliform, LAB, and Bifidobacterium counts in fecal samples

The bacteria count (CFU/g) in feces from the 2 groups on days 0, 7 and 21 is shown in Fig. 4. The number of *E. coli* colonies tended to be smaller on day 21 in the control group than in the HK-LS HS-1 group (P=0.06). Although the number of *Bifidobacterium* colonies showed no difference between treatment groups, that of LAB

colonies was significantly increased ($P<0.05$) in the HK-LS HS-1 group than in the control group on day 21.

DISCUSSION

The beneficial effects of probiotics include preventing the growth of pathogenic bacteria, increasing digestive capacity, lowering the pH of the intestinal tract, and improving mucosal immunity to improve production and health of animals have been widely studied in livestock animals (Malmuthuge *et al.*, 2015; Uyeno *et al.*, 2015). Additionally, because the live *Lactobacillus* are difficult to preserve and the effect is not constant, attempts have been made to produce "heat-killed" *Lactobacillus*. Several effects of the oral administration of heat-killed LAB as a supplement, such as induction of IL-12 which leads to a T helper 1 type immune response, suppression of IgE production against naturally fed food allergy, and improvement in the health-related quality of life, have also been reported previously in mice (Lin *et al.*, 2007; Ishikawa *et al.*, 2010), humans (Hirose *et al.*, 2006; Cheon *et al.*, 2011; Arimori *et al.*, 2012; Sawada *et al.*, 2016) and farm animals such as pig (Arimori *et al.*, 2012), chicken (Rikimaru *et al.*, 2017; Khonyoung and Yamauchi, 2019), and fishery (Tung *et al.*, 2010; Dawood *et al.*, 2015). Additionally, regarding HKLS HS-1 applications for farm animals, previous report revealed that daily gain in body weight and feed conversion were improved after supplementation for pigs (Shidara, 2012). Khonyoung and Yamauchi (2019) also examined the effects of oral HK-LS HS-1 supplementation on the growth performance of broiler chickens and found that body weight and feed efficiency increased in the HK-LS HS-1 group than without supplementation, which might be due to the morphological change in the hypertrophied intestinal absorptive epithelial cells on the villus apical surface. However, information on the application of "heat killed" LAB for cattle is still not available. Thus, the present study was conducted to confirm the effects of the daily oral administration of HK-LS HS-1 on

the incidence of diseases and fecal counts of both LAB and coliforms in the early stage of newborn calves. Results of our study showed the effects of HK-LS HS-1 supplementation for the first time in calves. We found a significant increase in LAB CFU/g at each successive sampling point and a low number of calves not requiring medical treatment for diarrhea during the HK-LS HS-1 administration period in the treatment group were found. Based on these observations, it was therefore suggested that the beneficial effects of HK-LS HS-1 reflected by their fecal conditions, particularly on the digestive health of newborn calves.

In the present study, measurements of hematology and blood chemistry were conducted to monitor the states of nutritional intake, immunoglobulins, and some harmful (side) effects of HK-LS HS-1 supplemented calves, as compared with the control calves. It was indicated that serum TP greater 5.5 g/dl of calves between 24 hr and 7 days of age is considered an accurate indicator of the serum IgG concentration of the animal; in case of Ht is also measured as a proxy for hydration status (House *et al.*, 2020). In the present study, the TP concentrations at Day 0 (starting day of the experiment of each calf) were more than 6.0 g/dl; the concentrations of Alb, Glb, and the A/G ratio were not significantly different between the two groups during the experimental period. In the colostrum, Alb functions as a transport protein; it is absorbed through the intestine of calves, and the concentrations of Alb and TP reflect the immunity of calves and can be used diagnostically (Quezada-Tristan *et al.*, 2014). Additionally, serum IgA concentrations, which also reflect the colostrum intake after birth, were not different between the two groups, with both having similar concentrations and decreasing patterns, as previously report (Molla, 1978). Therefore, it was assumed that the immunity of the examined calves in the two groups were at the same levels as in the present study. In the

present study, metabolic evaluation revealed significant differences in some serum biochemical parameters between the calves receiving HK-LS HS-1 and those not within the normal reference ranges, for example, in levels of FFA on day 0, Glu and vitamin A on day 7, and GGT on day 21, although the reasons for these significant differences are unknown. The differences might reflect the milk intake status between birth and day 0 (the day of start of the experiment) in FFA; the health status between day 0 and day 7 of the calves as shown in Table 1 (although the number of treatments was not significantly different). The GGT concentrations of both groups decreased in a time-dependent manner, and a significant difference was observed between the groups on day 21 sampling. As previously indicated (Thompson and Pauli, 1981; Perino *et al.*, 1993; Wesselink *et al.*, 1999), the GGT concentration of newborn calves that have fed on the colostrum is usually extremely high (300 <U/l) and decreases in a time-dependent manner. In the present study, the mean first sampling day (Day 0) after calving was 5.8 days in the HK-LS HS-1 group and 7.6 days in the control group, in which the GGT concentrations were more than 300 U/l. Therefore, we assume that the GGT concentrations on Day 0 from both groups also reflect a sufficient intake of colostrum after birth, and these differences might reflect the different day interval from birth to start of the experiment of the calves or the health and nutritional status of the calves during
the experimental period.

APPs are secreted during the acute-phase response, as part of the innate immune response to different stimuli such as infection and inflammation (Murata *et al.*, 2004; Eckersall and Bell, 2010). Haptoglobin and SAA are the most prominent APPs in cows [4], and HG is a major APP in ruminants in which the serum HG concentration of healthy cattle is less than 20 mg/l but can increase to 2 g/l within 2 days of infection (Eckersall

and Bell, 2010). Based on previous reports regarding HG, the 2 calves that showed high HG concentrations (more than 500 μg/ml) were excluded from the experiment. Interestingly, the SAA concentrations of the excluded calves were also extremely higher (151 and 175 μg/ml) than the other examined 10 healthy calves that showed less than 20 mg/l in HG but higher SAA concentrations in both groups (HK-LS HS-1 group, 39.0 μg/ml; control group, 39.9 μg/ml) on day 0. In cattle, the SAA3 isoform has been detected in high concentrations in the colostrum (Orro *et al.*, 2008; Berg *et al.*, 2011); thus, the high levels of SAA in the blood of calves may be reasonable after ingesting the colostrum, as observed in the 2 excluded calves that showed extremely higher concentrations. Daily HK-LS HS-1 administration for 21 days after calving had etiotropic effects on the animal's health, particularly diarrhea in the present study, and significantly reduced the number of calves diagnosed with and treated for diarrhea. These results imply that the enteric inflammation is not as severe in the treatment group (also as shown in APP results) compared with the control group that received medical treatments. Therefore, the SAA may also be a possible marker for acute inflammation especially for newborn JB calves. Further clinical research is necessary to obscure the correlation of both HG and SAA in newborn calves.

Recently, the first week of life has been reported to be an important period for the intestinal colonization of calves by LAB spp. (Takino *et al.*, 2017). Moreover, the administration of LAB spp. and *Bifidobacterium* to newborn calves during the first week of life increases weight gain and feed conversion ratio, and decreases diarrhea incidences (Abe *et al.*, 1995; Malmuthuge *et al.*, 2015). These effects are most pronounced in pre-weaned calves, suggesting that probiotic supplements are more effective when the gut microbiota is being established and less effective when the microbiome has stabilized

(Abe *et al.*, 1995; Malmuthuge *et al.*, 2015). Our hypothesis was that supplementation of HK-LS HS-1 at age 7 days will enable LAB to establish a community in the intestinal tract and increase in numbers. Although the number of *E. coli* colonies tended to be smaller in the control group than in the HK-LS HS-1 group on day 21, possibly due to more frequent administrations of antidiarrheal in the control group as shown in Table 1, our results indicated a significant increase in log CFU/g of LAB in the feces collected on Day 21 from HK-LS HS-1 group. Therefore, it was suggested from the results that the HK-LS HS-1 supplementation to calves from approximately at age 7 days would enable LAB to establish a community in the intestinal tract and increase in concentration similar to live probiotics. Previously, the concept of "biogenics" was defined as "food ingredients which beneficially affect the host by directly immunostimulating or suppressing mutagenesis, tumorigenesis, peroxidation, hypercholesterolemia, or intestinal putrefaction" and suggested the administration of non-viable probiotic bacteria to obtain some "probiotic" effects (Mitsuoka, 2000; Ohshima *et al.*, 2016). It was also proposed a concept "paraprobiotic" defined as "non-viable microbial cells (intact or broken) or crude cell extracts, which, when administered in adequate amounts, confer a benefit on the human or animal consumer" (Taverniti and Guglielmetti, 2011). On the other hand, prebiotics are defined as "non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity of one or a limited number of microbes in the gut" (Uyeno *et al.*, 2015). Our findings suggest that HK-LS HS-1 may have etiotropic effects (based on the clinical observations) on the health of calves with affecting the number of intestinal LABs in the early stage of pre-weaning. Hence, HK-LS HS-1 is also expected to function as a biogenic/paraprobiotic in JB calves. Further research is necessary to clarify the functional mechanisms of HK-LS HS-1 for

calves.

In conclusion, the present study revealed the potential benefit of HK-LS HS-1 to improve the intestinal LAB, improving the etiotropic effects of the calf, and reducing the number of medical treatments compared with control calves. Further research is required to elucidate the mechanism of action of HK-LS HS-1 as a biogenic/paraprobiotic that improves immunological functions to newborn calves, which may include a change in the number of fecal anaerobic bacteria such as *Lactobacilli*.

FIGURE LEGENDS

Figure 1. Effects of heat-killed *Lactobacillus sakei* HS-1 (HK-LS HS-1) supplementation on periodic changes in hematology and serum total protein (mean ± SD) of calves.

Figure 2. Periodic alterations in serum biochemical parameters (mean ± SD) of calf supplemented with or without heat-killed *Lactobacillus sakei* HS-1 (HK-LS HS-1) (significant difference between the supplement and control groups *P<0.05)

Figure 3 Periodic alterations in serum haptoglobin (HG) and serum amyloid A (SAA) (mean ± SD) with heat-killed *Lactobacillus sakei* HS-1 (HK-LS HS-1) and control groups

Figure 4 Population of fecal aerobic bacteria colony (*Escherichia coli*) and anaerobic bacteria colonies (lactic acid bacteria, *bifidobacteria*) (mean ± SD) in the heat-killed *Lactobacillus sakei* HS-1 (HK-LS HS-1) group and control group (*P=0.06, **P<0.05).

Table 1. Efficacy of heat-killed *Lactobacillus sakei* HS-1 (HK-LS HS-1) supplementation on calf number requiring medical treatment and frequencies of medical administrations

Figure 1

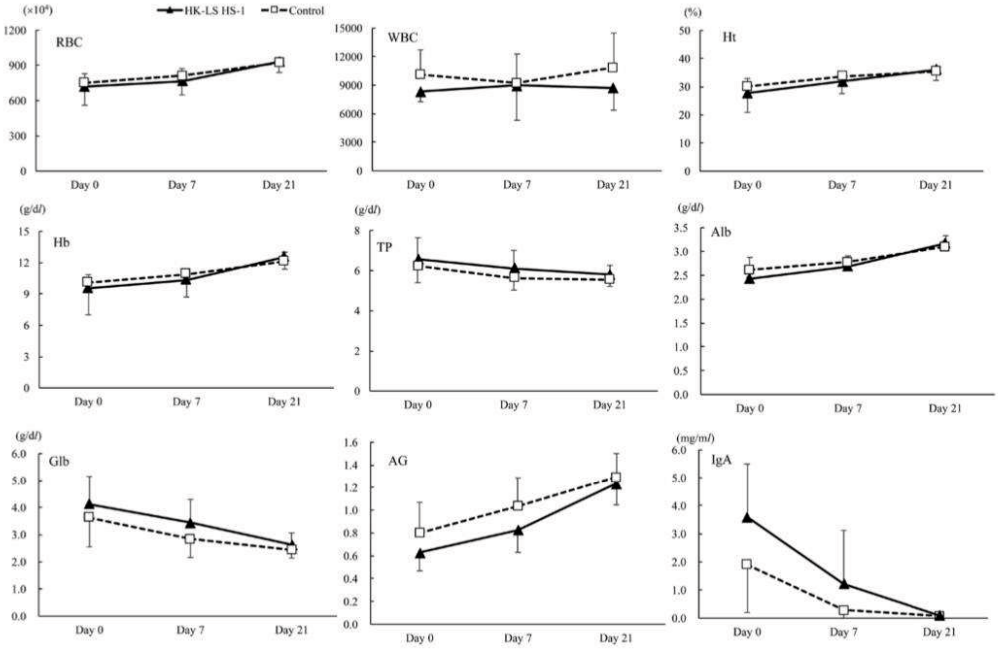
(×10⁴)
HK-LS HS-1
Control
RBC
1200
900
600
300
0
Day 0
Day 7
Day 21
WBC
15000
12000
9000
6000
3000
0
(%)
Ht
50
40
30
20
10
(g/dl)
Hb
15
12
9
6
3
TP
8
6
4
2
Alb
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Glb
AG
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
(mg/ml)
IgA

Figure 2

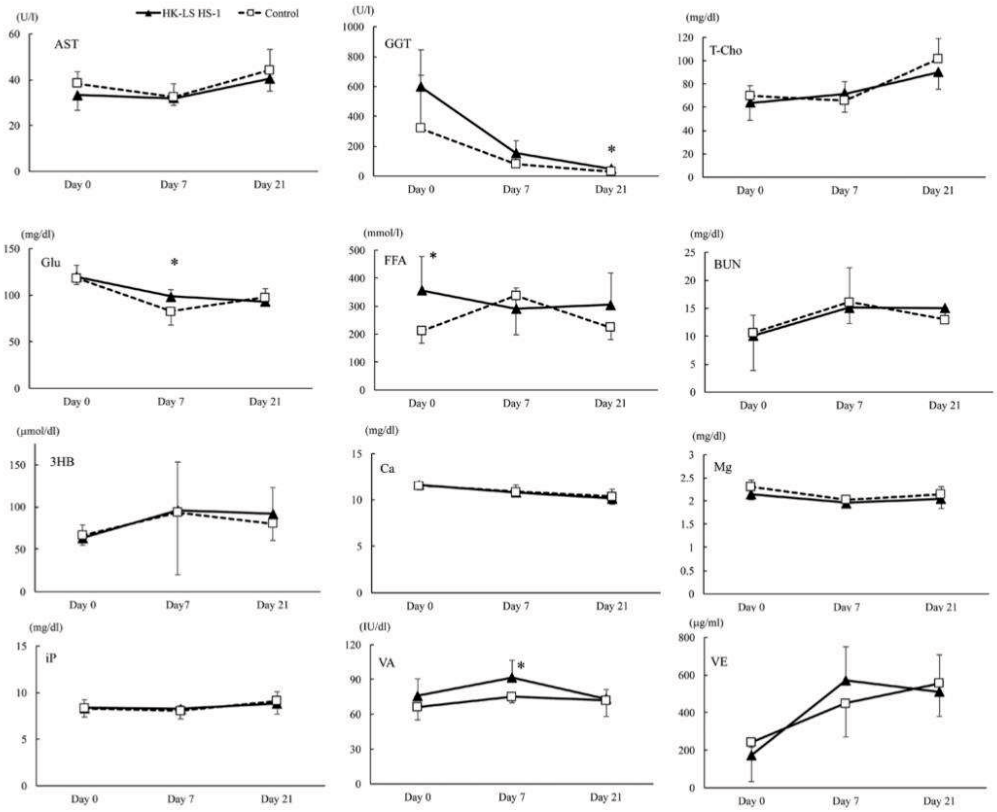
HK-LS HS-1
Control
(U/l)
AST
GGT
(mg/dl)
T-Cho
Glu
(mmol/l)
FFA
BUN
(μmol/dl)
3HB
Ca
Mg
iP
(IU/dl)
VA
(μg/ml)
VE
Day 0
Day 7
Day 21
Day7
Dav 0
Dav 7
Dav 21
*

Figure 3

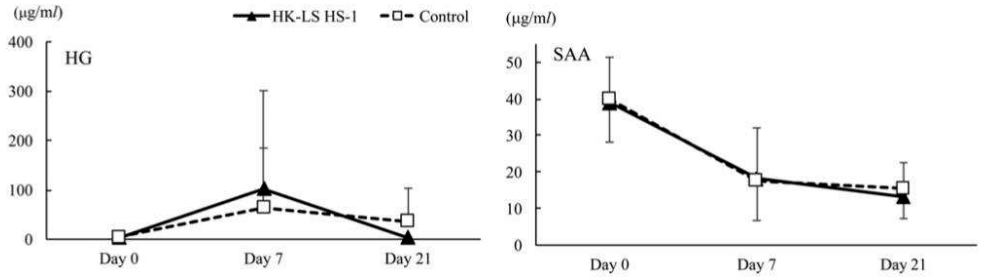
(μg/ml)
HK-LS HS-1
Control
HG
400
300
200
100
0
Day 0
Day 7
Day 21
(μg/ml)
SAA
50
40
30
20
10
0
Day 0
Day 7
Day 21

Figure 4

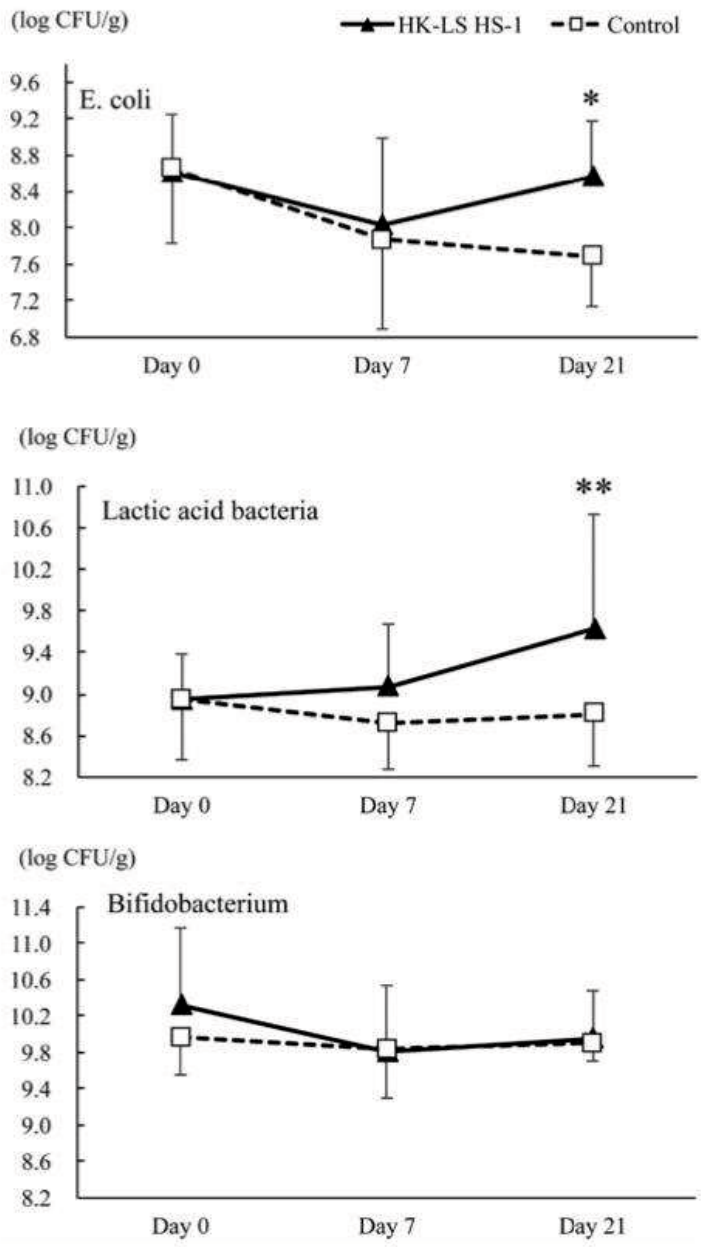
(log CFU/g)
HK-LS HS-1
Control
E. coli
*
9.6
9.2
8.8
8.4
8.0
7.6
7.2
6.8
Day 0
Day 7
Day 21
(log CFU/g)
Lactic acid bacteria
**
11.0
10.6
10.2
9.8
9.4
9.0
8.6
8.2
Day 0
Day 7
Day 21
(log CFU/g)
Bifidobacterium
11.4
11.0
10.6
10.2
9.8
9.4
9.0
8.6
8.2
Day 0
Day 7
Day 21

Table 1 Efficacy of HK-LS HS-1 supplementation on calf number requiring medical treatment and frequencies of medical administrations.

	HK-LS HS-1 (n=5)		Control (n=5)	
	Day 0–Day 7	Day 8–Day 21	Day 0–Day 7	Day 8–Day 21
No. of calf with treatment (%)	2	4	2	4
Total	4		5	
Mean No. of treatments	0.6 ± 0.8	4.6 ± 3.7	1.8 ± 3.1	9.0 ± 7.2
Oral antidiarrheal	2	20	6	43
Antibiotics injection	1	3	3	1
Total (Day 0–Day 21)	5.2 ± 3.9*		10.6 ± 5.3*	
Cost of treatment (Yen)	3,619.6 ± 2,616.1**		7,234.2 ± 3,577.2**	

*Significant difference of tendency between the HK-LS HS-1 supplementation group and control group (P＝0.07)

**Significant difference of tendency between the HK-LS HS-1 supplementation group and control group (P＝0.07)

Chapter 2

Mitigation of sterigmatocystin exposure in cattle by difructose anhydride III feed supplementation and detection of urinary sterigmatocystin and serum amyloid A concentrations

ABSTRACT

We evaluated the effects of supplementing cattle feed with difructose anhydride III (DFA III) by measuring urinary sterigmatocystin (STC) concentrations using 20 Japanese Black cattle aged 9–10 months from one herd. DFA III was supplemented for 2 weeks for 10 animals, and non-treated animals served as controls. The natural STC concentration in the dietary feed was 0.06 mg kg−1(mixture of roughage and concentrate) at the beginning of the study (Day 0). The urine STC concentration was measured using liquid chromatography with tandem mass spectrometry 1 d prior to DFA III administration, 9 and 14 d thereafter, and 9 d following supplementation cessation, concomitant with the measurement of serum amyloid A (SAA). The number of heifers in which STC was detected in the urine was low (10 %) in the DFA III group compared to that (60 %) in the control group on Day 9. After 9 d following supplementation cessation (Day 23), STC concentrations were significantly lower (P = 0.032) in the DFA III group than in the control group, although there was no difference in the number of heifers in which urinary STC was detected or in SAA concentrations between the two groups. Our findings demonstrate the effect of DFA III on reducing the urinary concentration of STC in Japanese Black cattle.

INTRODUCTION

The contamination of agricultural commodities with mycotoxins is a major worldwide challenge in agriculture and livestock production (Fink-Gremmels, 2008). Recently, more attention has been paid to the impact of mycotoxins, as global warming has exacerbated exposure and thereby enhanced the risk for harmful effects on both human and animal health (Liew and Mohd-Redzwan, 2018; Raduly *et al.*, 2020). The contamination of food and feed chains with *Aspergillus*-derived mycotoxins poses a significant global challenge (Raduly *et al.*, 2020). Sterigmatocystin (STC) is generally considered an *Aspergillus*-derived mycotoxin but can be produced by some fungi of the genus *Penicillium* as well. This mycotoxin is considered the end product of a biosynthetic pathway in some fungal species, such as *Aspergillus versicolor* and *Aspergillus nidulans*, and is also a well-known precursor for the synthesis of aflatoxin (AF) B1 (Hsieh *et al.*, 1973; Wilkinson *et al.*, 2004; Versilovskis and de Saeger, 2010). Currently, there is no consensus on the maximum tolerable limit of STC in food or feed; the European Food Safety Authority (EFSA) classified STC as a possible human carcinogen (Group 2B). This classification was based on research data indicating that STC has carcinogenic, mutagenic, neurotoxic, immunogenic, and estrogenic effects in vitro and in vivo (EFSA Panel on Contaminants in the Food Chain, 2013; Kusunoki *et al.*, 2011). Several incidences of STC contamination in food and feed (e.g., grains, grain based products, maize, and rice) have been also reported from Japan (Kobayashi *et al.*, 2018; Nomura *et al.*, 2018; Kobayashi *et al.*, 2019; Yoshinari *et al.*, 2019). In our previous study, in which STC contamination levels in cattle feed were monitored, we measured urinary STC concentrations using liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Fushimi *et al.*, 2014b) and found that monitoring urinary STC levels helps to evaluate the contamination level in cattle herds. Additionally, we have also assessed the

efficiency of mycotoxin detoxification supplements in dietary feed to impair the intestinal adsorption of mycotoxins (Takagi *et al.*, 2011; Hasunuma *et al.*, 2012; Fushimi *et al.*, 2014a).

Several approaches for the prevention of *Aspergillus*-derived mycotoxins in both preharvest and postharvest stages have been reported, such as appropriate agricultural practice and crop management, the introduction of a non-toxigenic antagonistic fungal strain into the field prior to harvest, adequate storage management, and the application of fungicidal agents and other protective silage additives at the postharvest stage. As these measures are only partly effective, it has become common practice to apply adsorbing agents (denoted as mycotoxin adsorbents, MAs), such as mineral clays, probiotics and prebiotics (particularly yeast), yeast cell fractions, lactic acid bacteria, and mycotoxin-degrading enzymes, to animal feed (Sabater-Vilar *et al.*, 2007; Kutz *et al.*, 2009; Awad *et al.*, 2010; Wambacq *et al.*, 2016). Generally, MAs consist of a mixture of a mineral clay carrier, yeast cell wall preparations, and in some cases enzymes or living microorganisms (probiotics) that can adsorb and detoxify mycotoxins (Liew and Mohd-Redzwan, 2018; Vila-Donat *et al.*, 2018). Previously, we have not observed any decrease in STC concentration in the urine after adding a mineral clay carrier mixed with enzymes as a detoxification supplement in cattle feed (Fushimi *et al.*, 2014b), although clay is generally known to be an effective detoxification agent against AFs (Raduly *et al.*, 2020).

In addition, considering the dietary exposure of animals to complex mixtures of mycotoxins inherent to a professional mixed diet, there is a growing interest in the health promoting benefits of prebiotics and non-digestible oligosaccharides, such as mannan-oligosaccharides (Heinrichs *et al.*, 2003; Franklin *et al.*, 2005), fructooligosaccharides (Donovan *et al.*, 2002), and lactulose (Fleige *et al.*, 2007), to reduce the incidence of diseases in animals (Fleige *et al.*, 2009). In particular, oligosaccharides have been shown to interact with intestinal epithelial cells, decreasing their inflammatory reaction to dietary

challenges, such as mycotoxins or physiological stressors including heat stress, by upregulating tightjunction protein expression and modulating the intestinal immune responses (Akbari *et al.*, 2015, 2017a, b; Varasteh *et al.*, 2018). We previously reported the benefits of difructose anhydride III (DFA III), a unique non-digestible disaccharide present in commercial roasted chicory and manufactured from inulin through microbial fermentation (Sato *et al.*, 2007; Teramura *et al.*, 2015), which seems to have these properties. When DFA III was used as an anti-mycotoxin supplement, the concentration of zearalenone (ZEN) in the urine of cattle declined, as in indication of reduced systemic bioavailability in the presence of DFA III. However, the exact mechanism, which is likely related to the protective effect of DFA III on the intestinal barrier, has to be elucidated (Toda *et al.*, 2018). Based on these findings, we hypothesized that DFA III could be an effective anti-mycotoxin supplement against STC. Hence, the aim of this study was to evaluate the effects of DFA III supplementation on the concentration of STC in the urine as an indicator of the impact of DFA III on mycotoxin absorption. In addition, serum amyloid A (SAA), one of the most reliable positive acute-phase proteins mainly produced by the liver and other tissues including the intestines (Berg *et al.*, 2011; Zhang *et al.*, 2018), was also monitored to assess inflammation status and its relationship with changes in STC concentrations.

MATERIALS AND METHODS

All experiments were conducted according to the guidelines and regulations for the protection of experimental animals and guidelines stipulated by Yamaguchi University, Japan (no. 40, 1995; approved on 27 March 2017).

Chemicals and solvents

STC was purchased from MP Biomedicals (Heidelberg, Germany). Stock solutions of 1 μgmL−1 STC in acetonitrile were stored in the dark at 4 ◦C. High-performance liquid chromatography (HPLC)-grade methanol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A β-Glucuronidase/arylsulfatase solution was purchased from Merck (Darmstadt, Germany). Sodium acetate was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Tris was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Japanese Black cattle herds and sample collection

The details of the animals and collected serum and urine samples used in the present study were as described in our previous study (Toda *et al.*, 2018). In brief, 20 Japanese Black heifers (10 months-old, 250–300 kg) from one beef herd raised in Kagoshima Prefecture, Japan, were included in this experiment. The herd consisted of 500 beef cattle fed purchased concentrates and rice straw. The herd of beef cattle in this study was divided over 10 barns, each containing 2 beef cattle, and a total of 20 beef cattle were sampled in the order in which they entered. Roughage and concentrate were stored at ambient temperature in feed sheds and silos, respectively. The STC level in the dietary feed of the herd was first measured before beginning the experiment using LC-MS/MS, as previously reported (Fushimi *et al.*, 2014b). In brief, representative samples of stored straw (2 g) and concentrate (10 g) were homogenized and chopped into small pieces. To

each sample, 20 mL of 84 % acetonitrile was added, shaken for 1 h, and centrifuged for 10 min at 500 × g at room temperature. The supernatant (10 mL) was loaded into a MultiSep 226 AflaZon+ multifunctional column (Romer Labs, Union, MO, USA). A total of 1 mL of the eluent was mixed with 1 mL of acetic acid (1 + 100) and centrifuged for 5 min at 500 × g, and 10 μL of the supernatant was injected into an API3200 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization (ESI) interface and a Prominence HPLC System (Shimadzu Corp., Kyoto, Japan). All conditions of the LC-MS/MS system were the same as previously described (Fushimi *et al.*, 2014b). The detection limit for each analyte was set as 0.01 mg kg−1, and the mean STC recovery rates were 90.5 %–93.5 %. The mean concentration of STC in the mixture of roughage and concentrate fed to the heifers was found to be 0.06 mg kg−1. Heifers were selected from the experimental herd and divided into two groups differing in feed supplementation as follows: the DFA III group (n = 10) was fed 40 g of DFA III per day (20 g twice) mixed with concentrate, while the control group (n = 10) was fed a DFA III-free diet. As a method of allocation, 10 random numbers were generated by a computer: five barns were randomly allocated to the DFA III supplementation group, and five barns were allocated to the control group. The DFA III dose used herein is the dose recommended for the prevention of hypocalcemia in dairy cows (Sato *et al.*, 2007; Teramura *et al.*, 2015). A total of 2 h after the cattle were fed in the morning, urine samples were collected by massaging the pudenda, and blood samples were collected from the jugular veins into silicon-coated tubes. Sampling was performed at the start of DFA III supplementation (Day 0), on Day 9 and Day 14 (i.e. the last day of DFA III supplementation), and on the last day of the experimental period (Day 23), as shown in Fig. 1. All samples were immediately placed in a cooler containing dry ice, also protecting it from UV light, and immediately transported to the laboratory. All urine and blood samples were centrifuged at 1000 and 2000×g, respectively, for 10 min at room

temperature and then frozen at −30 ◦C until STC and SAA analyses. The primary outcome of this study was the STC value at Day 23. The secondary outcomes were the STC and SAA values at each measurement (Days 0, 9, 14) and the STC detectable frequency.

Urine sample analysis

First, STC concentrations were determined with LC-MS/MS using an API 2000 system (Applied Biosystems) equipped with an ESI interface and an HPLC system (Agilent 1200 Series; Agilent Technologies, Santa Clara, CA, USA) as previously described (Fushimi *et al.*, 2014b). Briefly, all urine samples (0.5 mL) were mixed with 3 mL of 50 mM ammonium acetate buffer (pH 4.8) and 10 μL of glucuronidase/arylsulfatase solution and incubated for 12 h at 37 ◦C. Following incubation, the solution was loaded into a C18 solid-phase extraction column (Strata; Phenomenex, Torrance, CA, USA) preconditioned with 3 mL of MeOH and 2 mL of tris buffer, followed by the addition of 2 mL of tris buffer and 3 mL of 40 % MeOH. After elution with approximately 1 mL of 80 % MeOH, the volume was adjusted to exactly 1 mL. Next, 20 μL of the solution was injected into the LC-MS/MS system. Chromatographic separation was performed on an Inertsil ODS-3 column (4.6i.d. × 100 mm, 5 μm; GL Sciences, Tokyo, Japan) at 40 ◦C. A mobile phase consisting of methanol/water/acetic acid (97 : 3 : 0.01, v : v : v), was used (200 μL/min) to separate the analyte in the isocratic mode. Measurements were performed for 15 min. The conditions of measurements with LC-MDS/MS were the same as those in our previous study (Fushimi *et al.*, 2014b). The limit of detection (LoD) was 0.2 ng/mL. The concentration of urinary creatinine was determined using a commercial kit (Sikarikit-S CRE; Kanto Chemical Co., Inc.) according to the manufacturer's instructions and assessed using a 7700 Clinical Analyzer (Hitachi High-Tech, Tokyo, Japan). STC concentrations in the urine were expressed as a ratio to creatinine (pg STC/mg creatinine). In urine samples

with no peaks using LC/MS/MS analysis, the concentration was set to zero (pg STC/mg creatinine).

Measurement of serum amyloid A concentration

All SAA concentrations in the collected samples on Days 0, 9, and 14 were measured using a Pentra C200 automated biochemical analyzer (HORIBA ABX SAS, Montpellier, France) with an SAA reagent specialized for animal serum or plasma (VET-SAA "Eiken" reagent; Eiken Chemical Co., Ltd., Tokyo, Japan). In addition, the concentration of SAA was calculated based on a standard curve made by a VET-SAA calibrator Set (Eiken Chemical Co., Ltd.). Following this, the concentrations of SAA were compared both between and within the groups. In addition, the numbers of heifers in which the concentration of SAA decreased in each sampling period were compared between the two groups.

Statistical analysis

The sample size of this study was selected based on our previous report (Fushimi *et al.*, 2014b). Since this is a pilot study, statistical power was calculated using post-hoc power analysis. All STC and SAA concentrations are expressed as means, standard errors of the mean (SEMs), median, and interquartile range (IQR). For STC value comparison on Day 23 between groups, which is the primary evaluation parameter, the Mann–Whitney U test was used as an ordinal scale in which zero was substituted for the measured value below the lower limit of detection. For the secondary evaluation parameter, the summary statistics of the STC and SAA values at each measurement time point, as well as STC detection frequency, were calculated. For these items, comparison tests were not performed due to difficulties in power and sample size. A p value < 0.05 was considered statistically significant. All statistical analyses were performed using Bell

Curve for Excel software (Social Survey Research Information Co., Ltd., Tokyo, Japan), and power analyses were performed using G*Power 3.1.9 (Franz Faul, Universität Kiel, Germany).

RESULTS

During the study period, no significant clinical differences were observed between the DFA III and control groups.

Concentration of sterigmatocystin with or without difructose anhydride III supplementation

Table 1 shows the results of the analysis of the concentrations of STC in the urine and the number of heifers in which the STC concentration was above the limit of detection (LoD) during the study period, with or without DFA III supplementation. The STC concentrations in the urine on Day 0 showed that both groups exhibited almost the same level of STC contamination. The number of heifers in which STC was quantifiable in the urine was 1 (10 %) in the DFA III group as compared to six (60 %) in the control group on Day 9. On Day 14, however, no STC was detected in urine samples from either group, possibly due to low-level STC contaminated parts within the same lot of feed (straw) eaten prior to sampling. A total of 9 d after terminating supplementation (Day 23), STC concentration was significantly lower ($P = 0.032$, power = 0.741) in the DFA III group than in the control group, although there was no difference in the number of heifers in which urinary STC was detected between the two groups. Representative chromatograms from the LCMS/MS assay are shown in Fig. 2.

Concentration of serum amyloid A with or without difructose anhydride III supplementation

Individual cattle from which all samples could not be obtained three times were omitted from these measurements (DFA III group: n = 1; control group n = 2). The concentrations of SAA (mg/L, mean ± SEM) in the DFA III group (n = 9) on Days 0, 9,

and 14 during the experimental period were 11.8±3.1,5.8±1.2, and 8.1±2.3, respectively, whereas those in the control group (n = 8) were 3.8 ± 0.8,3.2 ± 1.3, and 2.2 ± 0.3, respectively, with no significant differences among the sampling days within each group. In addition, no differences were observed in the numbers of heifers in which the concentration of SAA decreased among the sampling periods, i.e., from Day 0 to Day 9 (DFA III: 8 of 9, control: 5 of 8) and from Day 0 to Day 14 (DFA III: 4 of 9, control: 1 of 8).

DISCUSSION

We have previously reported the benefits of measuring urinary mycotoxin concentrations in cattle to assess the rate of exposure, using zearalenone and STC as examples (Takagi *et al.*, 2011; Fushimi *et al.*, 2014b). We could also show that this approach can be used to assess the efficacy of commercially available MA (Fushimi *et al.*, 2014a). Therefore, considering the desirable effect of DFA III as a feed supplement in reducing the concentration of ZEN in the urine and the hypothesis that this effect was related to the positive effect of DFA III on intestinal barrier functions, we hypothesized that DFA III might also be effective against STC.

Rice straw is considered one of the most important roughages used in the production of beef cattle in Japan. Generally, STC is a major mycotoxin produced in rice. However, the harmful or chronic effects of STC on cattle are not well understood, and there are no regulations or control measures regarding this toxin in Japan. As pointed out in a recent review by Vila-Donat *et al.* (2018), it is important to assess anti-mycotoxin agents by assessing the feed with natural contaminants using real-life scenarios, paying attention to their efficacy and safety, as well as their potential for interactions with critical nutrients, such as vitamins and minerals. Clay is a widely studied agent used in mycotoxin detoxification, especially in reducing the toxicity of AFs (Raduly *et al.*, 2020). STC has a molecular structure rather similar to that of AFs and acts as an intermediate in the biosynthetic pathway of AFs (EFSA Panel on Contaminants in the Food Chain, 2013). Therefore, it is assumed that clay-based MAs might show comparable effects against STC. However, no experimental studies to support this hypothesis have been performed and negative results were found with a clay-based commercial product in one of our previous studies (Fushimi *et al.*, 2014b). Therefore, in the current study, we monitored the concentration of STC in the urine of cattle to assess the beneficial effects of adding DFA

III as a feed supplement. DFA III is currently used as a prebiotic agent and in cases of hypocalcemia in cattle, due to its positive effect on calcium absorption (Teramura *et al.*, 2015). Our results suggest that DFA III might effectively prevent the adsorption of STC in the intestines of cattle, although the results could not be substantiated by statistical analysis due to the limited number of samples. Natural contaminated materials were used in the current study, and at this rather low exposure rate, STC concentrations in urine were not quantifiable in all samples.

When STC is ingested, it is metabolized in the liver and lungs by various cytochrome P450 enzymes into different hydroxymetabolites, and then both conjugated parent STC and its hydroxylated metabolites are excreted via bile and urine (EFSA Panel on Contaminants in the Food Chain, 2013). In a previous report, we indicated that STC is extensively conjugated in the liver, presumably to glucuronic acid, since only trace amounts of free mycotoxin could be detected in the urine (Fushimi *et al.*, 2014b). In this study, however, although the ratio of parent STC reaching systemic circulation remains unknown, our results reconfirmed that even at low natural concentrations of STC (0.06 mg/kg dry matter) detection in the urine of cattle is possible. The hydroxymetabolites could not be identified in this study, partly due to a lack of reference data. However, forthcoming studies should at least aim to capture glucuronidated STC excreted in urine by applying appropriate sample preparation steps.

Most commercial MAs act either by binding mycotoxins on their surfaces (adsorption) or by degrading or transforming them into less toxic metabolites (biotransformation) (Fleige *et al.*, 2007). We previously reported that the use of a commercial MA (mixed adsorption and biotransformation agents) in cattle significantly reduced the concentration of ZEN in the urine but failed to reduce the levels of STC in the urine. As we assume that DFA III reduces the bioavailability and subsequently the measurable concentration in urine by a different mechanism, we decided to assess the

effect of DFA III on STC, which structurally differs from ZEN. Although the number of results in this study is limited, as we used a naturally contaminated diet, results provide a strong indication that DFA III affects the systemic availability and hence the urinary concentration of STC. These results are of importance, as they indicate that DFA III acts differently from the clays used for aflatoxin adsorption. In line with the extensive data on the beneficial effects of non-digestible oligosaccharides on intestinal functions and barrier integrity, DFA III might have a comparable effect. This is supported by the current finding that next to ZEN, DFA III impacted the systemic availability of a structurally entirely different mycotoxin, such as STC.

The intestinal tract is the first organ susceptible to contamination by mycotoxins. It has been suggested that the intestinal barrier plays a crucial role in limiting the absorption of mycotoxins and that exposure to mycotoxins causes inflammatory responses, epithelial cell death, and tight-junction damage in the intestine, leading to a disruption of intestinal barrier function (Liew and Mohd-Redzwan, 2018; Akbari *et al.*, 2015, 2017a, b; Zhai *et al.*, 2019). Moreover, disruption of the intestinal barrier might enhance the translocation of bacterial toxins from the intestine, inducing systemic inflammation (Rao, 2009; Zhai *et al.*, 2019). To assess the level of systemic inflammation in cattle, SAA is often used as a biomarker, as its serum concentration increases in response to various inflammatory stimuli, such as infections, trauma, and toxicity (Berg *et al.*, 2011). However, our results showed that DFA III might not affect the concentration of SAA. This finding might be attributed to the rather low STC concentration in feed (0.06 mg/kg), which might be below the level that causes a systemic inflammatory response, or to the rather short half-life of SAA. Significant changes in SAA levels in the blood are primarily seen at the onset of inflammation, and hence this initial effect might have been overlooked by the current experimental protocol, as the first sampling date was after 9 d of exposure.

Taken together, the results of this study support the hypothesis that oligosaccharides,

such as DFA III, which are now widely being used as prebiotics, can be successfully used as mycotoxin-mitigating substances. The benefit of this approach is a combined effect on adsorption, here measured by declining urinary STC excretion as an indicator of the internal dose, and the known beneficial effects of the prebiotic DFA III on intestinal barrier integrity and gut health. Further field studies with a larger sample size are warranted to establish a database for the assessment of DFA III as a dietary supplement to reduce the absorption of mycotoxins in cattle and other animals. It is also important to further consider the systemic bioavailability of STC in ruminating cattle to improve our understanding of STC contamination and cocontamination with other mycotoxins and to assess the potential risk to cattle health.

FIGURE LEGENDS

Figure 1. Experimental protocol for DFA III supplementation in dietary feed and sampling of blood and urine.

Figure 2. Representative liquid chromatography-tandem mass spectrometry chromatograms for (a) the sterigmatocystin (STC) standard (1 ng/mL); (b) a clear peak of STC contamination in the urine sample (arrow). The horizontal axis of Figures represents time (minutes).

Figure 1

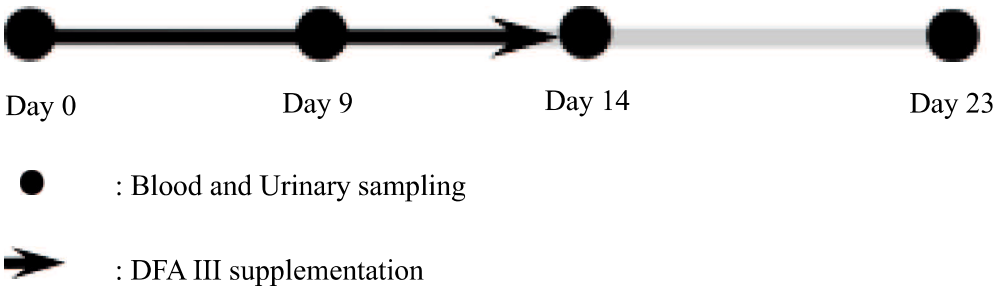
Day 0
Day 9
Day 14
Day 23
: Blood and Urinary sampling
: DFA III supplementation

Figure 2

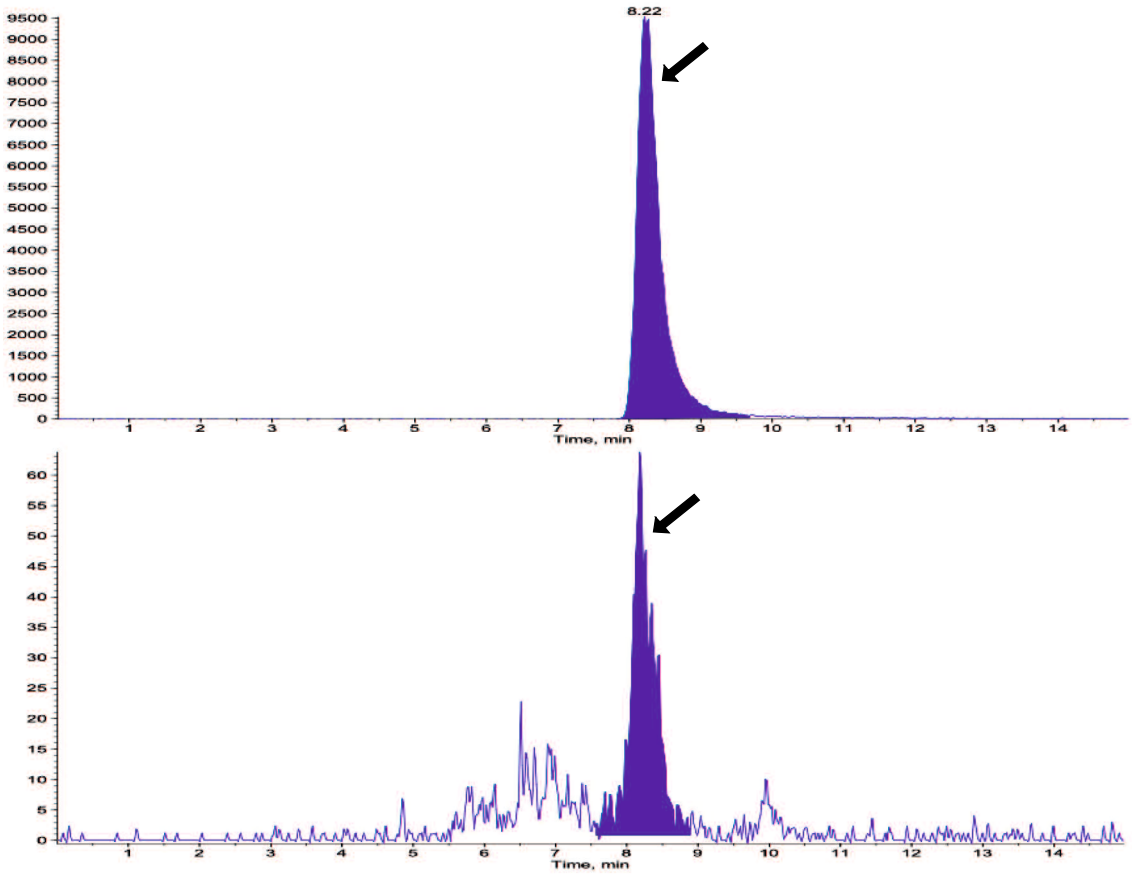

Table 1. Results of the analysis of the number of heifers in which the concentration of STC was above the limit of detection (LoD) during the study period, as well as the concentration of STC (measured as pg/mg creatinine) in the urine (mean ± standard error of the mean, SEM) with or without DFA III supplementation.

	Day 0	**Day 9**	**Day 14**	**Day 23**
DFA III (n=10)				
No. of heifers (%)	3 (30%)	1 (10%)	0 (0%)	5 (50%)
Concentration[#]				
Mean ± SEM	14.3 ± 8.0	10.5	0	16.8 ± 6.3[*]
Median value	0	0	0	10.3
Interquartile range	21.5	0	0	26.4
Control (n=10)				
No. of heifers (%)	2 (20%)	6 (60%)	0 (0%)	7 (70%)
Concentration				
Mean ± SEM	10.9 ± 7.6	87.1 ± 27.3	0	67.5 ± 16.8[*]
Median value	0	95.6	0	74.2
Interquartile range	0	141.0	0	79.1

*, $P = 0.032$; [#], Concentration in urine samples with no peaks using LC/MS/MS analysis was set as 0 (pg STC/mg creatinine) in the present study. STC, sterigmatocystin; DFA III, difructose anhydride III.

OVERALL DISCUSSIONS

In Chapter 1, dietary supplementation with heat-killed *Lactobacillus sakei* HS-1 (HK-LS HS-1) was revealed to improve calf growth performance. The number of medications administered was significantly lower in the supplement group than in the control group. In addition to the supplement group, LAB was significantly increased. The results indicated that HS-1 is potentially beneficial for improving intestinal microbes and reducing the number of medical treatments.

Dietary supplementation with HS-1 was based on clinical observation; thus, it was necessary to pursue a more detailed mechanism. Furthermore, the addition period should be expanded not only to calves but also to the stage of fattening and breeding. Moreover, it should be determined whether it is effective in a stressful environment. In particular, whether the effect is recognized even under stressful environments such as the weaning of calves should be verified.

In Chapter 2, the effect of DFA III on reducing the urinary concentration of STC in Japanese Black cattle and whether it can be used as a mycotoxin mitigating substance was assessed. Based on the present results, DFA III may have a function to improve the tight junction function between intestinal epithelial cells.

The results support the hypothesis that oligosaccharides, such as DFA III, which are now widely used as prebiotics, can be successfully used as mycotoxin-mitigating substances. The benefit of this approach is a combined effect on adsorption, measured by declining urinary STC excretion as an indicator of the internal dose, and the known beneficial effects of the prebiotic DFA III on intestinal barrier integrity and gut health. Further field studies with a larger sample size are warranted to establish a database for the assessment of DFA III as a dietary supplement to reduce the absorption of mycotoxins in cattle and other animals. The systemic bioavailability of STC in

ruminating cattle should be considered to improve our understanding of STC contamination and co-contamination with other mycotoxins and to assess the potential risk to cattle health.

In conclusion, the results of these field trials indicate that the two types of feed supplementations, which are expected to improve and restore the intestinal environment of cattle, have been confirmed to be effective in preventing disease outbreaks and reducing mycotoxin intake. Therefore, the feed supplementations were shown to be a useful approach in improving productivity in cattle production.

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