

Effects of dietary supplementation with Difructose Anhydride III on blood short-chain fatty acids concentrations and metabolic profiles in Japanese Black breeding cattle with chronic exposure to low-level zearalenone

(低濃度 ZEN 汚染下の黒毛和種繁殖牛に対する飼料中 Difructose Anhydride III 添加が血液中短鎖脂肪酸濃度と代謝プロファイルに与える影響)

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Effects of dietary supplementation with Diffructose Anhydride III on blood short-chain fatty acids concentrations and metabolic profiles in Japanese Black breeding cattle with chronic exposure to low-level zearalenone

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LIST OF ABBREVIATION

AG	: Albumin/Globulin Ratio
Alb	: Albumin
AMH	: Anti-Müllerian Hormone
BUN	: Blood Urea Nitrogen
Ca	: Calcium
Cre	: Creatinine
DFA III	: Difructose Anhydride III
ELISA	: Enzyme-Linked Immunosorbent Assay
FFA	: Free Fatty Acids
GC/MS	: Gas Chromatography-Mass Spectrometry
GGT	: Gamma-Glutamyl Transferase
Glu	: Glucose
GOT	: Glutamate-Oxaloacetate Transaminase
IP	: Inorganic Phosphatase
JB	: Japanese Black
LC-MS/MS	: Liquid Chromatography–Tandem Mass Spectrometry
MAs	: Mycotoxin Adsorbent
Mg	: Magnesium
SAA	: Serum Amyloid A
SCFA	: Short-Chain Fatty Acids
SEM	: Standard Error of Mean
STC	: Sterigmatocystin
T-Cho	: Total Cholesterol
TG	: Triglycerides
TP	: Total Protein
Vit. A	: Vitamin A
Vit. E	: Vitamin E
ZEN	: Zearalenone
ZOL	: Zearalenol
3HB	: 3-Hydroxybutyrate

α -ZOL : α -zearelenol
 β -ZOL : β -zearelenol

ABSTRACT

In the first chapter, evaluate the effects of dietary supplementation with difructose anhydride (DFA III) and non-supplementation by comparing serum levels of short-chain fatty acids (SCFAs) in Japanese Black (JB) cattle. Serum sample collected from two herds of female JB cattle ($n = 30$, aged 9–10 months). SCFA concentrations were measured using gas chromatography on days 0 (before DFA III supplementation), 9, and 14 after DFA III supplementation. Isobutyric acid and enanthic acid showed a significantly different trend, which might be related to the distinct fermentation status of supplemental DFA III in the intestines. These results suggest that the trend observed in the intestinal tract following DFA III administration may be associated with changes in the environment of intestinal bacterial flora, which could partially reflect the effects of DFA III supplementation on cattle. Supplementing with DFA III for a minimum of two weeks has an effect on the trend of blood SCFA concentrations in cattle. Our results provide evidence supporting the effects of DFA III on the intestinal environment and intestinal barrier function.

In the second chapter, evaluate the impact of DFA III supplementation on serum biochemical parameters and the intestinal environment under low-level chronic dietary exposure to zearalenone (ZEN) in JB breeding cows. Twenty-five JB cows were selected from a breeding farm with confirmed natural feed contamination using urine ZEN concentration as an indicator of exposure. Blood samples were collected before DFA III supplementation (day 0) and on days 20 and 40 post-supplementation. Serum biochemical parameters and concentrations of SCFA were measured. During the studies, dietary ZEN concentration increased, yet improvements were observed in liver function, nutritional status, immune response, and inflammatory markers. Interestingly, after DFA III was administered, the concentration of butyrate in the serum increased significantly. These findings suggest that DFA III may positively influence intestinal microflora and enhance intestinal barrier function, which could contribute to improved health and nutritional status in cattle exposed to low-level chronic dietary ZEN contamination. DFA III supplementation may be an effective approach to mitigate the adverse effects of low-level mycotoxin exposure in systems used for livestock production.

GENERAL INTRODUCTION

In recent years, there has been a trend towards increased global use of prebiotics as a safe alternative to decrease animal diseases and improve animal health. Prebiotics are defined as substrates that are selectively utilized by host microorganisms, conferring a health benefit (Gibson *et al.*, 2017). These may include non-digestible oligosaccharides, such as mannan oligosaccharides (Franklin *et al.*, 2005), fructo-oligosaccharides (Donovan *et al.*, 2002; Wang *et al.*, 2020), galacto-oligosaccharides, lactose, and inulin-type fructan (Fleige *et al.*, 2007; Hughes *et al.*, 2022). Oligosaccharides interact directly with intestinal epithelial cells, enhancing intestinal barrier function and modulating immunological responses (Akbari *et al.*, 2017a). Prebiotics positively influence the bacterial flora of the gastrointestinal tract by increasing the population of *Bifidobacteria* and *Lactobacillus*, thereby reducing the incidence of several different diseases in animals (Fleige *et al.*, 2007; Heinrichs *et al.*, 2009). Diffructose anhydride III (DFA III) is a non-digestible disaccharide derived from inulin via the microbial fermentation of chicory root (Cheng *et al.*, 2022). Previous studies have shown that DFA III enhances paracellular calcium (Ca) absorption in cattle (Teramura *et al.*, 2015a). We previously reported on the efficacy of dietary supplementation with DFA III as a prebiotic for improving the health and intestinal microbiota in calves, showing that supplementation with DFA III, combined with a replacer, reduced the onset of diseases such as fever and diarrhea (Matsumoto *et al.*, 2009; Takagi *et al.*, 2011).

Short-chain fatty acids (SCFAs), such as acetic, propionic, and butyric acids, are produced through the microbial fermentation of non-digestible carbohydrates, a process which is stimulated by prebiotic intake (Lee *et al.*, 2018). SCFAs contribute to improved gut microbiota composition (Lee *et al.*, 2018), reduce inflammation (Zhang *et al.*, 2023), and inhibit mycotoxin absorption (Crudo *et al.*, 2021). SCFAs are products of non-digestible carbohydrate fermentation by anaerobic gastrointestinal bacteria, but can also be produced endogenously through metabolism in body tissues or ingested through the diet (He *et al.*, 2023). Proper microbiota colonization in the gastrointestinal system and the production of SCFAs may play a vital role in the regulation of the inflammatory response (Akhtar *et al.*, 2022; He *et al.*, 2023), maintaining intestinal barrier integrity (Laviano *et al.*, 2023), and serving as indicators of host health status (Li *et al.*, 2022). A

previous experimental study in rats indicated that cecal SCFA concentrations in the DFA III-supplemented group were higher than those in the non-supplemented group under the same feeding conditions (Kikuchi *et al.*, 2004; Tamura *et al.*, 2006). Although a large volume of SCFA is produced through the microbial fermentation of plant cellulose in the rumen, the dynamics of SCFA levels in intestinal fermentation following supplementation with DFA III in cattle feeds remain unknown.

Mycotoxin contamination in animal feed, which is caused by secondary metabolites from filamentous fungi such as *Fusarium* spp., remains a persistent challenge in livestock production. We previously established a urinary concentration monitoring system for zearalenone (ZEN), which can screen samples to confirm its presence in beef and dairy cattle (Widodo *et al.*, 2023, 2024). Mycotoxins, such as ZEN, can induce acute or chronic disruptions in growth, reproduction, immune regulation, and metabolic function in animals (Fink-Gremmels, 2008; Xu *et al.*, 2022). While some mycotoxins are inactivated by the rumen flora, which functions as an initial barrier, others pass through the rumen, disrupt rumen flora, or are transformed into biologically active metabolites. Due to their antimicrobial activity, these compounds can disrupt the rumen flora and modulate the host immune system even at low doses (Gallo *et al.*, 2020). Common mycotoxin adsorbents used to counteract these effects include mineral clays (Nadziakiewicz *et al.*, 2019), yeast cell wall derivatives (Xu *et al.*, 2023), enzymes, probiotics such as lactic acid bacteria (Baralić *et al.*, 2023), and prebiotics such as oligosaccharides (Adegbeye *et al.*, 2020). We previously reported that our urinary ZEN concentration monitoring system could be used to estimate ZEN contamination levels in feed and to track intestinal ZEN adsorption following DFA III supplementation (Toda *et al.*, 2018). Additionally, we demonstrated that this supplementation preserves the integrity of the intestinal barrier in cattle, and could serve as a method of protecting against chronic low-dose mycotoxin contamination in cattle diets (Toda *et al.*, 2018; Sasazaki *et al.*, 2021). However, no studies have simultaneously assessed the effect of dietary DFA III supplementation under low-level ZEN contamination on inflammatory biomarkers, serum biochemistry, or the concentrations of circulating SCFA parameters in Japanese Black (JB) herds.

In the context of the author's aforementioned research, this study focuses on the practical application of DFA III as a supplement to evaluate the microbial environment,

intestinal barrier integrity, and metabolite profile, including nutritional and inflammatory status, to mitigating the adverse effects of mycotoxin contamination in JB cattle. Chapter 1 describes a study investigating the mechanism underlying the effect of DFA III supplementation on cattle. This study compared the serum SCFA levels in DFA III-supplemented cattle and non-supplemented control cattle and evaluated the fermented status of DFA III in the intestine. Chapter 2 presents the results of the evaluation of the effects of DFA III supplementation on serum biochemical parameters, including anti-Müllerian hormone (AMH), serum amyloid A (SAA), and nutritional indicators, in JB cattle with chronic dietary ZEN contamination. We further investigated changes in the intestinal environment by measuring serum SCFA concentration before and after DFA III supplementation to clarify its effects on intestinal barrier function under chronic mycotoxin contamination.

CHAPTER I

Measurement of Serum Short-Fatty Acid Concentration in Cattle After Oral Administration of Difructose Anhydride III

ABSTRACT

We previously reported the mitigation effects of difructose anhydride III (DFA III) on mycotoxins, such as zearalenon and sterigmatocystin, based on the urinary concentrations of these molecules in calves. This study was aimed at evaluating the effects of dietary supplementation of DFA III and the fermented status of DFA III in the intestine by comparing serum levels of short-chain fatty acid (SCFAs) in DFA III-supplemented cattle with those in non-supplemented control cattle. Serum SCFA concentrations were measured in 30 Japanese Black heifers, aged 9–10 months, from two herds, using gas chromatography on days 0 (before DFA III supplementation), 9, and 14 after DFA III supplementation. A notably different trend was observed for isobutyric acid and enanthic acid, which may reflect the different fermentation status of supplementary DFA III in the intestine. Our results indicate the possibility that this trend observed in the intestinal tract following DFA III administration is associated with changes in the environment of intestinal bacterial flora, which may partially reflect the effects of DFA III supplementation on cattle. DFA III supplementation for at least 2 weeks affects the trend of blood SCFA concentrations in cattle. Our results provide evidence supporting the effects of DFA III on the intestinal environment and intestinal barrier function.

INTRODUCTION

As the use of antibiotics in animal production may contribute to the resistance of human pathogens, alternatives, such as probiotics and prebiotics, have recently been proposed to overcome the challenges associated with the reduction of antibiotic usage (Heinrichs *et al.*, 2003; Jouany and Morgavi, 2007; Timmerman *et al.*, 2005). Therefore, there is growing interest in the health-promoting effects of prebiotics, such as mannanoligosaccharides (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). Prebiotics have been reported to positively influence the bacterial flora of the gastrointestinal tract (by increasing the population of *Bifidobacteria* and *Lactobacillus*), thereby, reducing the incidence of diseases in animals (Fleige *et al.*, 2007; Heinrichs *et al.*, 2009). A recent consensus regarding indigestible saccharides is that they stimulate short-chain fatty acids (SCFAs), including acetic, propionic, and butyric acids, which may improve the intestinal microbiota composition (Lee *et al.*, 2018; Wang *et al.*, 2021).

In addition to the problem of resistant pathogens due to antibiotic abuse, contamination of agricultural commodities with mycotoxins, which are secondary fungal metabolites, is also a major global concern in agriculture and livestock production (Fink-Gremmels, 2008). Consumption of mycotoxin-contaminated products causes acute and chronic effects in humans and animals; thus, contamination of food, feed, and ingredients by mycotoxins poses considerable health risks (Fink-Gremmels, 2008, Liu *et al.*, 2013). Therefore, mitigation strategies against mycotoxins are required at livestock production sites worldwide. To date, mycotoxin adsorbents (MAs) applied in animal feed generally consist of a mixture containing a mineral clay carrier, yeast cell wall preparations, and, in some cases, enzymes or living microorganisms (probiotics). Recently, direct interactions of the oligosaccharides with intestinal epithelial cells have been reported, which indicates that these oligosaccharides improve and protect the integrity of the intestinal barrier and modulate the immune responses of epithelial cells (Akbari *et al.*, 2015; Akbari *et al.*, 2017a, 2017b). Difructose anhydride III (DFA III) is a naturally occurring, non-digestible disaccharide in commercially roasted chicory manufactured from inulin through microbial fermentation (Tamura *et al.*, 2004; Yokota *et al.*, 1991). We previously reported

the efficacy of DFA III supplementation as a prebiotic for the improvement of the health and intestinal microbiota in calves (Matsumoto *et al.*, 2009; Takagi *et al.*, 2011). We showed that it protected the integrity of the intestinal barrier in cattle and can be used as an alternative to protect against chronic low-dose mycotoxin contamination from cattle diets (Toda *et al.*, 2018, Sasazaki *et al.*, 2021). A previous report with experimental animals (rats) indicated that cecal SCFA concentrations in the DFA III-supplemented group were higher than those in the non-supplemented group under the same feeding conditions (Kikuchi *et al.*, 2004; Tamura *et al.*, 2006). Although a large volume of SCFAs is produced through microbial fermentation of plant cellulose in the rumen, the dynamics of SCFAs in intestinal fermentation following the addition of DFA III as a supplement to cattle feeds are unknown. Because DFAIII is not degraded in the rumen and is fermented by intestinal microorganisms, the increase in SCFA concentrations in the intestinal tract due to the addition of DFA III and the accompanying change in SCFA concentrations in the blood due to absorption from the intestinal tract were hypothesized.

As a first step to clarify the mechanism underlying the effect of DFA III supplementation on cattle, this study was conducted to compare serum SCFA levels in DFA III-supplemented cattle and non-supplemented control cattle and to evaluate the fermented status of DFA III in the intestine.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Animal Experiment Ethic Committee of Yamaguchi University, Japan (Approval no. 40, 1995 dated 27-03-2017).

Study period and location

The research was conducted in December 2017 on two Japanese Black cattle farms in Kagoshima Prefecture, Japan.

Chemicals and solvents

Difructose anhydride III was donated by Nippon Beet Sugar Manufacturing Co. Ltd., Obihiro, Japan. We prepared a mixture of short fatty acids-acetic acid, propionic acid, isobutyric acid, butyric acid, 2-methylbutanoic acid, isovaleric acid, valeric acid, caproic acid, 2-methylhexanoic acid, and enanthic acid (Sigma Aldrich, Tokyo, Japan) to measure SCFAs in the serum samples using gas chromatography-mass spectrometry (GC/MS) according to previous reports on the concentrations of SCFAs in serum samples or rumen fluids of cattle (Chibisa *et al* 2020; Li *et al.*, 2022; Wang *et al.*, 2021).

Japanese black (JB) cattle herds and sample collection

The details regarding animals and collected serum samples used in the present study were as described in previous studies by Toda *et al.* (2018) and Sasazaki *et al.* (2021). Briefly, JB female heifers from two beef herds (Herd 1: $n = 10$; Herd 2: $n = 20$; approximately 10 months old and weighing 250–300 kg) raised in Kagoshima Prefecture, Japan, were included in this experiment. Herds 1 and 2 consisted of 370 and 500 JB cattle, respectively. Herds 1 and 2 were fed concentrate and rice straw. The composition of the dietary feed provided to the two herds is summarized in Table-1 (Toda *et al.*, 2018). Two groups of heifers were randomly selected from each experimental herd and assigned to two treatment groups that differed in feed supplementation as follows: Diffructose anhydride III group (Herd 1: $n = 5$; Herd 2: $n = 10$) fed 40 g DFA III/days (20 g, each feeding time) mixed with concentrate, and the control group (Herd 1: $n = 5$; Herd 2: $n = 10$) fed without DFA III supplementation. The dose of DFA III used in the study is the

recommended dose for prevention of hypocalcemia in dairy cows (Sato *et al.*, 2007; Teramura *et al.*, 2015b), which may affect the functions of the tight junction.

Table 1. Composition of feed provided to the two herds kept for fattening purposes.

Herd	Forage feed, kg	Formula Feed						TDN, %	CP, %
		Total, kg	Bran, %	Cereal, %	Oil meal seed, %	Other, %			
Herd 1	Straw 2.0, Timothy grass 2.0	3	24	46	16	14	>70.0	>16	
Herd 2	Straw 2.0, Oats 2.0	2	27	56	7	10	>71.5	>14	

TDN: total digestible nutrients, CP: crude protein. This table is taken from Toda *et al.* (2018).

Blood samples were collected from the jugular vein in silicone-coated tubes after two hours of morning feeding. In previous studies by Toda *et al.* (2018), Sasazaki *et al.* (2021), although we performed blood sampling at the start of DFA III supplementation (day 0), on days 9 and 14 (day 14, i.e., on the last day of DFA III supplementation) after treatment initiation and on the last day of the experimental period on day 23, the blood samples collected on days 0, 9, and 14 were used for the measurement of SCFA concentrations and 14 were used for the measurement of SCFA concentrations in the present study during the DFA III-supplemented periods.

All samples were immediately placed in a cooler containing ice gel for protection from light and were transported to the laboratory. Blood samples were centrifuged at 2000× *g* for 10 min at 20–25°C. Serum samples were frozen at –80°C until SCFA concentrations were analyzed.

Gas chromatography-mass spectrometry analysis of SCFAs in serum samples

The analytical procedures used for measuring the concentrations of SCFAs in the plasma were as described by Furuhashi *et al.* (2018). One milliliter of plasma with fibrin removed was placed into a 2 mL polypropylene (PP) tube with a zirconia ball. Plasma was added to 1 mL of 10% isobutanol and shaken using a shaker (TissueLyser II, Qiagen, Tokyo, Japan). The sample was centrifuged at 21,000× *g* for 5 min, and 675 µL of the supernatant was transferred to another PP tube. 3-methyl pentanoate was used as an internal standard, and 125 µL of 20 mM NaOH and 400 µL of chloroform were added to

the solution. The solution was subsequently mixed using a vortex mixer. Four hundred microliters of the upper layer (aqueous phase) was transferred into another PP tube, followed by centrifugation ($21,000\times g$, 2 min). A solution of isobutanol (80 μL), pyridine (100 μL), and ultrapure water (70 μL) was added. A boiling stone was placed in the tube to avoid bumping. For derivatization using isobutyl esterification, 50 μL of isobutyl chloroformate was added to the solution in the PP tube. Thereafter, 150 μL of hexane was added to the tube and the mixture was shaken using a vortex mixer. After centrifugation at $21,000\times g$ for 2 min, the upper layer was collected and placed in a glass vial. The derived SCFAs were measured using GC/MS (GC-2030 equipped with QP2020 NX and AOC-20i Plus, Shimadzu Corp., Kyoto, Japan). Samples were injected into a DB-1HT column (30 m length, 0.25 mm id, 0.10 μm film thickness, Agilent Technologies, Tokyo, Japan). The injector and detector temperatures were 260°C and 280°C , respectively. The column temperature was increased incrementally as follows: 40°C for 5 min, 180°C at a rate of $10^{\circ}\text{C}/\text{min}$, and then 310°C at $30^{\circ}\text{C}/\text{min}$. The temperature was maintained for 3 min.

Statistical analysis

We examined the differences in time-series trends in blood SCFA concentrations between the control and DFA III-supplemented (DFA III) groups. A linear mixed model analysis was conducted for each blood SCFA as the dependent variable, the number of individual cows nested in the pasture as a random effect, and time (days 0, 9, 14), group (control, DFA III), its interaction (time*group), and day 0 value of the dependent variable as fixed effects. The estimated mean (least squares [LS] mean) and its 95% confidence interval (CI) were calculated for each time point and the amount of change relative to day 0 for each group was determined. At this time, a comparison test between the DFA III and control groups was performed to determine the amount of change. For each group, a test on day 0 was also performed. A two-sided $p < 0.05$ was considered statistically significant. All statistical analyses were performed using Statistical Package for the Social Sciences for Windows version 24.0 (IBM Japan, Tokyo, Japan).

RESULTS

Among the 90 serum samples (Herd 1: 30; Herd 2: 60), four samples from Herd 1 and 15 samples from Herd 2 were excluded from the data analyses because no GC/MS peaks were detected. This may be due to impaired derivatization during sample processing before GC/MS analysis. Finally, data from 26 samples from Herd 1 and 45 samples from Herd 2 were included (analysis included 15 cows in the control group and 14 cows in the DFA III group).

There was no statistically significant difference in the amount of change in acetic, propionic, butyric, 2-methylbutanoic, isovaleric, valeric, caproic, and 2-methylheptanoic acids between the control and DFA III groups. However, the values for all these fatty acids in control and DFA III groups showed a significant decrease on day 14 compared with those on day 0.

Table-2 shows the results for control and DFA III supplementation. There were no significant differences in the total change in blood concentrations of SCFAs between groups on day 9 or day 14 (difference in changes [DFA III-control]; day 9: Least squares mean = 2.535 [95% CI: -15.933, 21.003], $p = 0.784$; day 14: 3.137 [-16.432, 22.707], $p = 0.749$). Pre-and post-tests in each group showed a significant reduction in fatty acid levels on days 9 and 14 compared to those on day 0 in both the groups (changes from day 0; control: day 9: -21.612 [-39.966, -8.258], $p = 0.002$; day 14: -47.615 [-61.835, -33.394], $p < 0.001$; DFA III: day 9: -19.077 [-31.829, -6.325], $p = 0.004$; day 14: -44.477 [-57.968, -30.986], $p < 0.001$).

Table-3 shows a substantial difference between DFA III supplementation and control groups. The changes in isobutyric acid concentrations on day 9 were significantly greater in the DFA III group than in the control group (difference in changes [DFA III-control]; LS mean = 0.415 [95% CI: 0.045, 0.785], $p = 0.029$). Within each group, there was no statistically significant variation between day 0 and day 9 in both groups; however, the values for the control group showed a decreasing tendency (-0.245 [-0.513, 0.023], $p = 0.072$), whereas those for the DFA III group showed an increasing tendency (0.170 [-0.086, 0.425], $p = 0.189$). The decrease in enanthic acid concentration on day 14 was significantly greater in the DFA III group than in the control group (difference in changes [DFA III-control]; LS mean = -0.129 [95%CI: -0.246, -0.013], $p = 0.031$). Within each

group, a statistically significant decrease was observed on day 14 compared with that on day 0 (changes from day 0; control: -0.341 [$-0.426, -0.256$], $p < 0.001$; DFA III: -0.470 [$-0.550, -0.390$], $p < 0.001$).

Table 2. Concentrations (μM) of short chain fatty acids in the serum of control and DFA III group animals.

	Control				DFA III			
	LS mean	95%CI		<i>p</i> -value	LS mean	95%CI		<i>p</i> -value
Acetic acid								
Day 0	50.110	44.986	55.235	-	49.241	44.340	54.143	-
Day 9	42.396	37.004	47.789	0.042	44.897	39.775	50.020	0.225
Day 14	30.944	24.936	36.952	<0.001	32.905	27.234	38.577	<0.001
Propionic acid								
Day 0	16.337	14.158	18.516	-	16.702	14.615	18.789	-
Day 9	13.631	11.345	15.917	0.092	14.846	12.667	17.025	0.223
Day 14	9.953	7.394	12.513	<0.001	9.742	7.333	12.150	<0.001
Isobutyric acid								
Day 0	1.108	0.918	1.299	-	0.974	0.794	1.154	-
Day 9	0.863	0.667	1.059	0.072	1.143	0.954	1.333	0.189
Day 14	0.677	0.453	0.902	0.004	0.687	0.478	0.896	0.038
Butyric acid								
Day 0	30.337	27.897	32.778	-	30.858	28.52	33.197	-
Day 9	22.007	19.448	24.566	<0.001	20.348	17.902	22.794	<0.001
Day 14	13.52	10.653	16.388	<0.001	14.971	12.273	17.669	<0.001
2-Methylbutanoic acid								
Day 0	3.047	2.705	3.39	-	2.907	2.581	3.234	-
Day 9	2.481	2.123	2.84	0.025	2.529	2.188	2.869	0.113
Day 14	1.915	1.516	2.313	<0.001	1.789	1.41	2.167	<0.001
Isovaleric acid								
Day 0	1.032	0.902	1.163	-	0.949	0.826	1.073	-
Day 9	0.792	0.656	0.928	0.013	0.804	0.675	0.934	0.109
Day 14	0.576	0.425	0.727	<0.001	0.615	0.471	0.76	<0.001
Valeric acid								
Day 0	4.077	3.751	4.402	-	1.197	1.116	1.277	-
Day 9	3.345	3.003	3.686	0.004	0.829	0.745	0.913	<0.001
Day 14	2.435	2.054	2.816	<0.001	0.636	0.543	0.729	<0.001
Caproic acid								
Day 0	1.185	1.101	1.268	-	1.197	1.116	1.277	-

Day 9	0.88	0.793	0.968	<0.001	0.829	0.745	0.913	<0.001
Day 14	0.671	0.572	0.77	<0.001	0.636	0.543	0.729	<0.001
2-Methylhexanoic acid								
Day 0	1.557	1.386	1.727	-	1.636	1.472	1.8	-
Day 9	1.018	0.84	1.197	<0.001	0.988	0.817	1.158	<0.001
Day 14	0.748	0.547	0.948	<0.001	0.763	0.574	0.951	<0.001
Enanthic acid								
Day 0	0.753	0.696	0.809	-	0.789	0.735	0.844	-
Day 9	0.59	0.531	0.649	<0.001	0.52	0.463	0.576	<0.001
Day 14	0.412	0.344	0.479	<0.001	0.319	0.257	0.381	<0.001

LS mean: Least square mean, 95%CI: 95% confidence interval, *p*-value: Versus Day 0, DFA III: Diffructose anhydride, *Significant differences between DFA III and control groups.

DISCUSSIONS

Previously, with experimental animals and humans, oligosaccharides, such as DFA III and DFA IV, extracted from inulin were reported to increase the number of health-promoting bacteria and decrease harmful bacteria in the host gastrointestinal tract and increase the concentrations of SCFAs, including acetic acid, which may alter the intestinal microbiota toward a healthier composition (Lee *et al.*, 2018; Wang *et al.*, 2021). In addition, treatment of such oligosaccharides was proposed to restore the impaired epithelial barrier functions in the small intestinal epithelial cells (Ali *et al.*, 2022; Lee *et al.*, 2018). We have also reported the effects of DFA III supplementation on calf/cattle, where we showed that supplementing DFA III with milk replacer reduced the onset of diseases, such as fever and diarrhea, during the pre-weaning period (Matsumoto *et al.*, 2009; Takagi *et al.*, 2011), and the effect of supplementing DFA III on feed-related epithelial barrier functions to reduce the absorption of mycotoxins, such as ZEN and STC, from the intestinal tract by measuring their urinary concentrations (Toda *et al.*, 2018; Sasazaki *et al.* 2021). As a stepping-stone to elucidate the effects of DFA III supplementation on cattle health and intestinal defense function, in this study, we aimed to confirm whether the addition of DFA III (40 g/days) to the diet increased the production of fatty acids in the intestinal tract and caused a difference in the serum concentrations of SCFAs after absorption from the intestinal tract. The results of the present study indicate that although there was no significant difference in the total SCFA concentrations between the DFA III and control groups, DFA III supplementation may affect the composition of SCFAs produced in the intestinal tract. We observed significant reductions in serum SCFA levels on days 9 and 14 compared with those on day 0 in both groups. The heifers used in this study were newly introduced to each herd for fattening immediately before the start of the experiment. Therefore, it is conceivable that the difference in the composition of the feed before and after the introduction led to a decrease in the concentrations of SCFAs at the beginning of DFA III supplementation and during the subsequent test period.

DFA III is an indigestible oligosaccharide enzymatically synthesized from inulin (Kikuchi *et al.*, 2004). *In vitro* experiments conducted on the small intestines of rats (Mineo *et al.*, 2002; Suzuki *et al.*, 1998) and on the duodena of cows (Teramura *et al.*, 2015b) have shown that the absorption of calcium through the paracellular pathway can

be accelerated by agents that act on tight junctions. DFA III promotes paracellular transport by reducing transepithelial electrical resistance and by enhancing the transport of paracellular markers (Suzuki & Hara., 2004, 2006), with alterations in claudin-1, a component of tight junctions and actin filaments in Caco-2 cells (Suzuki & Hara, 2006). Tight junctions are crucial in paracellular nutrient transport and barrier functions in the intestine.

Table 3. Data for change in the concentrations of short-chain fatty acids between control and DFA III group animals

	Difference [DFA III - Control]			
	LS mean	95%CI		<i>p</i> -value
Acetic acid				
Δ Day-9 (from 0)	3.370	−6.898	13.638	0.513
Δ Day-14 (from 0)	2.830	−8.047	13.708	0.604
Propionic acid				
Δ Day-9 (from 0)	0.850	−3.519	5.219	0.698
Δ Day-14 (from 0)	−0.577	−5.205	4.052	0.804
Isobutyric acid				
Δ Day-9 (from 0)	0.415	0.045	0.785	0.029*
Δ Day-14 (from 0)	0.145	−0.248	0.538	0.464
Butyric acid				
Δ Day-9 (from 0)	−2.18	−7.069	2.709	0.375
Δ Day-14 (from 0)	0.929	−4.254	6.112	0.721
2-Methylbutanoic acid				
Δ Day-9 (from 0)	0.188	−0.494	0.869	0.583
Δ Day-14 (from 0)	0.014	−0.709	0.737	0.969
Isovaleric acid				
Δ Day-9 (from 0)	0.095	−0.163	0.353	0.463
Δ Day-14 (from 0)	0.123	−0.151	0.396	0.373
Valeric acid				
Δ Day-9 (from 0)	−0.064	−0.231	0.104	0.451
Δ Day-14 (from 0)	−0.047	−0.225	0.131	0.600
Caproic acid				
Δ Day-9 (from 0)	−0.064	−0.231	0.104	0.451
Δ Day-14 (from 0)	−0.047	−0.225	0.131	0.6
2-Methylhexanoic acid				
Δ Day-9 (from 0)	−0.11	−0.452	0.231	0.52
Δ Day-14 (from 0)	−0.065	−0.427	0.297	0.721
Enanthic acid				
Δ Day-9 (from 0)	−0.107	−0.217	0.003	0.056
Δ Day-14 (from 0)	−0.129	−0.246	−0.013	0.031*

LS mean: Least square mean, 95%CI: 95% confidence interval, *p*-value: DFA III vs. Control, *Significant differences between DFA III and control groups.

Previously, we reported that DFA III, when supplemented in the diet of cattle, could be successfully used as a mycotoxin-mitigating substance, against mycotoxins, such as zearalenone (Toda *et al.*, 2018) and sterigmatocystin (Sasazaki *et al.*, 2021), due to the known beneficial effects of prebiotic DFA III on the integrity of the intestinal barrier and gut health. DFA III has been suggested to exhibit extremely low degradability and fermentability by microflora in the rumen, which strongly suggests that it bypasses the rumen and reaches the intestinal tract (Sato *et al.*, 2006). Recent findings in broilers suggested that crosstalk occurs between nutrients and epithelial barrier function through the dynamic regulation of tight junctions and enhanced intestinal barrier function mediated by DFA IV (Lee *et al.*, 2018). Therefore, in this study, we examined whether dietary DFA III changes the serum concentrations of SCFAs, reflecting the fermentation of SCFAs in the intestinal tract and their absorption from the intestine. The results indicated that although there were no significant differences in change in total SCFAs between groups on day 9 or day 14, the changes in the concentration of isobutyric acid on day 9 were significantly greater in the DFA III group than in the control group ($p = 0.029$). In addition, within each group, there was no statistically significant variation between days 0 and 9 in both groups; however, the control group showed a tendency of decrease in concentration ($p = 0.072$) and the DFA III group showed a tendency of increase in concentration ($p = 0.189$). In addition, the decrease in enanthic acid on day 14 was significantly greater in the DFA III group than in the control group ($p = 0.031$). SCFAs, primarily acetate, butyrate, and propionate, are predominantly produced by fermenting dietary fibers in the gut using anaerobic bacteria and have been shown to possess anti-inflammatory potential by inhibiting the production of inflammatory cytokines. They help reduce LPS-induced pathological damage to the intestine (Lee *et al.*, 2018) or mammary gland (Ali *et al.*, 2022). Based on a recent report, the serum concentration of isobutyric acid in dairy cattle ($n = 18$) was in the 1.59–18.63 $\mu\text{M/L}$ range; the large inter-cow variation in SCFA content suggests that SCFA molecules may be closely associated with the metabolism in individual animals, as all animals were managed in the same herd with the same feeding regime (Li *et al.*, 2022). In this study, lower concentrations of isobutyric acid than those reported previously were observed, possibly due to differences in dietary feed quality and quantity between dairy Holstein cows and JB heifers. As mentioned above, DFA III has been suggested to have extremely

low degradability and fermentability by microflora in the rumen, which strongly suggests that it bypasses the rumen and reaches the intestinal tract (Sato *et al.*, 2006). Li *et al.* (2022) also reported that the isobutyric acid concentration in ruminal fluids after dietary inulin (original source of DFA III) supplementation did not change. Iso-acids, such as isobutyric acid, are formed during the fermentation of amino acids, and higher ruminal concentrations of iso-acids have been reported after dietary supplementation with yeast, which may stimulate the growth of amylolytic bacteria that preferentially use peptides and amino acids due to their proteolytic activity (Garcia Diaz *et al.*, 2018). Alternatively, from the results of monensin supplementation in dairy cattle, a significant decrease in enanthic acid concentrations in rumen fluids was reported, suggesting a greater development of cellulolytic bacteria in the rumen (Mammi *et al.*, 2021). Therefore, it may be speculated that our results reflect the changes in bacterial flora in the intestine after the fermentation of DFA III. Further research is necessary to elucidate the effects of DFA III on cattle.

It is concluded that DFA III supplementation for at least 2 weeks affects the trends of blood SCFA concentrations in cattle. As we previously reported, oligosaccharides, such as DFA III, that are now widely used as prebiotics, can be successfully used as mycotoxin-mitigating substances with beneficial effects on intestinal barrier integrity and gut health (Sasazaki *et al.*, 2021). Our results support the effects of DFA III on the intestinal environment and barrier function. By further accumulating and integrating field- and *in vitro* data, new mycotoxin control methods that can be applied to livestock production sites will be developed.

CHAPTER II

Effect of Dietary Difructose Anhydride III Supplementation on the Metabolic Profile of Japanese Black Breeding Herds with Low-Level Chronic Exposure to Zearalenone in the Dietary Feed

ABSTRACT

Mycotoxin contamination in animal feed can cause acute or chronic adverse effects on growth, productivity, and immune function in livestock. This study aimed to evaluate the impact of difructose anhydride III (DFA III) supplementation on serum biochemical parameters and intestinal environment in Japanese Black (JB) breeding cows under low-level chronic dietary exposure to zearalenone (ZEN). Using urinary ZEN concentration as an indicator of exposure, 25 JB cows were selected from a breeding farm with confirmed natural feed contamination. Blood samples were collected before DFA III supplementation (day 0), and on days 20 and 40 post-supplementation. Serum biochemical parameters and short-chain fatty acid concentrations were measured. During the studies, dietary ZEN concentration increased, yet improvements were observed in liver function, nutritional status, immune response, and inflammatory markers. Notably, serum butyrate concentration significantly increased following DFA III administration. These findings suggest that DFA III may positively influence intestinal microflora and enhance intestinal barrier function, which could contribute to improved health and nutritional status in cattle exposed to low-level chronic dietary ZEN contamination. DFA III supplementation may represent a promising strategy for mitigating the effects of low-level mycotoxin exposure in livestock production systems.

INTRODUCTION

Mycotoxin contamination in animal feed, caused by secondary metabolites from filamentous fungi such as *Fusarium* spp., is a persistent challenge in livestock production. These toxins, including zearalenone (ZEN), can induce acute or chronic disruptions in growth, reproduction, immune regulation, and metabolic function in animals (Fink-Gremmels, 2008; Xu *et al.*, 2022). To address this, there is a growing need for effective detection tools and nutrition strategies that mitigate the biological effects of dietary mycotoxins. We previously established a urinary concentration monitoring system for ZEN, a mycotoxin known for its endocrine disrupting effects in animals. This system combines enzyme-linked immunosorbent assay (ELISA) screening with liquid chromatography–tandem mass spectrometry (LC–MS/MS) verification (Widodo *et al.*, 2023, 2024) and has been applied to breeding cattle herds: Japanese Black (JB) for beef and Holstein for dairy production. While some mycotoxins are inactivated by rumen flora as the first barrier, others pass through the rumen, disrupt rumen flora, or are transformed into biologically active metabolites. Due to their antimicrobial activity, they can disrupt rumen flora and modulate the host immune system even at low doses (Gallo *et al.*, 2020). Common adsorbent agents used to counteract these effects include mineral clays (Nadziakiewicz *et al.*, 2019), yeast cell wall derivatives (Xu *et al.*, 2023), enzymes, probiotics such as lactic acid bacteria (Baralić *et al.*, 2023), and prebiotics such as oligosaccharides (Adegbeye *et al.*, 2020). Prebiotics are specific substrates utilized by host microbes to confer health benefits (Zaura & Twetman, 2019). Oligosaccharides have been shown to interact directly with intestinal epithelial cells, enhancing intestinal barrier function and modulating immunological responses (Akbari *et al.*, 2017b). Non-digestible oligosaccharides, such as mannan oligosaccharides (Franklin *et al.*, 2005), fructooligosaccharides (Donovan *et al.*, 2002), and lactulose, have attracted interest for their potential to reduce disease incidence in animals by promoting beneficial gut flora by increasing the populations of *Bifidobacterium* spp. (Fleige *et al.*, 2007).

Short-chain fatty acids (SCFAs), such as acetic, propionic, and butyric acids, are produced through microbial fermentation of non-digestible carbohydrates and are known to be stimulated by prebiotic intake (Lee *et al.*, 2018). SCFAs contribute to improved gut microbiota composition (Lee *et al.*, 2018), reduce inflammation (Zhang *et al.*, 2023), and

inhibit mycotoxin absorption (Crudo *et al.*, 2021). SCFAs are products of non-digestible carbohydrate fermentation by anaerobic gastrointestinal bacteria. They are also produced endogenously through metabolism in body tissues and ingested through the diet (He *et al.*, 2023). Proper microbiota colonization in the gastrointestinal system and SCFAs may play a vital role in regulating inflammatory response (Akhtar *et al.*, 2022; He *et al.*, 2023), maintaining intestinal barrier integrity (Laviano *et al.*, 2023), and serving as indicators of host health status (Li *et al.*, 2022). In light of increasing concerns about antimicrobial resistance due to the overuse of antibiotics in livestock, alternatives such as probiotics and prebiotics are gaining prominence for their health-promoting properties.

Difructose anhydride III (DFA III), or di-D-fructofuranose-1,2':2,3'-dianhydride, is a non-digestible disaccharide derived from inulin via microbial fermentation of chicory root (Cheng *et al.*, 2022). Previous studies have shown that DFA III enhances paracellular calcium (Ca) absorption in cattle (Teramura *et al.*, 2015). In earlier work, we demonstrated that oral DFA III supplementation in JB calves, used as a prebiotic, improved gut flora composition and overall health status (Matsumoto *et al.*, 2009; Takagi *et al.*, 2011). We have also reported that our urinary ZEN concentration monitoring system was used to estimate ZEN contamination levels in feed and to track intestinal ZEN adsorption following DFA III supplementation (Toda *et al.*, 2018). With the serum samples derived from the same herd (Toda *et al.*, 2018), we recently reported that DFA III administration modulated blood SCFA concentrations in JB heifers, suggesting improvements in the intestinal environment and barrier function (Priyo *et al.*, 2023). Based on these findings, we hypothesized that DFA III supplementation could mitigate the adverse effects of mycotoxin contamination, improve the intestinal barrier integrity, and reduce systemic inflammation in cattle. To our knowledge, no studies have simultaneously assessed the effect of dietary DFA III supplementation on mycotoxin contamination, inflammatory biomarkers, serum biochemistry, and circulating SCFA concentrations in JB herds.

This study aimed to examine the effects of DFA III supplementation on serum biochemical parameters such as anti-Mullerian hormone (AMH), which represents antral follicle reserve in the ovary, and serum amyloid A (SAA), which is an acute-phase protein and nutritional indicator in JB cattle with chronic dietary ZEN contamination. We also investigated changes in the intestinal environment by measuring serum SCFA

concentration before and after DFA III supplementation to clarify its effects on intestinal barrier function under chronic mycotoxin contamination.

MATERIAL AND METHODS

Ethical Approval

The guidelines and rules for the protection of experimental animals at Yamaguchi University in Yamaguchi, Japan (No. 110, 11 November 2008) were followed during the conduct of this study. All farmers who participated in the study provided informed consent.

Chemicals and Solvents

DFA III was donated by Nippon Beet Sugar Manufacturing Co., Ltd. (Obihiro, Japan). ZEN was purchased from MP Biomedicals (Heidelberg, Germany). A stock solution of ZEN (1 μ L in methanol) was stored in a dark area at 4 °C. For the measurement of SCFAs using GC/MS, a mixture of acids was prepared with slight modifications based on a previous study (Priyo *et al.*, 2023). The mixture included formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, 2-methylvaleric acid, isovaleric acid, valeric acid, isocaproic acid, hexanoic acid (caproic acid), 2-methylhexanoic acid, and heptanoic acid (Sigma Aldrich, Tokyo, Japan).

Preliminary ZEN Contamination Screening

In December 2021, after the rice harvest, preliminary screening was conducted at a JB cattle breeding farm to evaluate ZEN contamination in dietary roughage (rice straw and/or whole crop silage). Urinary ZEN levels were monitored in three neighboring herds (1, 2, and 3) located in the Kyushu region of Japan. A veterinarian provided routine care and consultation to the farmers. Under consistent management practices, all animals were housed indoors and fed concentrate and roughage separately. Sampling dates were coordinated across herds. Urine samples were collected from two cows with similar body weights in each herd via natural urination after gentle perineal massage, following a previously described method (Widodo *et al.*, 2023). According to a previous study (Widodo *et al.*, 2023, 2024), two samples per herd were considered sufficient for estimating feed contamination levels. All concentrates administered to the cattle were commercially sourced and examined for mycotoxin contamination during manufacturing. Collected urine and blood samples were immediately cooled, protected from light, centrifuged, and frozen. The frozen samples were then transported to the laboratory and

stored at $-30\text{ }^{\circ}\text{C}$ for ZEN and Cre analysis. Among the three herds, Herd 3 showed relatively higher urinary ZEN concentrations than Herds 1 and 2. Therefore, it was re-sampled in May 2022 for further ZEN and blood biochemistry analysis.

Animals and Management

The study was conducted between November and December 2022 using Herd 3 (female JB cattle). A total of 25 JB cows (mean age $\pm$ SEM, 6.2 ± 0.8 years; approximate body weight range, 400–600 kg) were enrolled. Ambient temperatures during the study period ranged from 7 to $21\text{ }^{\circ}\text{C}$. DFA III was administered at a dose of 40 g/day (20 g per feeding, twice daily), mixed with concentrate. The dosage used was based on a previous study (Sasazaki *et al.*, 2021). All cows were fed a mixture of wrapped sorghum silage and barnyard millet as roughage, harvested from their herd's pastureland, and supplemented with commercial concentrate formulated for JB breeding cows (Kurupita, Minami Hihon Kumiai Siryo Co., Ltd., Kagoshima, Japan). The concentrate contained digestive tract materials (46%), grain (32%), vegetable oil cakes (8%), and other components (14%), and was administered twice daily. Its nutritional composition included total digestible nutrients $> 71.5\%$, crude protein $> 15\%$, crude fat $> 3\%$, crude fiber $> 10\%$, crude ash $> 10\%$, Ca $> 0.8\%$, and phosphorus $> 0.4\%$. The herd had a documented history of poor reproductive performance. Before starting the experiment, ovarian status was assessed, and ZEN levels were measured in urine samples from four cows (two per test group) using ELISA. The mean urinary ZEN concentrations (pg/mg Cre, $\pm$ SEM) were 4575.3 ± 927.2 and 4733.8 ± 42.3 , respectively, confirming chronic low-level ZEN contamination in feed. This herd was thus selected as a model to evaluate the effects of DFA III supplementation on urinary ZEN, AMH, SAA, and blood biochemical parameters. A schematic diagram outlining the study design, including the preliminary screening process, is shown in Figure 1.

Urine and Blood Sample Collection

During the DFA III supplementation period, both urine (from two identical cows) and blood samples ($n = 25$) were collected at three time points: day 0 (prior to DFA III supplementation), day 20 (after 20 days of supplementation), and day 40 (10 days post-supplementation). Blood samples were collected from the jugular vein using a 10 mL

plastic syringe, while urine samples (approximately 10 mL) were collected via perineal massage into 15 mL tubes. All samples were immediately placed in an ice-filled container to maintain low temperature and shielded from light during transport to the laboratory. Blood samples were centrifuged at $1409\times g$ for 10 min. The resulting serum was divided and stored at $-80\text{ }^{\circ}\text{C}$ for SCFA analysis and at $-30\text{ }^{\circ}\text{C}$ for other biochemical tests. Urine samples were centrifuged at $1917\times g$ for 5 min, transferred to 1.5 mL microtubes, and stored at $-30\text{ }^{\circ}\text{C}$ until analysis.

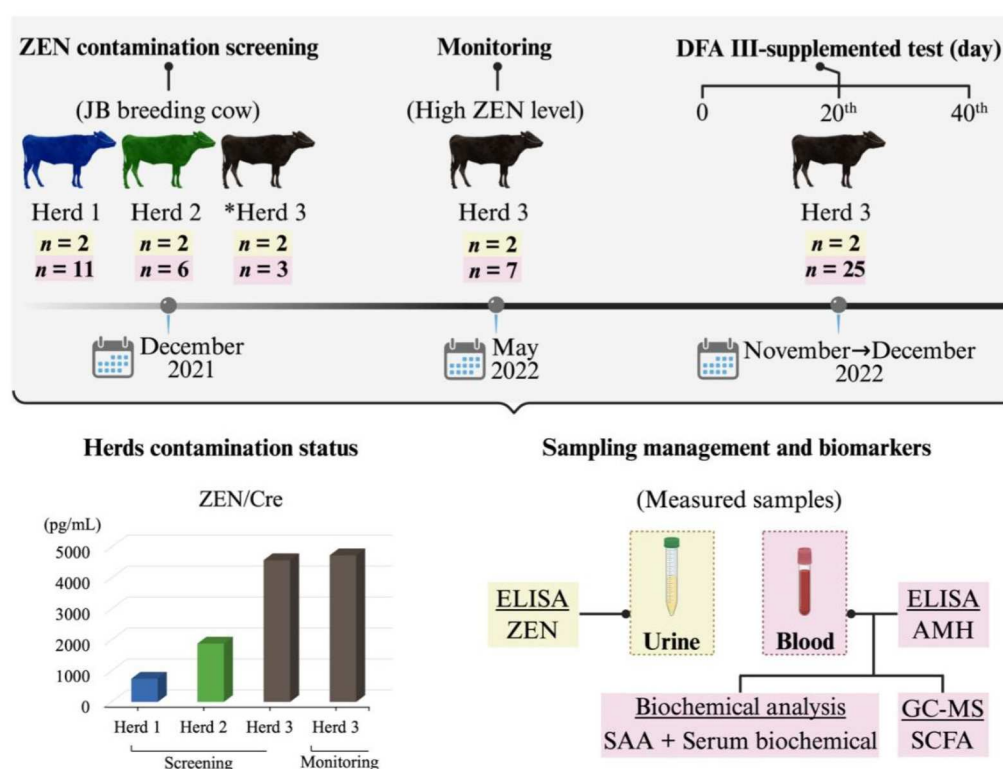


Figure 1. Schematic representation of the experimental design, sampling history, biomarkers, and ZEN contamination status in this study. *: High levels of urinary ZEN contamination were detected; →: Indicates the period between months; ZEN: zearalenone; Cre: Creatinine; JB: Japanese Black; DFA III: difructose anhydride III; AMH: anti-Mullerian Hormone; SAA: serum amyloid A; GC-MS: gas chromatography–mass spectrometry; SCFA: short-chain fatty acid.

Measurement of ZEN, AMH, and SAA

Urinary ZEN was measured using a commercially available kit (RIDASCREENR Zearalenon; R-Biopharm AG, Darmstadt, Germany) with minor modifications to the manufacturer's protocol. Briefly, 10 μL of β -glucuronidase/arylsulfatase solution was added to 3 mL sodium acetate buffer (50 mM, pH 4.8) containing 0.1 mL of five-fold

diluted urine. After 15 h incubation at 38 °C, the mixture was purified using C18 solid-phase extraction (SPE) columns (Strata; Phenomenex, Torrance, CA, USA) preconditioned with 3 mL of methanol followed by 2 mL of 20 mM Tris buffer (pH 8.5)/methanol (80:20). The column was then washed with an additional 2 mL of Tris buffer (pH 8.5)/methanol (80:20) and 3 mL of methanol (40%), followed by centrifugation for 10 min at 39× g to dry the column. Elution was performed slowly (15 drops/min) with 1 mL of methanol (80%). The eluate was evaporated at 60 °C using a centrifugal evaporator and redissolved in 50 µL methanol. Subsequently, 450 µL of sample dilution buffer was added, thoroughly mixed, and an aliquot of 50 µL was used for the ELISA assay. Absorbance was measured at 450 nm using a microplate spectrophotometer, and urinary ZEN concentrations were calculated using RIDA RSOFTWin (R-Biopharm, Art No. Z9999). Urinary Cre concentrations were determined using a commercial kit (Sikarikit-S CRE, Kanto Chemical, Tokyo, Japan) and a Hitachi 7700 clinical autoanalyzer (Hitachi High-Tech, Tokyo, Japan). ZEN concentrations were normalized to urinary Cre (pg/mg Cre) as previously described (Widodo *et al.*, 2023). Serum AMH concentrations were measured using an ELISA kit (Ansh Labs, Webster, TX, USA) with 50 µL of undiluted serum. The assay's limit of detection was 11 pg/mL, and the coefficient of variation was 2.9%. SAA concentrations were measured using an automated biochemical analyzer (Penta C200, HORIBA ABX SAS, Montpellier, France) with a reagent specialized for animal serum or plasma (VET-SAA 'Eiken' reagent; Eiken Chemical Co., Ltd., Tokyo, Japan). Final concentrations were calculated using a standard curve generated with the VET-SAA calibrator set (Eiken Chemical Co., Ltd., Tokyo, Japan).

Biochemical Analysis for Metabolic Profile Evaluation

The following concentrations were measured using the Labospect 7080 autoanalyzer (Hitachi, Tokyo, Japan) to evaluate metabolic profiles and conduct biochemical analyses: glutamic oxaloacetic transaminase (GOT), gamma-glutamyl transferase (GGT), free fatty acids (FFA), total cholesterol (T-Cho), blood urea nitrogen (BUN), glucose (Glu), Ca, inorganic phosphatase (IP), magnesium (Mg), triglycerides (TG), vitamin A (Vit. A), vitamin E (Vit. E), 3-Hydroxybutyrate (3HB), total protein (TP), and albumin (Alb), following previously established protocols (Watanabe *et al.*, 2008).

Blood serum was collected within 40 days of DFA III supplementation to monitor hepatic such as GOT and GGT and renal function such as BUN, nutritional status total such as T-Cho, Glu, TG, FFA, Vit. A, Vit. E, 3HB, TP, and Alb, and mineral intake Ca, IP, and Mg.

Measurement of SCFA with GC/MS

Serum SCFA concentrations were analyzed as previously reported (Priyo *et al.*, 2023). Briefly, 1 mL of plasma was added to a 2 mL polypropylene tube containing a zirconia ball and 1 mL of 10% isobutyl alcohol. The sample was homogenized for 6 min using a TissueLyser II (Qiagen, Tokyo, Japan). After 5 min of centrifuging ($21,000\times g$) the sample, 675 μ L of the supernatant was transferred to a clean polypropylene tube. An internal standard (3-methylpentanoate) was added, followed by 400 μ L of chloroform and 125 μ L of 20 mM NaOH. After vortex mixing and centrifugation, the upper layer was collected and transferred to a glass vial. Derivatization was performed by adding 80 μ L of isobutyl alcohol, 100 μ L of pyridine, 70 μ L of milli-Q water, and a boiling chip to the vial. Additionally, 50 μ L of isobutyl chloroformate was added, and the mixture was vortexed. After derivatization, 150 μ L of hexane was added, and the mixture was again vortexed and centrifuged at $21,000\times g$. The upper organic layer was collected for analysis, and 1 μ L injected into GC/MS.

The derivatized SCFAs were measured using a GC-2030 gas chromatograph equipped with a QP2020 NX mass spectrometer and AOC-20i Plus autosampler (Shimadzu Corp., Kyoto, Japan). Separation was performed on a DB-5MS column (30 m length, 0.25 mm id, 0.25 μ m film thickness; Agilent Technologies, Tokyo, Japan). The injector and detector temperatures were set at 260 °C and 280 °C, respectively. The column oven was programmed to heat from 40 °C (5 min) to 180 °C at a rate of 10 °C/min, then to 310 °C at a rate of 30 °C/min, followed by a final hold. Then, SCFA concentrations were analyzed by both selective ion monitor (SIM) and scan modes. Extracted ion chromatograms for individual SCFAs in SIM of GC/MS measurements are shown in Supplemental Table S5.

Statistical Analysis

A time-series analysis (days 0–40) was conducted for biochemical and SCFA data. As the results did not follow a log-normal distribution, natural log-transformation (ln)

values were used. A linear mixed-effects model was employed, with each parameter set as a dependent variable. Measurement time points (0, 20, and 40 days) were treated as fixed effects, and individual cattle as random effects. Log-transformed estimates (mean and 95% CI) were back-transformed to obtain values on the original scale, representing the rate of change. Statistical comparisons were conducted using the mixed-effects model to determine whether significant changes occurred on days 20 and 40 relative to day 0. The Bonferroni correction was used to adjust for multiple comparisons. The two-sided $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS version 29.0 (IBM, Japan)

RESULTS

Study of Metabolic Profiles in Preliminary ZEN Contamination Screening

The urinary ZEN/creatinine (Cre) concentrations (pg/mL Cre, mean $\pm$ SEM) for Herds 1, 2, and 3 were 749.3 ± 19.9 , 1885.2 ± 578.6 , and 4575.3 ± 927.2 , respectively. All metabolic profile parameters were within the normal reference ranges, except for AMH and SAA, for which no established reference ranges exist. Significant differences were observed in FFA ($p = 0.000$), BUN ($p = 0.027$), Glu ($p = 0.002$), TG ($p = 0.039$), 3HB ($p = 0.000$), and TP ($p = 0.034$), indicating variability in nutritional status. The A/G ratio also differed significantly ($p = 0.013$), suggesting disparities in nutritional and immune status, particularly malnutrition in Herd 3. A significant difference in serum Mg concentrations ($p = 0.022$) was also noted, possibly due to differences in mineral supplementation or roughage composition among herds. No significant differences were observed in AMH, SAA, GOT, GGT, T-Cho, Ca, IP, Vit. A, and Alb. Based on these results, a recommendation was made to slightly increase protein and energy intake in the diet of Herd 3. Preliminary screening results exploring the relationship between ZEN contamination and serum biochemical parameters in three JB herds are shown in Supplemental Table S1.

A follow-up screening conducted in May 2022 (approximately 6 months later) for Herd 3 (Supplemental Table S2) showed no change in urinary ZEN concentration, confirming persistent ZEN contamination. However, significant differences in the indicators of nutritional status, such as FFA, BUN, Glu, 3HB, and TP concentrations, were confirmed. This indicated an improvement in the nutritional status of the herd. Re-monitoring revealed no change in urinary ZEN concentration, and chronic ZEN exposure at the same levels was confirmed during the two sampling periods. Therefore, we conducted a DFA III supplementation trial for Herd 3.

Effects of DFA III Supplementation

This study assessed the effects of DFA III supplementation on serum biochemistry and SCFA concentrations under conditions of chronic low-concentration ZEN contamination. Twenty-five cows from Herd 3 were included in the time-series analysis. Figure 2a shows the changes in urinary ZEN concentrations in the same two cows, while

Figure 2b shows mean ($\pm$ SEM) AMH and SAA concentrations during the period of DFA III supplementation. A statistically significant time-series change was observed in the AMH concentrations, with a significant decrease on days 20 and 40 relative to those of day 0. The change rates were 0.75 (95% CI: 0.71, 0.80; $p < 0.001$) on day 20 and 0.80 (95% CI: 0.75, 0.84; $p < 0.001$) on day 40 relative to day 0. No significant changes were observed in SAA concentrations during the supplementation period. Mean serum biochemical values are presented in Figure 3.

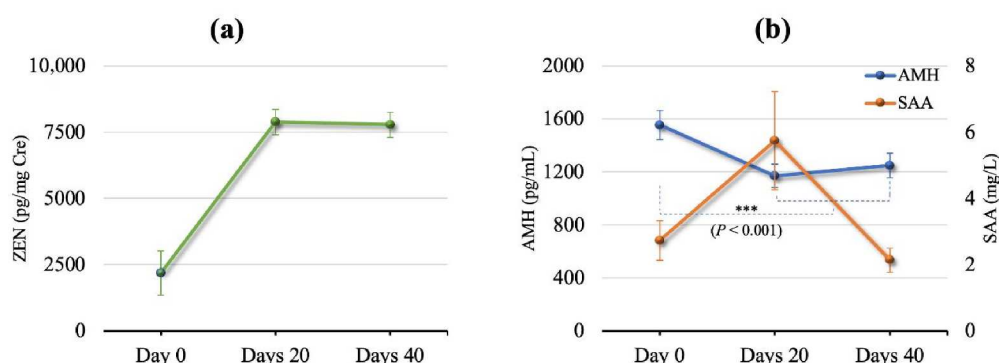


Figure 2. (a) ZEN/Cre concentration (mean $\pm$ SEM) in urine sample. (b) AMH and SAA concentrations in blood samples obtained on days 0, 20, and 40 after DFA III supplementation. *** indicates a significant difference ($p < 0.001$).

Parameters for Liver Function

GOT levels significantly increased on day 20 relative to day 0 ($p < 0.001$) and decreased by day 40 ($p < 0.001$), with no significant difference between the concentrations on days 0 and 40. The GGT concentrations for days 0 and 20 and days 20 and 40 were not significantly different, but a significant increase from day 0 to 40 was observed ($p = 0.042$).

Parameters for Nutritional Status

Several parameters, such as FFA, T-Chol, Glu, 3HB, and TP concentrations, demonstrated peak- or trough-shaped transitions from day 0 to day 40. FFA levels decreased on day 20 relative to day 0 ($p < 0.001$), increased by day 40 ($p < 0.001$), but remained statistically unchanged between days 0 and 40. The T-Chol concentration significantly decreased on day 20 relative to that of day 0 ($p < 0.001$), increased from day 20 to day 40 ($p < 0.001$), and significantly increased from day 0 to day 40. Glu and TP

showed similar trends. In contrast, the 3HB concentrations significantly increased on day 20 relative to day 0 ($p < 0.001$) and remained elevated on day 40 ($p = 0.034$). BUN concentrations did not differ significantly between days 0 and 20 but increased significantly from day 20 to day 40 ($p < 0.001$) and from day 0 to day 40 ($p < 0.001$).

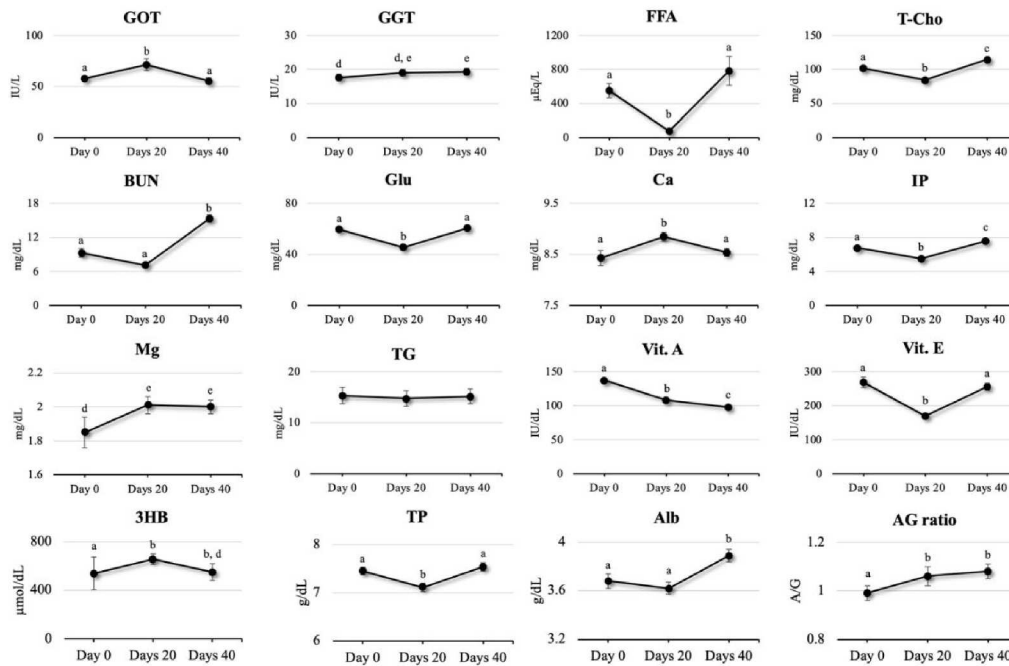


Figure 3. Nutritional condition (mean $\pm$ SEM) on days 0, 20, and 40 after difructose anhydride III supplementation. Hepatic function was assessed using glutamic oxaloacetic transaminase (GOT) and γ -glutamyl transferase (GGT). The nutritional condition was evaluated by determining the concentrations of free fatty acid (FFA), total cholesterol (T-Chol), blood urea nitrogen (BUN), glucose (Glu), triglyceride (TG), albumin (Alb), 3-hydroxybutyrate (3HB), and total protein (TP). Vitamin intake was determined using vitamin A (Vit. A) and vitamin E (Vit. E) concentrations. The mineral status was evaluated by determining the concentrations of calcium (Ca), inorganic phosphate (IP), and magnesium (Mg). The albumin-to-globulin (Alb: Glo) ratio was also determined. a–b; a–c, b–c indicates $p < 0.01$ and a–d; d–e indicates $p < 0.05$.

Parameters for Mineral Status

The Ca concentrations significantly increased on day 20 relative to the values on day 0 ($p = 0.004$), and no statistically significant changes were observed until day 40. IP levels significantly decreased from day 0 to day 20 ($p < 0.001$), increased from day 20 to day 40 ($p < 0.001$), and were overall higher from day 0 to day 40 ($p = 0.042$). The Mg concentrations significantly increased from day 0 to day 20 ($p = 0.029$) and remained elevated until day 40 ($p = 0.037$).

Parameters for Immune and Inflammation Status

Alb concentrations significantly decreased on day 20 compared to day 0 ($p < 0.001$) but significantly increased again by day 40 ($p < 0.001$), returning to baseline levels. The A/G ratio increased on day 20 relative to that of day 0 ($p = 0.007$), remained stable through day 40, and was significantly higher overall from day 0 to day 40 ($p < 0.001$).

Parameters for Vitamin Status

Vit. A level significantly decreased on days 20 and 40 compared with that on day 0 ($p < 0.001$), with further decline between days 20 and 40 ($p = 0.007$). Overall, Vit. A level declined significantly from day 0 to day 40 ($p < 0.001$). Vitamin E (Vit. E) concentration decreased on day 20 relative to that of day 0 ($p < 0.001$) but significantly increased between days 20 and 40 to a value close to that of day 0 ($p < 0.001$). Time-series trends of all biochemical parameters (linear mixed-model analysis) are presented in Supplemental Table S3.

Parameters for SCFA Status

Figure 4 presents SCFA trends over time, and Supplemental Figure S1 presents representative chromatograms on gas chromatography–mass spectrometry (GC/MS) of the SCFA standards analyzed in the present study. No time-series changes were observed in acetic acid and valeric acid concentrations after DFA III supplementation. Propionic acid, isobutyric acid, and isocaproic acid levels decreased significantly on days 20 and 40 relative to those of day 0 ($p < 0.001$). Butyric acid concentrations dropped significantly on day 20 relative to those of day 0 ($p = 0.011$), then returned to near baseline by day 40 ($p < 0.001$). 2-methylvaleric acid significantly decreased from day 20 to day 40 relative to those of day 0 ($p < 0.001$). Hexanoic (Caproic) acid concentrations significantly decreased only on day 40 relative to those of day 20 ($p = 0.018$). 2-Methylhexanoic acid and heptanoic acid significantly decreased only on day 40 relative to those of day 0 ($p < 0.001$). Heptanoic acid concentrations decreased significantly from day 0 to 20 ($p < 0.001$) and from day 20 to 40 ($p = 0.005$). Full SCFA analysis is provided in Supplemental Table S4.

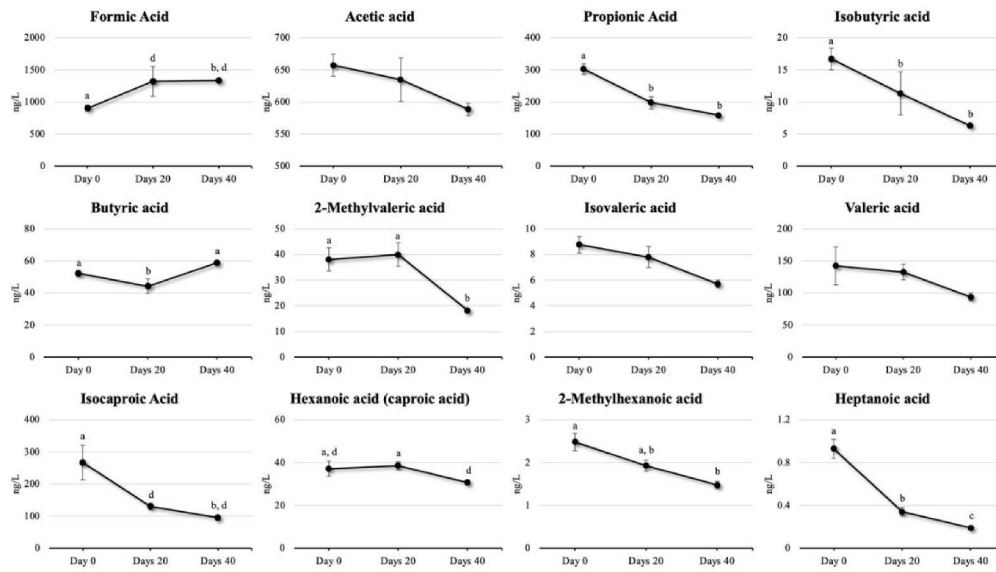


Figure 4. Concentrations of short-chain fatty acids (SCFAs) (mean $\pm$ SEM) on days 0, 20, and 40 after difructose anhydride III supplementation. a–b; a–c, b–c indicates $p < 0.01$ and a–d indicates $p < 0.05$.

DISCUSSIONS

DFA III supplementation in cattle has traditionally been implemented post-parturition to prevent milk fever and improve productivity by promoting Ca and Mg absorption from the intestine. It has been shown to be safe and effective, and it is also added to the colostrum for newborn calves to support intestinal IgG absorption. Based on previous findings from dairy cattle, the present study aimed to verify the effects of DFA III supplementation in JB cattle. DFA III has been shown to reduce diseases in JB calves when added to milk replacements, and it can also inhibit the absorption of mycotoxins such as ZEN and sterigmatocystin (STC) when administered to JB heifers' feed (Toda *et al.*, 2018; Sasazaki *et al.*, 2021). Based on this, this pilot study was conducted to extend investigations into the efficacy of DFA III supplementation in breeding cattle and was a field trial to verify the effect of DFA III supplementation in JB breeding cows, focusing on metabolic profile testing via blood biochemical analysis. A preliminary evaluation included blood biochemical screening and urinary ZEN monitoring in three JB breeding herds under a similar feeding regimen (Widodo *et al.*, 2023), identifying a chronically contaminated herd for further study.

The present study revealed significant differences in the concentrations of minerals, such as Ca, Mg, and IP, in JB cattle. Concentrations of all minerals increased from days 0 to 20 or from days 0 to 40, suggesting an increased absorption rate of minerals during DFA III supplementation. Several effects of oral DFA III administration, including increased intestinal Ca absorption (Mineo *et al.*, 2006), Mg absorption (Escartín *et al.*, 2024), and increased hemoglobin and iron concentrations (Nakamori *et al.*, 2010), have been reported in humans, cows, and rats. Supplementation with DFA III has been linked to improved health in JB calves based on serum biochemical parameters (Matsumoto *et al.*, 2009; Takagi *et al.*, 2011) and reduced the urinary concentrations of ZEN and STC in JB heifers (Sasazaki *et al.*, 2021; Toda *et al.*, 2018). Mechanistically, DFA III may facilitate paracellular transport by decreasing transepithelial electrical resistance and improving paracellular marker movement, with changes in Caco-2 cells' claudin-1, an element of tight junctions, and actin filaments (Suzuki & Hara, 2006; Teramura *et al.*, 2015b). This suggests that DFA III contributes to intestinal barrier integrity (Inokuchi *et al.*, 2009), as corroborated by the mineral profile improvements observed here. Even

though there was no control group in this study, the observed alterations happened without any change in feed composition, which supports the conclusion that the results show DFA III effects.

Metabolic evaluation revealed significant differences in all serum biochemical parameters, except for TG concentrations. Liver function markers, such as GOT and GGT, were significantly different, primarily because GOT concentration decreased. Additionally, there were significant differences in nutritional conditions, such as FFA, T-Cho, Glu, Vit. A, Vit. E, TP, and Alb, with most parameters increasing within 40 days following DFA III supplementation.

Previous studies confirm that 1–2 months of DFA III administration improves health and nutritional conditions of calves (Htun *et al.*, 2018; Matsumoto *et al.*, 2009; Takagi *et al.*, 2011). This indicates that DFA III supplementation as a prebiotic helps restore liver function and improve the nutritional status in JB cows contaminated with ZEN (during the supplementation test period, when urine ZEN concentrations increased). In this study, the urinary ZEN concentration was measured three times during the 40 days of supplementation. Despite unchanged feed, the urinary ZEN concentration tripled on average in the second and third samples compared to the sample collected when the supplementation started. This indicated an increase in the degree of ZEN infiltration in the diet. The results of each biochemical test during that period seemed to reflect the urinary ZEN concentrations. GGT, an indicator of liver function, significantly increased. Contrastingly, the FFA, T-Cho, BUN, Glu, and TP concentrations, which are indicators of nutritional status, significantly decreased, while 3HB concentrations significantly increased. However, the above-mentioned effects were alleviated from 20 to 40 days after the start of DFA III supplementation. A significant decrease in GGT and improvement in various nutritional status indicators were also confirmed, along with a significant decrease in 3HB concentration, which is an indicator of malnutrition. Furthermore, a significant increase in the A/G ratio, an indicator of immune and inflammatory status, was observed within 40 days of treatment. Compared with the results of a preliminary study involving three herds with different urinary ZEN concentrations, the results of this study did not reveal an effect of DFA III supplementation on the inhibition of ZEN absorption from the intestine, as indicated by the urinary ZEN concentrations. However, the results of several serum biochemistry tests suggest improved nutritional and inflammatory statuses of the

herds. Previous findings from DFA III administered for 2 weeks (ZEN concentrations in roughage and concentrate were 0.27 and 0.22 mg/kg, respectively) in 9- to 10-month-old JB cattle demonstrated reduced urinary ZEN and zearalenol (ZOL) concentrations (Toda *et al.*, 2018).

LC–MS/MS on the day prior to DFA III administration, on days 9 and 14 during supplementation, and again 9 days after its discontinuation, revealed lower ZEN metabolites in the DFA III group and improved IP concentration on the 23rd day (8.4 versus 7.7 mg/dL). From these results, we report that DFA III reduces the concentration of mycotoxins that reach the systemic circulation and are excreted in urine and suggest that this preventive effect may be related to the improvement of tight junction-dependent intestinal barrier function (Toda *et al.*, 2018). The following findings were emphasized by a comprehensive reexamination of the previously described studies (Toda *et al.*, 2018). The effect of DFA III on urinary ZEN concentration was observed approximately 10 days after the start of DFA III dietary supplementation and persisted up to 9 days after the 2-week administration period was discontinued. No significant difference was observed between the urinary ZEN concentration and total ZEN concentration; however, a significant difference was confirmed in the concentrations of the urinary ZEN metabolites α -zearelenol (α -ZOL) and β -zearelenol (β -ZOL). These results suggest that some degree of intestinal barrier function was maintained even after DFA III administration was discontinued, although the effect declined over the following 9 days.

AMH concentrations declined significantly between days 20 and 40, consistent with prior findings on the impact of ZEN on ovarian granulosa cells of pre-antral follicles and its influence on antral follicle counts (Widodo *et al.*, 2023). Mycotoxin contamination, particularly ZEN and its metabolites, is known to disturb the antral follicle, interrupt the endogenous estrogenic response during the pre-ovulatory stage, decrease ovarian follicle maturation, and impair the steroidogenic function of ovarian granulosa cells (Silva *et al.*, 2021). In our previous study, we found that natural ZEN contamination may negatively correlate with AMH concentration in JB breeding cattle (Widodo *et al.*, 2023, 2024). In the current study, urinary ZEN concentrations increased by day 20, coinciding with a reduction in AMH levels from day 0 to 20, followed by stabilization through day 40. These results support our previous findings regarding the relationship between urinary ZEN and AMH concentrations (Widodo *et al.*, 2023). However, previous data indicate

that AMH concentrations require approximately 2 months to recover in response to ZEN contamination (Widodo *et al.*, 2023), and the present results suggest that 30-day DFA III supplementation may not be sufficient to restore AMH concentrations affected by ZEN contamination. On the other hand, based on the results of the present study alone, it remains unclear whether the intestinal barrier protection conferred by DFA III helped mitigate the effects of ZEN exposure. It was speculated that the intestinal barrier function induced by DFA III supplementation may have been exerted after the inflammatory state of the intestinal epithelial cells was improved. Thus, it is possible that a delayed effect may occur following the initiation of DFA III supplementation. Further research is needed to elucidate the mechanism underlying the effect of DFA III supplementation. No significant differences in SAA concentrations were observed during the 1 month of the DFA III supplementation period. The gastrointestinal microbiota plays a key role in immune system development and function, modulating both local and systemic inflammatory responses through interactions with epithelial cells, intestinal permeability, dendritic cells, and T and B immune cells (Hughes *et al.*, 2022). Mycotoxin co-contamination, particularly ZEN, has been shown to induce an inflammatory response in the intestines (Jia *et al.*, 2020; Ma *et al.*, 2022) and liver (Dolenšek *et al.*, 2021) as well as disrupt immunity (Mohamed *et al.*, 2024; Zhou *et al.*, 2021). Inulin supplementation has been reported to mitigate both intestinal and systemic inflammation by increasing the production of SCFAs, especially butyrate (Hughes *et al.*, 2022). Our previous findings also suggested that supplementation with DFA III may affect intestinal SCFA concentrations and improve intestinal barrier function in cattle (Priyo *et al.*, 2023). Notably, SAA served as an effective marker of inflammation in highly ZEN-contaminated herds (urinary ZEN/Cre > 20,000 pg/mL Cre) in prior studies (Widodo *et al.*, 2023, 2024). In contrast, the current study involved cattle exposed to relatively lower ZEN concentrations than in a previous report (Widodo *et al.*, 2023), suggesting that there may be no change in the SAA concentration after DFA III supplementation. Therefore, SAA may not be a sensitive indicator for monitoring inflammation, especially in the setting of chronic infiltration with low concentrations of ZEN. Interestingly, the concentration of Alb, another marker associated with inflammation, increased following DFA III supplementation. Therefore, the results of this study showed that DFA III supplementation may improve inflammation in vivo in a chronic, low-concentration, ZEN-contaminated

environment. In the present study, serum SCFA concentrations increased on day 20 and declined by day 40 following DFA III supplementation. This pattern most likely results from DFA III's fermentation in the gastrointestinal tract and its subsequent absorption into the bloodstream.

In cows, ruminal microorganisms have difficulty digesting DFA III. Studies of microflora in the rumen have indicated its low fermentability and degradability, which strongly implies that it passes through the rumen and enters the intestinal tract (Teramura *et al.*, 2015), especially the duodenum, 1 h after oral ingestion (Nakai *et al.*, 2007). SCFAs, including acetate, propionate, and butyrate, are metabolites produced during bacterial fermentation of dietary fiber in the intestinal tract (Liu *et al.*, 2021) and are known to regulate and maintain the integrity of epithelial intestinal barrier function and prevent inflammation and systemic immune responses via evolutionarily conserved pathways, including G protein-coupled receptor signaling and histone deacetylase inhibition (Mann *et al.*, 2024). The anti-inflammatory role of butyrate is mediated through its direct effects on the differentiation of intestinal epithelial cells, phagocytes, B cells, and plasma cells, as well as regulatory and effector T cells. Intestinally derived SCFAs also directly and indirectly affect immunity at extraintestinal sites, such as the liver, lungs, reproductive tract, and brain, and have been implicated in various disorders, including infections and intestinal inflammation (Mann *et al.*, 2024). In our previous report, DFA III supplementation altered serum SCFA profiles, significantly increasing isobutyric acid and decreasing valeric acid concentrations before and 2 weeks after supplementation (Priyo *et al.*, 2023). In the present study, butyric acid concentrations decreased on day 20 of supplementation but significantly increased by day 40. Similar findings were reported by (Mineo *et al.*, 2006), who observed no significant differences in lactic acid, acetic acid, propionic acid, and butyric acid concentrations between the control and DFA III groups. Likewise, isobutyric acid levels in ruminal fluids remained constant upon supplementation with inulin, which is the source of DFA III (Li *et al.*, 2022). Isobutyric acid, derived from the fermentation of amino acids, can stimulate the growth of amylolytic bacteria that prefer to use peptides and amino acids because of their proteolytic activity (Garcia Diaz *et al.*, 2018). Additionally, the rumen increases the proliferation of cellulolytic bacteria, which is another reason for the decline in acetic acid concentrations (Mammi *et al.*, 2021). Alternatively, DFA III supplementation did not increase specific

SCFAs or other organic acids, suggesting a relatively low energy for growth in rats (Tamura *et al.*, 2006). Additionally, DFA III supplementation did not increase the total SCFA level but increased the anaerobic bacteria population inside of cecum in rats (Mineo *et al.*, 2006). Therefore, the results obtained in this study can be considered comparable to previous reports on DFA III supplementation tests (Escartín *et al.*, 2024), and the difference in the increase or decrease in SCFA concentration observed in some cases may be due to the difference in the feed given.

Future research should investigate shifts in gut microbiota composition and population to better understand the fermentation behavior and metagenomics of DFA III in the intestinal tract. There are certain limitations to this study. Due to the herd's feeding management style, a control group without DFA III supplementation could not be included, potentially limiting the ability to definitively attribute observed effects of DFA III supplementation. However, the significant increase in mineral concentrations was considered an indirect indicator of the effects of DFA III supplementation, supported by findings from two preliminary metabolite profile tests in three JB breeding herds. In this study, statistically significant differences were observed in several parameters, including biochemistry results and SCFA concentrations, after DFA III supplementation within 40 days. Nonetheless, further studies incorporating a control group and correlation analysis of ZEN concentrations, biochemistry results, and SCFA profiles are warranted to enhance the robustness and interpretability of these findings.

In conclusion, a 40-day DFA III supplementation in cattle herds chronically exposed to low-level ZEN contamination may improve liver function, enhance nutritional status and immune response, and influence blood SCFA concentration trends. DFA III, as a prebiotic, has beneficial effects on the integrity of the intestinal barrier and overall intestinal health. These findings suggest that DFA III supplementation could help mitigate the adverse effects of dietary ZEN contamination. Further accumulation and integration of field data are warranted to establish this strategy as a viable mycotoxin control approach in livestock farming.

SUPPLEMENTARY TABLE

Table Supplementary 1. Results of urinary ZEN concentration and serum biochemical analysis from 3 JB breeding herds as a preliminary monitoring.

Parameters	Herd 1 (n=11)	Herd 2 (n=6)	Herd 3 (n=3)	P- value	Reference value*
ZEN (pg/mg/Cre)	749.3 ± 19.9 (n=2)	1885.2 ± 578.6 (n=2)	4575.3 ± 927.2 (n=2)		
AMH (pg/mL)	952.2 ± 185.1	806.2 ± 107.7	627.0 ± 110.8	0.584	15–2000
SAA (mg/L)	2.5 ± 0.7	1.6 ± 0.4	1.2 ± 0.3	0.471	<6.5
GOT (IU/L)	66.1 ± 2.4	57.2 ± 3.5	57.8 ± 2.7	0.078	35–80
GGT (IU/L)	15.6 ± 1.0	12.8 ± 1.2	15.5 ± 0.5	0.181	11–25
FFA (μEq/L)	97.9 ± 6.9 ^a	190.1 ± 25.1 ^b	73.7 ± 4.8 ^a	0.000	155–350
T-Cho (mg/dL)	97.2 ± 4.6	120.8 ± 9.8	105.6 ± 15.1	0.090	100–180
BUN (mg/dL)	8.8 ± 0.5 ^a	11.7 ± 1.1 ^b	7.9 ± 1.9 ^{a,b}	0.027	6–27
Glu (mg/dL)	63.7 ± 1.4 ^a	58.1 ± 4.4 ^a	42.7 ± 5.8 ^b	0.002	45–75
Ca (mg/dL)	9.5 ± 9.5	9.2 ± 0.1	9.2 ± 0.1	0.056	9.7–12.4
IP (mg/dL)	6.7 ± 0.3	6.6 ± 0.1	6.2 ± 0.2	0.650	5.6–6.5
Mg (mg/dL)	2.1 ± 0.0 ^{a,b}	2.1 ± 0.1 ^a	2.5 ± 0.1 ^b	0.022	1.8–2.3
TG (mg/dL)	23.5 ± 2.0 ^a	19.1 ± 2.4 ^{a,b}	12.9 ± 1.1 ^b	0.039	0–14
Vit. A (IU/dL)	134.1 ± 4.9	138.8 ± 7.1	137.7 ± 9.3	0.838	>80
Vit. E (IU/dL)	190.1 ± 20.8 ^a	396.3 ± 37.9 ^b	318.7 ± 40.7 ^b	0.000	>105
3HB (μmol/L)	419.0 ± 22.2 ^a	508.5 ± 59.4 ^a	1115.9 ± 268.6 ^b	0.000	0–1000
TP (g/dL)	6.8 ± 0.1 ^a	6.7 ± 0.1 ^{a,b}	6.3 ± 0.1 ^b	0.034	5.7–8.1
Alb (g/dL)	3.5 ± 0.1	3.3 ± 0.1	3.6 ± 0.0	0.171	2.1–3.6
AG ratio	1.1 ± 0.4 ^a	1.0 ± 0.1 ^a	1.3 ± 0.4 ^b	0.013	0.7–1.2

ZEN: zearalenone; AMH: anti-Müllerian hormone; SAA: serum amyloid A; GOT: glutamate-oxaloacetate transaminase; GGT: gamma-glutamyl transpeptidase; FFA: free fatty acid; T-Cho: total cholesterol; BUN: blood urea nitrogen; Glu: glucose; Ca: calcium; IP: inorganic phosphorus; Mg: magnesium; TG: triglycerides; Vit. A: vitamin A; Vit. E: vitamin E; 3HB: 3-hydroxybutyrate; TP: total protein; Alb: albumin; AG: albumin/globulin ratio. *Reference ranges of biochemical analysis reported by Watanabe *et al.* (2013).

Table Supplementary 2. Results of urinary ZEN concentration and serum biochemical analysis for herd 3 as a preliminary monitoring.

Parameters	Herd 3-1st (n=3)*	Herd 3-2nd (n=7)**	P-value	Reference value***
ZEN (pg/mg/Cre)	4575.3 ± 927.2 (n=2)	4733.8 ± 42.3 (n=2)		
AMH (pg/mL)	627.0 ± 110.8	1603.0 ± 279.8	0.061	15–2000
SAA (mg/L)	1.2 ± 0.3	7.5 ± 4.9	0.435	<6.5
GOT (IU/L)	57.8 ± 2.7	62.7 ± 4.8	0.542	35–80
GGT (IU/L)	15.5 ± 0.5	23.5 ± 3.1	0.141	11–25
FFA (μEq/L)	73.7 ± 4.8	436.1 ± 146.0	0.003	155–350
T-Chol (mg/dL)	105.6 ± 15.1	104.4 ± 9.4	0.945	100–180
BUN (mg/dL)	7.9 ± 1.9	14.5 ± 0.6	0.003	6–27
Glu (mg/dL)	42.7 ± 5.8	67.0 ± 4.0	0.010	45–75
Ca (mg/dL)	9.2 ± 0.1	8.4 ± 0.3	0.113	9.7–12.4
IP (mg/dL)	6.2 ± 0.2	8.2 ± 0.5	0.032	5.6–6.5
Mg (mg/dL)	2.5 ± 0.1	2.1 ± 0.1	0.063	1.8–2.3
TG (mg/dL)	12.9 ± 1.1	13.3 ± 1.8	0.880	0–14
Vit. A (IU/dL)	137.7 ± 9.3	128.3 ± 5.5	0.391	>80
Vit. E (IU/dL)	318.7 ± 40.7	355.0 ± 40.4	0.610	>105
3HB (μmol/L)	1115.9 ± 268.6	286.8 ± 27.6	0.001	0–1000
TP (g/dL)	6.3 ± 0.1	7.5 ± 0.2	0.003	5.7–8.1
Alb (g/dL)	3.6 ± 0.0	3.8 ± 0.1	0.395	2.1–3.6
AG ratio	1.3 ± 0.4	1.1 ± 0.1	0.081	0.7–1.2

* Conducted in December 2021. ** conducted at May 2022, all analyzed by T-test. ZEN: zearalenone; AMH: anti-Müllerian hormone; SAA: serum amyloid A; GOT: glutamate oxaloacetate transaminase; GGT: gamma-glutamyl transpeptidase; FFA: free fatty acid; T-Chol: total cholesterol; BUN: blood urea nitrogen; Glu: glucose; Ca: calcium; IP: inorganic phosphorus; Mg: magnesium; TG: triglycerides; Vit. A: vitamin A; Vit. E: vitamin E; 3HB: 3-hydroxybutyrate; TP: total protein; Alb: albumin; AG: albumin/globulin ratio. ***Reference ranges of biochemical analysis reported by Watanabe *et al.* (2013).

Table Supplementary 3. The time-series trends of all the biochemical test results (linear mixed-model analysis).

Parameter	Day	LS mean	95% CI		P-value
AMH	Δ Day 20 (from Day 0)	0.75	0.71	0.80	<0.001
	Δ Day 40 (from Day 0)	0.80	0.75	0.84	<0.001
	Δ Day 40 (from Day 20)	1.06	1.00	1.13	0.178
SAA	Δ Day 20 (from Day 0)	1.31	0.67	2.53	>0.999
	Δ Day 40 (from Day 0)	0.95	0.49	1.85	>0.999
	Δ Day 40 (from Day 20)	0.73	0.38	1.41	>0.999
GOT	Δ Day 20 (from Day 0)	1.21	1.11	1.32	<0.001
	Δ Day 40 (from Day 0)	0.95	0.87	1.04	0.753
	Δ Day 40 (from Day 20)	0.79	0.72	0.86	<0.001
GGT	Δ Day 20 (from Day 0)	1.04	0.97	1.12	0.672
	Δ Day 40 (from Day 0)	1.09	1.02	1.17	0.042
	Δ Day 40 (from Day 20)	1.05	0.98	1.12	0.584
FFA	Δ Day 20 (from Day 0)	0.16	0.11	0.23	<0.001
	Δ Day 40 (from Day 0)	1.18	0.81	1.71	>0.999
	Δ Day 40 (from Day 20)	7.57	5.19	11.03	<0.001
T-Cho	Δ Day 20 (from Day 0)	0.83	0.78	0.89	<0.001
	Δ Day 40 (from Day 0)	1.13	1.06	1.21	<0.001
	Δ Day 40 (from Day 20)	1.36	1.28	1.45	<0.001
BUN	Δ Day 20 (from Day 0)	0.80	0.67	0.97	0.077
	Δ Day 40 (from Day 0)	1.81	1.49	2.18	<0.001
	Δ Day 40 (from Day 20)	2.25	1.86	2.71	<0.001
Glu	Δ Day 20 (from Day 0)	0.75	0.68	0.83	<0.001
	Δ Day 40 (from Day 0)	1.01	0.92	1.11	>0.999
	Δ Day 40 (from Day 20)	1.34	1.22	1.47	<0.001
Ca	Δ Day 20 (from Day 0)	1.05	1.02	1.09	0.004
	Δ Day 40 (from Day 0)	1.02	0.99	1.05	0.854
	Δ Day 40 (from Day 20)	0.96	0.94	1.00	0.079
IP	Δ Day 20 (from Day 0)	0.81	0.75	0.88	<0.001
	Δ Day 40 (from Day 0)	1.11	1.02	1.21	0.042
	Δ Day 40 (from Day 20)	1.37	1.26	1.49	<0.001
Mg	Δ Day 20 (from Day 0)	1.10	1.03	1.19	0.029
	Δ Day 40 (from Day 0)	1.10	1.02	1.18	0.037
	Δ Day 40 (from Day 20)	1.00	0.93	1.07	>0.999
TG	Δ Day 20 (from Day 0)	0.91	0.68	1.22	>0.999
	Δ Day 40 (from Day 0)	0.99	0.74	1.33	>0.999
	Δ Day 40 (from Day 20)	1.09	0.82	1.46	>0.999
Vit. A	Δ Day 20 (from Day 0)	0.79	0.73	0.84	<0.001
	Δ Day 40 (from Day 0)	0.71	0.66	0.75	<0.001
	Δ Day 40 (from Day 20)	0.90	0.84	0.96	0.007
Vit. E	Δ Day 20 (from Day 0)	0.63	0.57	0.70	<0.001
	Δ Day 40 (from Day 0)	0.96	0.87	1.07	>0.999
	Δ Day 40 (from Day 20)	1.52	1.37	1.69	<0.001
3HB	Δ Day 20 (from Day 0)	1.84	1.39	2.43	<0.001

TP	Δ Day 40 (from Day 0)	1.44	1.09	1.90	0.034
	Δ Day 40 (from Day 20)	0.78	0.59	1.03	0.249
	Δ Day 20 (from Day 0)	0.95	0.93	0.97	<0.001
Alb	Δ Day 40 (from Day 0)	1.01	0.99	1.03	0.661
	Δ Day 40 (from Day 20)	1.06	1.04	1.08	<0.001
	Δ Day 20 (from Day 0)	0.99	0.96	1.01	0.826
A/G	Δ Day 40 (from Day 0)	1.06	1.03	1.09	<0.001
	Δ Day 40 (from Day 20)	1.06	1.03	1.09	<0.001
	Δ Day 20 (from Day 0)	1.07	1.03	1.12	0.007
	Δ Day 40 (from Day 0)	1.09	1.04	1.14	<0.001
	Δ Day 40 (from Day 20)	1.02	0.98	1.06	>0.999

AMH: anti-Müllerian hormone; SAA: serum amyloid A; GOT: glutamate-oxaloacetate transaminase; GGT: gamma-glutamyl transpeptidase; FFA: free fatty acid; T-Cho: total cholesterol; BUN: blood urea nitrogen; Glu: glucose; Ca: calcium; IP: inorganic phosphorus; Mg: magnesium; TG: triglycerides; Vit. A: vitamin A; Vit. E: vitamin E; 3HB: 3-hydroxybutyrate; TP: total protein; Alb: albumin; AG: albumin/globulin ratio.

Table Supplementary 4. The time-series trends of all SCFA test results (linear mixed model analysis).

Parameter	Day	LS mean	95% CI		P-value
Formic acid	Δ Day 20 (from Day 0)	1.30	1.05	1.62	0.059
	Δ Day 40 (from Day 0)	1.56	1.25	1.93	<0.001
	Δ Day 40 (from Day 20)	1.20	0.97	1.48	0.285
Acetic acid	Δ Day 20 (from Day 0)	0.91	0.77	1.08	0.804
	Δ Day 40 (from Day 0)	0.90	0.76	1.06	0.610
	Δ Day 40 (from Day 20)	0.99	0.83	1.17	>0.999
Propionic acid	Δ Day 20 (from Day 0)	0.61	0.49	0.77	<0.001
	Δ Day 40 (from Day 0)	0.55	0.44	0.69	<0.001
	Δ Day 40 (from Day 20)	0.90	0.71	1.13	>0.999
Isobutyric acid	Δ Day 20 (from Day 0)	0.54	0.39	0.75	0.001
	Δ Day 40 (from Day 0)	0.40	0.29	0.56	<0.001
	Δ Day 40 (from Day 20)	0.74	0.53	1.02	0.188
Butyric acid	Δ Day 20 (from Day 0)	0.76	0.64	0.91	0.011
	Δ Day 40 (from Day 0)	1.13	0.94	1.35	0.532
	Δ Day 40 (from Day 20)	1.48	1.24	1.77	<0.001
2-Methylvaleric acid	Δ Day 20 (from Day 0)	1.02	0.78	1.33	>0.999
	Δ Day 40 (from Day 0)	0.53	0.40	0.69	<0.001
	Δ Day 40 (from Day 20)	0.51	0.39	0.67	<0.001
Isovaleric acid	Δ Day 20 (from Day 0)	0.86	0.63	1.17	0.977
	Δ Day 40 (from Day 0)	0.71	0.53	0.97	0.095
	Δ Day 40 (from Day 20)	0.83	0.61	1.13	0.683
Valeric acid	Δ Day 20 (from Day 0)	1.01	0.76	1.34	>0.999
	Δ Day 40 (from Day 0)	0.76	0.57	1.00	0.151
	Δ Day 40 (from Day 20)	0.75	0.57	0.99	0.139

Isocaproic acid	Δ Day 20 (from Day 0)	0.67	0.48	0.94	0.064
	Δ Day 40 (from Day 0)	0.51	0.36	0.72	<0.001
	Δ Day 40 (from Day 20)	0.77	0.55	1.08	0.371
Hexanoic acid (caproic acid)	Δ Day 20 (from Day 0)	1.09	0.93	1.27	0.821
	Δ Day 40 (from Day 0)	0.87	0.75	1.02	0.260
	Δ Day 40 (from Day 20)	0.80	0.69	0.94	0.018
2-Methylhexanoic acid	Δ Day 20 (from Day 0)	0.80	0.65	0.99	0.120
	Δ Day 40 (from Day 0)	0.62	0.51	0.77	<0.001
	Δ Day 40 (from Day 20)	0.78	0.63	0.95	0.051
Heptanoic acid	Δ Day 20 (from Day 0)	0.36	0.22	0.60	<0.001
	Δ Day 40 (from Day 0)	0.16	0.10	0.26	<0.001
	Δ Day 40 (from Day 20)	0.44	0.26	0.72	0.005

Table Supplementary 5. Extracted ion chromatogram for individual short fatty acids in GC/MS measurements.

Fatty acids	Extracted ion chromatogram (m/z)*
Acetic acid	56
Propionic acid	57
Isobutyric acid	71
Butyric acid	71
Valeric acid	85
Isocaproic acid	99
Hexanoic acid	99
2-Methylhexanoic acid	113
4-Methylhexanoic acid	113
Heptanoic acid	113
3-Methylpentanoic acid	99

*Extracted ion chromatograms were referred from Priyo *et al.* 2023.

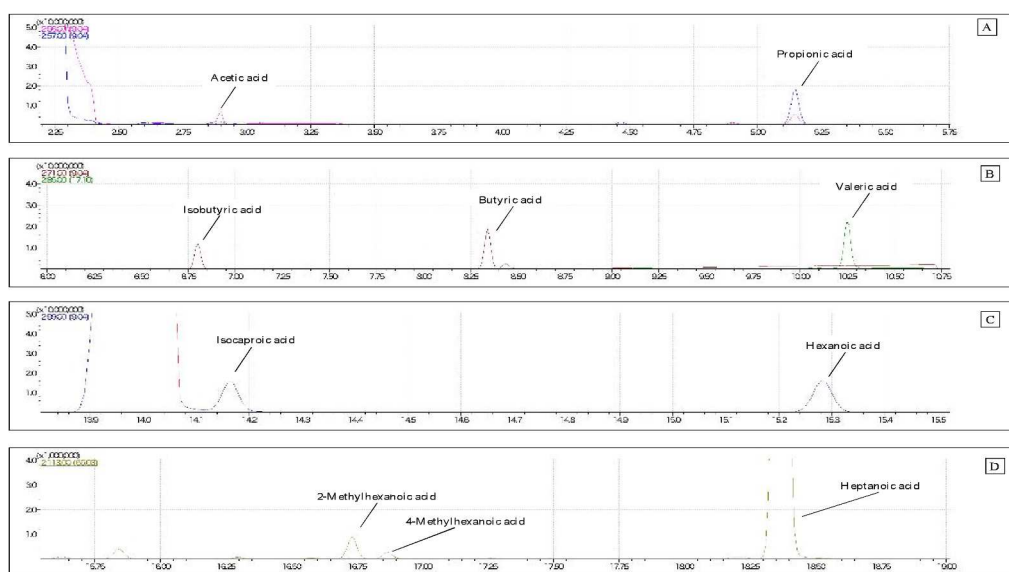


Figure Supplementary 1. Chromatograms of individual fatty acids in standard solution. A: $m/z=56$ and 57 , B: $m/z=71$ and 85 , C: $m/z=99$, and D: $m/z=113$. The horizontal axis shows retention time.

OVERALL DISCUSSIONS AND CONCLUSION

In Chapter 1, we confirmed that dietary supplementation of DFA III altered the trends in blood SCFA concentrations in JB cattle. We further observed significant reductions in serum SCFA levels following supplementation in both groups. The changes in the concentration of isobutyric acid and enanthic acid concentrations were greater in the DFA III group than in the control group. In Chapter 2, we further demonstrated that the 40-day DFA III supplementation in cattle herds resulted in significant differences in significantly different mineral levels, such as Ca, Mg, and IP, and nutritional status; liver function parameters, including GOT and GGT; immune response; and changes in SCFA concentration under chronically contaminated low-level ZEN.

Overall, these results support the hypothesis that prebiotics, specifically oligosaccharides, such as DFA III, can be successfully and usefully applied as feed supplements to improve the intestinal environment and barrier function, as measured by SCFA parameters. Following supplementation, the production of fatty acids in the intestinal tract increased, causing a difference in the serum concentrations of SCFAs after absorption from the intestinal tract. Therefore, it could be speculated that our results reflect the changes in bacterial flora in the intestine after the fermentation of DFA III. SCFA is a metabolite produced during bacterial fermentation in the intestinal tract, which functions to maintain intestinal barrier function and prevent inflammation. The tight junctions in epithelial cells play a crucial role in maintaining the intestinal barrier function. Additionally, DFA III supplementation helps to restore liver function and improve nutritional and inflammatory status indicators contaminated with ZEN, despite an increase in urine ZEN concentrations during the supplementation test period.

In conclusion, the results of this study indicate that DFA III supplementation functions effectively as a prebiotic, altering trends in blood SCFA concentration, enhancing the intestinal barrier, improving immune function, and mitigating the effects of dietary ZEN contamination in JB cattle. Therefore, DFA III supplementation was demonstrated to be a beneficial substrate approach for enhancing health in cattle production.

Future field studies should aim to measure and investigate the metagenomics of bacteria recovered directly from the intestinal environment to assess the effects of DFA III supplementation on factors related to increasing the health and productivity of cattle.

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