Measurement of serum short-chain fatty acid concentrations in cattle after oral administration of difructose anhydride III

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Abstract

Background and Aim: 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.

Materials and Methods: 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.

Results: 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.

Conclusion: Difructose anhydride 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.

Keywords: cattle, difructose anhydride III, short-chain fatty acid.

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 [1–3]. Therefore, there is growing interest in the health-promoting effects of prebiotics, such as mannanoligosaccharides [1, 4], fructooligosaccharides [5], lactulose [6], and fermentation products [7]. 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 [6, 7]. 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 [8, 9].

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 [10]. 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 [10, 11]. 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 [12–14]. Difructose anhydride III (DFA III) is a naturally occurring, non-digestible disaccharide in commercially roasted chicory manufactured from inulin through microbial fermentation [15, 16]. We previously reported the efficacy of DFA III supplementation as a prebiotic for the improvement of the health and intestinal microbiota in calves [17, 18]. 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 [19, 20]. 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 [21, 22]. 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.

Chemical 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-methylhexanelic 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 [9, 23, 24].

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*. [19], Sasazaki *et al*. [20]. 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 [19]. Two groups of heifers were randomly selected from each experimental herd and assigned to two treatment groups that differed in feed supplementation as follows: Difructose 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 [25, 26], which may affect the functions of the tight junction.

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*. [19], Sasazaki *et al*. [20], 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 in the present study during the DFA III-supplemented periods.

All samples were immediately placed in a cooler containing dry ice 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*. [27]. 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

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

Herd	Forage feed, kg	Formula feed						
		Total, kg	Bran, %	Cereal, %	Oil seed meal, %	Other, %	TDN, %	CP, %
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 adopted from Toda *et al.* [19].

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× *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× *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°C/min, and then 310°C at 30°C/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-methybutanoic, isovaleric, valeric, caproic, and 2-methylhezanelic acids between the control and DAF 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

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

Parameters	Control			DFA III		
	LS mean	95%CI	p-value	LS mean	95%CI	p-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	28.520 33.197	-
Day 9	22.007	19.448 24.566	<0.001	20.348	17.902 22.794	<0.001
Day 14	13.520	10.653 16.388	<0.001	14.971	12.273 17.669	<0.001
2-Methylbutanoic acid						
Day 0	3.047	2.705 3.390	-	2.907	2.581 3.234	-
Day 9	2.481	2.123 2.840	0.025	2.529	2.188 2.869	0.113
Day 14	1.915	1.516 2.313	<0.001	1.789	1.410 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 0.760	<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	0.880	0.793 0.968	<0.001	0.829	0.745 0.913	<0.001
Day 14	0.671	0.572 0.770	<0.001	0.636	0.543 0.729	<0.001
2-Metylhexanelic acid						
Day 0	1.557	1.386 1.727	-	1.636	1.472 1.800	-
Day 9	1.018	0.840 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
Enhatic acid						
Day 0	0.753	0.696 0.809	-	0.789	0.735 0.844	-
Day 9	0.590	0.531 0.649	<0.001	0.520	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=Difructose anhydride III

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).

Discussion

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 [8, 9]. In addition, treatment of such oligosaccharides was proposed to restore the impaired epithelial barrier functions in the small intestinal epithelial cells [8, 28]. 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 [17, 18], 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 [19, 20]. 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 (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

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

Parameters	Difference (DFA III-control)			
	LS mean	95%CI		p-value
Acetic acid				
⊿Day 9 (from 0)	3.370	−6.898	13.638	0.513
⊿Day 14 (from 0)	2.830	−8.047	13.708	0.604
Propionic acid				
⊿Day 9 (from 0)	0.850	−3.519	5.219	0.698
⊿Day 14 (from 0)	−0.577	−5.205	4.052	0.804
Isobutyric acid				
⊿Day 9 (from 0)	0.415	0.045	0.785	0.029*
⊿Day 14 (from 0)	0.145	−0.248	0.538	0.464
Butyric acid				
⊿Day 9 (from 0)	−2.180	−7.069	2.709	0.375
⊿Day 14 (from 0)	0.929	−4.254	6.112	0.721
2-Methylbutanoic acid				
⊿Day 9 (from 0)	0.188	−0.494	0.869	0.583
⊿Day 14 (from 0)	0.014	−0.709	0.737	0.969
Isovaleric acid				
⊿Day 9 (from 0)	0.095	−0.163	0.353	0.463
⊿Day 14 (from 0)	0.123	−0.151	0.396	0.373
Valeric acid				
⊿Day 9 (from 0)	−0.064	−0.231	0.104	0.451
⊿Day 14 (from 0)	−0.047	−0.225	0.131	0.600
Caproic acid				
⊿Day 9 (from 0)	−0.064	−0.231	0.104	0.451
⊿Day 14 (from 0)	−0.047	−0.225	0.131	0.600
2-Methylhexanelic acid				
⊿Day 9 (from 0)	−0.110	−0.452	0.231	0.520
⊿Day 14 (from 0)	−0.065	−0.427	0.297	0.721
Enhatic acid				
⊿Day 9 (from 0)	−0.107	−0.217	0.003	0.056
⊿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

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.

Difructose anhydride III is an indigestible oligosaccharide enzymatically synthesized from inulin [21]. *In vitro* experiments conducted on the small intestines of rats [29, 30] and on the duodena of cows [26] have shown that the absorption of calcium through the paracellular pathway can be accelerated by agents that act on tight junctions. Difructose anhydride III promotes paracellular transport by reducing transepithelial electrical resistance and by enhancing the transport of paracellular markers [31, 32], with alterations in claudin-1, a component of tight junctions and actin filaments in Caco-2 cells [32]. Tight junctions are crucial in paracellular nutrient transport and barrier functions in the intestine. 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 [19] and sterigmatocystin [20], due to the known beneficial effects of prebiotic DFA III on the integrity of the intestinal barrier and gut health. Difructose anhydride 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 [33]. 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 [8]. 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 [8] or mammary gland [28]. Based on a recent report, the serum concentration of isobutyric acid in dairy cattle (n = 18) was in the 1.59–18.63 µM/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 [24]. 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 [33]. Li *et al*. [24] 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 [34]. Alternatively, from the results of monensin supplementation in dairy cattle, a significant decrease in enenthic acid concentrations in rumen fluids was reported, suggesting a greater development of cellulolytic bacteria in the rumen [35]. 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.

Conclusion

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 [20]. 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.

Authors' Contributions

TWP, SU, OY, and MiT: Conceived and designed the study. SU, EK, OY, MO, DP, and MiT: Collected the samples and performed formal analysis. TWP, SU, EK, KT, HH, DM, OY, OSW, MO, MT, and MIT: Conducted the literature review and prepared tables. TWP, MT, and MiT: Drafted the manuscript, reviewed, and edited. All authors have read, reviewed, and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests and no person from Nippon Beet Sugar Manufacturing Co., Ltd. was involved at any stage of the study and publication.

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