Metabolite Profiling of Japanese Black Cattle Naturally Contaminated with Mycotoxin, and Clinical Evaluation of Fructo-Oligosaccharide (DFA III) Feed Supplementation for Mitigation of Zearalenone Exposure in Cattle by a Urinary Zearalenone Monitoring System

（カビ毒汚染牛群における代謝プロファイル評価および尿中濃度を指標とした Fructo-Oligosaccharide (DFA III)添加によるゼアラレノン浸潤低減効果）

The United Graduate School of Veterinary Science

Yamaguchi University

Katsuki TODA

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By

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We hereby recommend that the thesis prepared under supervision by Katsuki Toda, entitled “Metabolite Profiling of Japanese Black Cattle Naturally Contaminated with Mycotoxin, and Clinical Evaluation of Fructo-Oligosaccharide (DFA III) Feed Supplementation for Mitigation of Zearalenone Exposure in Cattle by a Urinary Zearalenone Monitoring System” should be accepted as fulfilling in part for the degree of Doctor of Philosophy.

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ABSTRACT

The objective of Chapter 1 of the present study was to evaluate the metabolic profile of cattle fed with or without zearalenone (ZEN) and sterigmatocystin (STC)-contaminated diets. To this end, we used a gas chromatography–mass spectrometry metabolomics approach. Urinary samples were collected from two individual fattening female Japanese Black (JB) cattle (n = 6 per herd; age: 23 months, weight: 550–600 kg). Herd 1 had persistently high urinary ZEN and STC concentrations because they were fed contaminated rice straw. Herd 2 received the same dietary feed as Herd 1, but with non-contaminated rice straw. In Herd 1, urine samples were collected 2 weeks after the contaminated rice straw had been replaced with uncontaminated rice straw (Herd 1N). The metabolites identified in the samples were subjected to principal component analysis (PCA) and ANOVA. The PCA revealed that the effects on cattle metabolites depended on the ZEN and STC concentrations. Therefore, the contamination of cattle feed with multiple mycotoxins may alter systemic metabolic processes, including metabolites associated with ATP generation, amino acids, glycine-conjugates, organic acids, and purine bases. The results obtained from Herd 1N indicated that the 2-week remedy period was not sufficient to improve the levels of urinary metabolites, suggesting that chronic contamination with mycotoxins may cause long-term harm to the systemic metabolism of cattle.

In the second study, the potential effect of difructose anhydride III (DFA III) supplementation in cattle feed was evaluated using a previously developed urinary-ZEN monitoring system. JB cattle from two beef herds aged 9–10 months were used. DFA III was supplemented for 2 weeks. ZEN concentrations in feed were similar between the

herds (0.27 and 0.22 mg/kg in roughage and concentrates, respectively and were below the maximum allowance in Japan. ZEN, α-zearalenol (α-ZOL), and β-ZOL concentrations in urine were measured using liquid chromatography-tandem mass spectrometry the day before DFA III supplementation began, 9 and 14 days later, and 9 days after supplementation ceased. Significant differences in ZEN, α-ZOL, β-ZOL, and total ZEN were recorded between the different sampling dates. The concentration of inorganic phosphate in the urine of DFA III-supplemented animals was significantly higher than in the controls on day 23 (8.4 vs. 7.7 mg/dL), suggesting that DFA III affects the tight junction of intestinal epithelial cells. This is the first evidence that DFA III reduces mycotoxin levels in the systemic circulation and urine, and that this effect may involve improved tight-junction-dependent intestinal barrier function. Additionally, our practical approach confirmed that urinary mycotoxin levels can be monitored to ascertain whether dietary supplements prevent mycotoxin adsorption.

GENERAL INTRODUCTION

Mycotoxins are secondary metabolites produced by specific filamentous fungi, molds. Contamination of agricultural commodities with mycotoxins is a major problem for livestock production worldwide (Fink-Gremmels, 2008). In humans and animals, consuming mycotoxin-contaminated food, feed, and ingredients causes significant health risks (Fink-Gremmels, 2008; Liu *et al*., 2013). The specific effects of mycotoxins include reduced feed intake, feed refusal, poor feed conversion, diminished body weight gain, increased disease incidence, reduced reproductive efficacy, and occasionally even cause for culling or death, all of which leads to economic losses (Binder *et al.*, 2007; Fink-Gremmels and Malekinejad, 2007). More than 300 mycotoxins have been described, but the most damaging to agriculture are aflatoxins, deoxynivalenol, and zearalenone (ZEN). In cattle production, some reports have described the acute and chronic *in vivo* effects of mycotoxins. Most of these have focused on clinical symptoms in field studies, while others have involved the experimental administration of mycotoxins to cattle and subsequent biochemical analysis. However, few investigations have involved risk evaluations focusing on the metabolic status of cattle with mycotoxin-contaminated feed, which may differ from that of healthy cattle, because all animals must control their *in vivo* environment to maintain homeostasis. Consequently, if an animal were to consume mycotoxin-contaminated feed, its metabolic profiles may vary due to the changes in its physical condition. However, several studies have reported that metabolomics can quantitatively describe the presence of low-molecular-weight endogenous metabolites in biological samples, such as the urine, plasma, tissue, and organs, or in the entire body of an organism (Wishart, 2005; Madsen *et al.*, 2010). Additionally, it is understood that

metabolomics may also provide information about the effects of disease, toxicants, and other stressors (Kind *et al.*, 2007; Huang *et al.*, 2008; Chorell *et al.*, 2009). With regard to this, we have detected the presence of zearalenone (ZEN) and sterigmatocystin (STC) in a contaminated cattle herd (Hasunuma *et al.*, 2012; Fushimi *et al.*, 2014) and stored their urine samples. Therefore, investigating the metabolomics of mycotoxin contamination in cattle feed under field conditions, particularly considering ZEN and STC contamination, using stored biological samples would be an interesting and novel approach.

Recently, several researchers have focused on whether prebiotics and non-digestible oligosaccharides, such as mannan-oligosaccharides (Heinrichs *et al.*, 2003; Franklin *et al.*, 2005), fructo-oligosaccharides (FOS) (Donovan *et al.*, 2002), and lactulose (Fleige *et al.*, 2007), can reduce the incidence of disease in animals (Fleige *et al.*, 2009). Another recent study carried out in experimental animals, as well as in pigs and poultry, found that oligosaccharides interact directly with intestinal epithelial cells, and that they improve and protect the integrity of the intestinal barrier and modulate the immune responses of epithelial cells (Akbari *et al.*, 2015, 2017a,b). In the recent years, prebiotics have been widely used at cattle production sites to maintain and improve the health of the cattle. Difructose anhydride (DFA) Ⅲ, which is a non-digestible disaccharide found in commercial roasted chicory, promotes mineral absorption in rats, humans, and cattle (Mineo *et al.*, 2002; Sato *et al.*, 2007; Tomita *et al.*, 2007). Additionally, we have already reported that it was a useful feed supplement in the form of a prebiotic to improve health and intestinal microbiota in calves (Takagi *et al.*, 2009, 2011). Hence, we hypothesized that the etiotropic effects of DFA III can be applied to cattle herds in practice and that its administration can improve and protect the integrity of the intestinal

barrier in cattle as an alternative way to protect against even a chronic, low-dose, mycotoxin contamination of cattle diets.

The main purpose of Chapter 1 in the present study was to investigate the metabolic effects of mycotoxin contamination in cattle feed under field conditions, with a particular focus on ZEN and STC. Another aim was to elucidate whether chronic intake of mycotoxin-contaminated feed can harm the systemic metabolism of cattle in the long term. Additionally, in Chapter 2, we investigated whether supplementary DFA III has beneficial effects on intestinal barrier function in cattle. To this end, we evaluated the impact of DFA III on mycotoxin absorption, as well as chronic, low-level mycotoxin exposure, by monitoring the urinary concentration of ZEN and its metabolites.

Chapter 1

Gas chromatography-mass spectrometry for metabolite profiling of Japanese black cattle naturally contaminated with zearalenone and sterigmatcystin

ABSTRACT

The objective of this study was to evaluate the metabolic profile of cattle fed with or without zearalenone (ZEN) and sterigmatocystin (STC)-contaminated diets using a gas chromatography-mass spectrometry metabolomics approach. Urinary samples were collected from individual animals (n = 6 per herd) from fattening female Japanese Black (JB) cattle herds (23 months old, 550–600 kg). Herd 1 had persistently high urinary ZEN and STC concentrations due to the presence of contaminated rice straw. Herd 2, the second female JB fattening herd (23 months old, 550–600 kg), received the same dietary feed as Herd 1, with non-contaminated rice straw. Urine samples were collected from Herd 1, two weeks after the contaminated rice straw was replaced with uncontaminated rice straw (Herd 1N). Identified metabolites were subjected to principal component analysis (PCA) and ANOVA. The PCA revealed that the effects on cattle metabolites depended on ZEN and STC concentrations. The contamination of cattle feed with multiple mycotoxins may alter systemic metabolic processes, including metabolites associated with ATP generation, amino acids, glycine-conjugates, organic acids, and purine bases. The results obtained from Herd 1N indicate that a two-week remedy period was not sufficient to improve the levels of urinary metabolites, suggesting that chronic contamination with mycotoxins may have long-term harmful effects on the systemic metabolism of cattle.

INTRODUCTION

Contamination of agricultural commodities with mycotoxins, which are secondary metabolites of fungi, is a major problem in agriculture and livestock production worldwide (Fin-Gremmels *et al.*, 2008). The consumption of mycotoxin-contaminated products is generally believed to cause acute and chronic effects in humans and animals; thus, the contamination of food, feed, and ingredients with mycotoxins presents significant health risks (Fin-Gremmels *et al.*, 2008; Liu *et al.*, 2013). In cattle production, several in vivo reports on the acute and chronic effects of mycotoxicosis have been published. Those reports are mainly based on clinical symptoms observed in field studies or following the experimental administration of mycotoxin to cattle, occasionally with concomitant biochemical analysis. However, risk evaluations concerning the metabolic status of cattle exposed to mycotoxin contamination are lacking (Fushimi *et al.*, 2014).

Recently, we reported the presence of mycotoxin contamination in the dietary feed (rice straw) of a cattle herd by demonstrating the presence of zearalenone (ZEN) and sterigmatocystin (STC), which are produced by two groups of fungi, *Fusarium* spp. and *Aspergillus* spp., and their secondary metabolites in urine using our established liquid chromatography-tandem mass spectrometry (LC-MS/MS) monitoring technique (Hasunuma *et al.*, 2012; Fushimi *et al.*, 2014). Moreover, we suggested that monitoring ZEN and STC levels in urine is a practical and useful way of evaluating the contamination status of a cattle herd and assessing the efficiency of mycotoxin adsorbent, which is supplemented in dietary feeds to impair the intestinal adsorption of mycotoxins (Takagi *et al.*, 2011; Hasunuma *et al.*, 2012; Fushimi *et al.*, 2014).

Because organisms must maintain homeostasis, metabolic profiles vary

continuously with changes in their physical condition. Metabolomics can provide a quantitative description of endogenous metabolites of low molecular mass present in biological samples such as urine, plasma, tissue, and organs, or in the whole body of an organism (Wishart *et al.*, 2005; Madsen *et al.*, 2010). Metabolomics can, therefore, provide information about the metabolic processes occurring in an organism, and has proven to be highly sensitive at evaluating the effects of disease (Kind *et al.*, 2007), toxicants (Huang *et al.*, 2008), and other stressors (Chorell *et al.*, 2009). Recently, there have been reports of using blood, urine, and milk samples to study the effects of diseases and toxicants on cattle (Kitagawa *et al.*,2003; Bertam *et al.*, 2011; Osorio *et al.*, 2012; Brand *et al.*, 2015; Sun *et al.*, 2015).

Conversely, techniques such as mass spectrometry combined with gas chromatography (GC/MS) (Michell *et al.*, 2008; Vallejo *et al.*, 2009), liquid chromatography (Lu *et al.*, 2007; Luo *et al.*, 2007), or capillary electrophoresis (Moraes *et al.*, 2011) have high specificity and sensitivity. GC/MS is potentially useful for metabolomics, because of its high sensitivity, peak resolution, and reproducibility (Xu *et al.*, 2010). However, for GC/MS analysis, compounds must be volatile and thermally stable; therefore, because most metabolites are polar and nonvolatile, they cannot be readily analyzed by GC/MS (Goodacre *et al.*, 2004; Xu *et al.*, 2010). Thus, metabolite profiling using GC/MS usually requires chemical derivatization of the polar functional groups of analytes to reduce their polarity and increase their thermal stability and volatility. To our knowledge, only a few studies have evaluated the effects of mycotoxin contamination through GC/MS analysis of naturally contaminated cattle herds. Thus, the main purpose of the present study was to investigate the metabolic effects of mycotoxin contamination in cattle feed under field conditions, especially with regard to ZEN and

STC contamination.

MATERIALS AND METHODS

Animals were cared for according to the Guide for the Care and Use of Laboratory Animals (Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan).

Chemicals and Reagents

N-methyl-*N*-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS) was purchased from Thermo Scientific (Pittsburgh, PA, USA). *O*-methylhydroxylammonium chloride (methoxylamine hydrochloride), pyridine, 2,2-dimethyl succinic acid, myristic acid d27, pesticide-analytical-grade chloroform and hexane, and high-performance liquid chromatography (HPLC)-analytical-grade methanol were purchased from Wako Pure Chemical (Tokyo, Japan). Urease was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample Collection and Processing

Two herds of female Japanese Black cattle (Herds 1 and 2; 23-month old, weighing 550–600 kg) maintained for fattening for meat in Kagoshima Prefecture, Japan, were used in this study. Herd 1 is known to have persistently high urinary ZEN and STC concentrations due to the contamination of rice straw. Herd 2 had the same dietary feed as Herd 1, but were fed with rice straw not contaminated with ZEA and STC. The content of the feed given to each herd is described in Table 1. Urine samples were collected from individual animals ($n = 6$ per herd) 2 h after the morning feed by massaging the perineum. Additional urine samples were collected from Herd 1, 2 weeks after replacing their rations

with newly un-contaminated rice straw (Herd 1N). ZEN and STC concentrations in the dietary straw, ZEN, and the concentrations of its metabolites; α-Zearalenol (α-ZOL), and β-Zearalenol (β-ZOL), in urine samples of cattle derived from each experimental group collected on the same day, including the results of the dietary straw and urine samples collected at 42 or 34 days before (for Herds 1 and 2, respectively), have been partially reported previously (Hasunuma *et al.*, 2012; Fushimi *et al.*, 2014a, b), and are shown in Table 2. These results indicate that the administration of the contaminated rice straw in Herd 1 lasted at least for 42 days. During the 2-week period, no clinical differences were observed, except for the physical appearance of the buttocks which may reflect the fecal discharge condition. All the samples were immediately placed in a cool box, protected from light, and were transported to the laboratory. Urine samples were centrifuged (Model 2410, Kubota Corp., Tokyo, Japan) at 500× *g* for 10 min at room temperature to remove debris and frozen at −30 °C until GC/MS analysis.

Preparation of Urine Samples for GC/MS Analyses

After thawing at room temperature, the urine samples were centrifuged (Model 6000, Kubota Corp., Tokyo, Japan) at 10,000 rpm for 10 min at 4 °C. Aliquots of urine samples (100 μL) were transferred into 2-mL polypropylene (PP) microtubes, and 100 μL urease (2 mg/mL) was added. Samples were incubated for 30 min at 37 °C with shaking. One-milliliter of methanol cooled with ice was added to the urine samples, which were then shaken on a vortex mixer, and centrifuged at 10,000 rpm for 10 min at 4 °C. Next, 900 μL of the upper layer was placed in another 2-mL PP microtube, spiked with 2,2-dimethyl succinic acid and myristic acid d27 for use as the internal standards, and then evaporated to dryness under a gentle stream of nitrogen at room temperature.

Derivatization was performed in two steps: oximation and silylation. To this end, the dried residue was dissolved in 10 μL of a pyridine solution of methoxylamine hydrochloride (40 mg/mL) by means of continuous shaking at 30 °C for 90 min, then silylation was carried out with 90 μL of MSTFA + 1% TMCS at 37 °C for 30 min. After derivatization, 100 μL of hexane was added to each sample, which were additionally diluted 10 times with hexane, and rapidly injected into the GC/MS.

GC/MS Analysis and Creatinine Levels in the Urine

Metabolites were analyzed on an Agilent Technologies 6890 Series gas chromatograph equipped with a 5973 MSD mass selective detector and a DB 5-ms capillary column (i.d. 0.25 mm × 30 m, 0.25-μm film thickness; J&W Scientific, Folsom, CA, USA). The injected sample volume was 1 μL. The injector and detector temperatures were 250 °C and 290 °C, respectively; the oven temperature program was as follows: 60 °C for 1 min, increased to 325 °C at 10 °C/min, and then held at 325 °C for 12 min. In the present study, we replicated the GC/MS analysis with the same urine to avoid experimental fluctuations and misinterpretations.

Creatinine concentrations in the urine were determined using a commercial kit (Sikarikit-S CRE; Kanto Chemical, Tokyo, Japan) according to the manufacturer's instructions and measured using a clinical autoanalyzer (7700 Clonical Analyzer; Hitachi High-Tech, Tokyo, Japan).

Data Processing

Metabolite peaks on the GC/MS chromatogram were drawn from the baseline to obtain the peak area, deconvoluted, and aligned using MetAlignTM (ver. 080311,

Wageningen University, Wageningen, The Netherlands). Data obtained from peak areas for individual target metabolites and normalized by the peak area of 2,2-dimethyl succinic acid as an internal standard were additionally normalized by the creatinine levels measured in individual cattle. The normalized values were analyzed by one-way analysis of variance (ANOVA). Then PCAs were performed for the metabolites that differed significantly by ANOVA among all groups, in order to evaluate differences in toxic effects among the groups. PCA score plots were used with component loading to evaluate how specific metabolites were affected by exposure to mycotoxins. Statistical analyses were performed with the R programming language (http://www.r-project.org/). $p < 0.05$ was considered significant.

RESULTS

GC/MS Analysis of Urine Samples Derived from Cattle Herds

Original Chromatograms

In the present study, we used GC/MS to identify 55 non-targeted endogenous metabolites related to the metabolism of ATP generation, amino acids, thyroid hormones, neurotransmitters, glycine-conjugation, organic acids, and purine bases, as well as dietary plant components. All identified data was analyzed by ANOVA and/or principal component analysis (PCA) to determine the effects of dietary mycotoxin contamination.

Pattern Recognition

Unfortunately, metabolic data from one sample of Herd 2 were not obtained because the derivatization step failed; therefore, data for this sample were not included in the PCA analysis. In the PCA analysis shown in Figure 1, the score plots of the first two principal components (PC1 and PC2) allowed us to visualize and compare the data for the three examined cattle herds.

Data for both Herd 1 and Herd 1N clustered on the upper left-to-right of the plot, and those for Herd 2 fell in the lower left of the plot (PC1 accounted for 36.3% of the variance and PC2 accounted for 14.4%). These results indicated that the metabolite profiles of Herd 1, 1N, and 2 clearly differed. Therefore, based on these results, clustering along PC1 represented the effects of mycotoxin (ZEN and STC) contamination intensity within the dietary feeds, and PC2 represents the effects common to the mycotoxin contamination group. The component loadings that significantly contributed to the clustering of each group along PC1 and/or PC2 are listed in Table 3.

Metabolites associated with ATP generation metabolism (xylitol, pantothenic acid, galactose, myo-inositol, aconitic acid, glucose, isocitric acid, lactate, lactose, lyxose, and xylitol), organic acid (hydroxyisovaleric acid, methyl succinic acid, ethylhydracrylic acid, and phenaceturic acid), amino acids (threonine, lysine, taurine, tyrosine, oxoproline, tryptophan, phosphocolamine, and serine), glycine-conjugates (butyrylglycine, hippurate, and methylbutyrylglycine), purine bases (ribofuranose, allantoin, β-pseudouridine, and uric acid), and dietary plant-derived metabolites (adonitol, threitol, cinnamate, hydroxyphenyllactate, glycolic acid, indol-3-acetic acid, galacturonic acid, hippuric acid, deoxytetronic acid, threonic acid, and gluconic acid) significantly contributed to different clusters for the three cattle herds on PCA (Table 3). We used a one-way ANOVA to determine significant differences in the levels of metabolites (creatinine modified area) among the three cattle herds. Results of the ANOVA indicated that there were significant differences in the amounts of 11 metabolites among the three herds, as shown in Table 3 and Figure 2.

DISCUSSION

Metabolomics can be efficiently utilized to clarify several simultaneous effects in a single examination, and can identify temporal variation in the metabolic responses of living organisms to physiological and pathological stress conditions (Seeling *et al.*, 2006; Uno *et al.*, 2012; Wan *et al.*, 2015). Although several studies have investigated the toxicity of acute exposure to ZEN or other kinds of mycotoxins in laboratory animals, only a few have obtained data from cattle urine samples, from which chronic contamination with ZEN and STC were identified. To our knowledge, the present metabolomics study reports, for the first time, the chronic effects of mycotoxin contamination on cattle metabolism.

Previous studies investigating the effects of ZEN have involved measuring and comparing levels of a single or several biochemical markers. Seeling *et al*. (2006) reported that the effects of Fusarium toxin intake at 8.21 mg DON/kg DM and 0.09 mg ZEN/kg DM were insufficient to induce any toxicological changes in serum biochemical parameters of protein metabolism or liver damage, such as serum aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), total protein (TP), or serum albumin (Alb) in cows. Additionally, we previously investigated serum samples from animals of Herds 1, 2, and 1N, and no significant differences were observed in protein metabolism (TP, Alb, albumin/globulin ratio, and blood urea nitrogen), liver function indicators (AST, GGT, and insulin like growth factor-1), or energy metabolism (total cholesterol and glucose), except for free fatty acids, which may be indicative of an energy imbalance (Fushimi *et al.*, 2014). However, based on a recent consensus regarding the evaluation of adverse effects of mycotoxins, traditional methods may not be sensitive enough to determine the systemic metabolic status of animals (Seeling *et al.*, 2006; Liu

et al., 2013).

Previously, ZEN exposure was shown to induce oxidative stress, and some common changes in systemic metabolism, including effects on cell membrane metabolism, protein biosynthesis, glycolysis, and gut microbiota metabolism in rodents (Liu *et al.*, 2013). Additionally, Santos and Fink-Gremmels (2014) performed genomic analyses in dairy cattle and reported that dysfunctional lipid metabolism and oxidative stress seemed to be key elements of the mycotoxin syndrome. Metabolomics studies with experimental animals have also shown that higher concentrations of metabolites in rat urine samples indicate high levels of metabolic waste or the ineffective use of nutrients, which are subsequently excreted (Sun *et al.*, 2015). Therefore, in the present study, we evaluated the metabolic status of animals considering the findings of previous studies as well as the general consensus.

Metabolism of ATP Generation

In the present study, seven detected metabolites were associated with gluconeogenesis, and significantly/strongly contributed to the separation along PC1 (positively), and four detected metabolites were along PC2 (negatively) in the PCA. Additionally, significant differences were observed for both urinary lactate and lactose among the three groups by ANOVA analysis. Our results indicate that mycotoxin exposure may affect gluconeogenesis/energy metabolism in cattle, as reported in experimental animals (Liu *et al.*, 2013). Xylitol is produced by the hydrogenation of xylose derived from hemicellulose, which is one of the main constituents of biomass, and can be extracted from the fibrous material of oats, cornhusks, and sugar cane bagasse (Saha, 2003). Carbohydrates in dietary feed are ultimately digested as glucose, galactose,

and fructose, which are absorbed from intestinal epithelial cells and utilized for gluconeogenesis in the liver at the end of several metabolic pathways. Lactose is a disaccharide derived from galactose and glucose, and is usually found in milk. In the present study, the levels of urinary xylitol, galactose, and glucose in the contaminated herds were significantly higher than those in the control herd; the inverse was found for urinary lactose and lactate, which were significantly higher in the control herd than in the contaminated herds. Lactate is the end-product of energy metabolism/glycolysis (Liu *et al.*, 2013). Previous studies involving experimental animals have indicated that the concentration of plasma lactate increases following ZEN exposure (Conkova *et al.*, 2011; Liu *et al.*, 2013). Gluconeogenesis has been shown to occur during long-term starvation; however, similar metabolic changes can occur when organisms encounter harmful stimuli (Lasram *et al.*, 2014). Additionally, previous studies on experimental animals have suggested that increased glucose utilization might be a major metabolic effect of mycotoxin exposure (Zhang *et al.*, 2010; Liu *et al.*, 2013). We previously reported that there was no difference in the serum glucose concentration between Herds 1 and 2 at the time of urine collection (74.8 ± 0.9 and 65.4 ± 3.5 mg/dL, respectively) (Fushimi *et al.*, 2014). Therefore, our results may indicate that increased gluconeogenesis and reduced glycolysis in animals of Herds 1 and 1N are similar as they attempt to maintain glucose concentration.

Based on the results of the PCA, urinary pantothenic acid, myo-inositol, aconitic acid, and isocitric acid were found to differ significantly between contaminated and control herds. Animals require pantothenic acid to synthesize coenzyme-A (CoA) (Novelli *et al.*, 1949). CoA is important in energy metabolism, and is needed for pyruvate to enter the tricarboxylic acid cycle (TCA cycle). Myo-inositol is an important structural

molecule for a number of secondary messengers in eukaryotic cells, including insulin signal transduction, cytoskeleton assembly, control of intercellular calcium concentration, and the breakdown of fats (Fisher *et al.*, 1992).

In the present study, a significant difference in methyl succinic acid, hydroxyisovaleric acid (HIVA), and uric acid was observed between the contaminated and control groups. The formation of 3-HIVA in mammals has been demonstrated in association with leucine degradation, and is normally present in small quantities in urine (Landaas *et al.*, 1974). In a previous study, increased concentrations of 3-HIVA were found in all urine samples from patients with ketoacidosis (Landaas *et al.*, 1974). This observation may suggest a mild ketotic condition in contaminated herds, even without any difference in serum glucose concentrations between contaminated and control herds. Analysis of beta-hydroxybutyrate must be performed in the future to clarify this point.

Amino Acid Metabolism

In the present study, significant differences in the essential amino acids, threonine, and lysine, and taurine were observed, resulting in the separation of contaminated and control groups along with PC1 in PCA. These results suggest that mycotoxin exposure alters amino acid metabolism in cattle. Threonine is converted to pyruvate via threonine dehydrogenase, and an intermediate in this pathway can undergo thiolysis with CoA to produce acetyl-CoA and glycine. Lysine is metabolized in mammals to produce acetyl-CoA, via initial transamination with α-ketoglutarate. Because both threonine and lysine are essential amino acids, they must be obtained from the diet, or are biosynthesized within the rumen in cattle. As the diet composition of the individual herds was almost the same, except for the contaminated rice straw, variation can be attributed to the different

levels of mycotoxin exposure. Previous studies have shown that ZEN can inhibit protein synthesis in experimental animals (Abid-Essefi *et al.*, 2004; Liu *et al.*, 2013), which might be due to stress-induced increases in energy expenditure, and thus the elevated consumption of amino acids. However, in the present study, urinary extraction was increased upon feeding with mycotoxin-contaminated diets, especially for taurine. Our results seem to contradict the results of other studies with experimental animals, but the causes of these phenomena are obscure. Another approach may be necessary to clarify the differences.

In the present study, levels of tyrosine and oxoproline were significantly different between contaminated and control herds. Notably, tyrosine is an important amino acid in many proteins, peptides, and even enkephalins, and is the precursor of thyroid hormones (Lemmon *et al.*, 2010). Oxoproline is a metabolite involved in the glutathione cycle, which is converted to glutamate. The liver represents an important pool for amino acid metabolism, and is vital for the decomposition and utilization of most amino acids in cattle, except for branched-chain amino acids (Haque *et al.*, 2012). Our results suggest that excessive protein synthesis occurs to maintain the concentration of thyroid hormone.

In the present study, levels of tryptophan, phosphocolamine, and serine significantly differed between the contaminated and control herds along PC1 of the PCA. Additionally, a significant difference was observed in phosphocolamine among the three herds. Tryptophan is a precursor of the neurotransmitters serotonin and melatonin. Phosphocolamine is an ethanolamine derivative that is used to construct two different types of phospholipids; glycerophospholipid and sphingomyelin. Serine, a non-essential amino acid, is important in metabolism, and participates in the biosynthesis of purines and pyrimidines. In addition, it is the precursor of several amino acids, including glycine,

cysteine, and tryptophan in bacteria, and plays an important role in the catalytic function of many enzymes. 5-hydroxytryptamine is a messenger generated from tryptophan, which can produce pleasurable emotions and affects nearly all types of brain activity (Chen *et al.*, 2012). Previous studies have shown that low levels of 5-hydroxytryptamine can lead to depression (Hale *et al.*, 2011). Thus, our results indicate that increased levels of neurotransmitter-related amino acids may explain the inappetence and depression observed in cattle exposed to ZEN and STC mycotoxin contamination.

Glycinconjugation

In the present study, three metabolites associated with glycine conjugates, hippurate, butyrylglycine, and methylbutyrylglycine, significantly contributed to the separation of contaminated and control herds along with PC1. The results of a previous study suggested that both hippurate and glycine conjugates are normally present in the urine of healthy cattle, and that the increased urinary excretion of glycine conjugates is related to detoxification (Kitagawa *et al.*, 2003). Hippurate is a normal component in urine and is formed by the conjugation of benzoic acid and glycine; it is thought that the amount of urinary hippurate may reflect the hepatic detoxification ability by means of glycine conjugation (Kitagawa *et al.*, 2003). Butyrylglycine and methylbutyrylglycine are acyl glycines, which are normally minor metabolites of fatty acids. In some cases, the levels of these metabolites in body fluids can be used to diagnose disorders associated with mitochondrial fatty acid beta-oxidation. The metabolism of xenobiotics is often divided into three phases: Phase I, modification; Phase II, conjugation; and Phase III, excretion. In subsequent Phase II reactions, these activated xenobiotic metabolites are conjugated with charged species such as glutathione, sulfate, glucuronic acid, and glycine. Therefore,

the results of the present study suggest that mycotoxin contamination (ZEN and STC exposure) in cattle may have affected detoxification during Phase II.

Purine Base Metabolism

Urine metabolites, such as purine derivatives, have been used to estimate levels of microbial protein synthesis in the rumen (Boudra *et al.*, 2012). In the present study, levels of ribofuranose, allantoin, β-pseudouridine, and uric acid differed significantly between contaminated and control herds. Additionally, significant differences were observed in ribofuranose among the three herds. Ribofuranose forms part of the RNA backbone, which is related to deoxyribose. Uric acid is a bi-product of the metabolic breakdown of purine nucleotides, and in most mammals, the enzyme uricase further oxidizes uric acid to allantoin. Hypouricemia may occur following exposure to drugs and toxic agents (Bomalaski *et al.*, 2004). The presence of allantoin in the urine can occur via non-enzymatic means, through high levels of reactive oxygen species; thus, allantoin is thought to be a marker of oxidative stress (Liu *et al.*, 2013). A previous report indicated that serological parameters of oxidative stress are the most practical parameters associated with dietary multiple mycotoxin contamination in dairy cattle (Santos and Fink-Gremmels, 2014). Allantoin is a product of purine metabolism, and a strong correlation between the urinary excretion of purine metabolites and allantoin has been reported (Shingfield *et al.*, 2009). Urinary purine metabolite excretion seems to be an indicator of microbial protein synthesis in ruminants (Chen *et al.*, 1995; Shingfield *et al.*, 2009). Additionally, Sun *et al.* (2015) reported a negative relationship between allantoin and nitrogen efficiency. Therefore, our results suggest that mycotoxin (ZEN and STC) exposure may oxidatively affect stress as well as nitrogen efficiency in the rumen of cattle.

Metabolites Possibly Derived from Dietary Components

In the present study, the levels of nine identified metabolites associated with feed materials differed significantly between contaminated and control herds, including adonitol, threitol, cinnamate, hydroxyphenyllactate, glycolic acid, indol-3-acetic acid, deoxytetronic acid, threonic acid, and gluconic acid. Additionally, significant differences were observed in the levels of galacturonic acid, hippuric acid, and glycolic acid among the three herds. Adonitol is a crystalline pentose alcohol formed via the reduction of ribose, which occurs in the cell walls of Gram-positive bacteria. Cinnamate is found naturally in a variety of plants. It is thought that hydroxyphenyllactate is derived from roughage. Glycolic acid is found in some sugar-crops. Indol-3-acetic acid is the most common naturally occurring plant hormone of the auxin class. Therefore, the above results may reflect the difference in dry matter content in dietary feed between the contaminated and control herds, and the subsequent nutrient utilization by the animals. Conversely, galacturonic acid is a sugar acid- oxidized form of galactose, and is the main component of pectin. Pectin consists of a complex set of polysaccharides, which are present in most primary cell walls of plants. As noted, because urinary galactose excretion contributed to the separation of contaminated and control herds, this may affect/reflect the significant differences in urinary galacturonic acid excretion observed in the present study. Hippuric acid is a carboxylic acid found in the urine of horses and other herbivores. Although rumen microorganisms may synthesize benzoic acid from a variety of sources, benzoic acid is not normally synthesized by animals, and ingested benzoic acid is excreted as hippuric acid in the urine of herbivorous animals following conjugation with glycine (Sharma *et al.*, 1972). Hippuric acid in bovine urine is strongly associated with its

concentration in dietary feed (Kitagawa *et al.*, 2003). Therefore, the different levels of urinary hippuric acid observed between the contaminated and control herds may reflect its volume in the straw feed.

In conclusion, the results of the present study indicate that the contamination of cattle feed with multiple mycotoxins, as confirmed by the measurement of ZEN and STC, may result in altered metabolic processes, including ATP generation, amino acid, and purine base metabolism. The results obtained from Herd 1N indicate that the two-week remedy period for contaminated feed was not sufficient to modify most of the urinary metabolites, suggesting that chronic contamination of mycotoxins may have long-term adverse effects on systemic metabolism in cattle. This suggests that monitoring endogenous metabolites in urine samples (metabolomics) is useful for evaluating the effects of cattle exposure to multiple mycotoxins. Further field studies are required to develop a basic tool to evaluate the impact of chronic exposure to low-dose mycotoxins on cattle health and productivity. Additionally, because of the limitations of using GC/MS as the analytical tool for metabolomics analysis, extensive sample treatment and analytical validation must be performed.

FIGURE LEGENDS

Fig. 1. Principal component analysis (PCA) score plots of PC1 versus PC2 from target metabolite profiles of urinary samples derived from three groups of cattle. Herd 1: known to have persistently high urinary ZEN and STC concentrations due to contaminated rice straw, Herd 1N: urine samples were collected from Herd 1, 2 weeks after replacing the newly not-contaminated rice straw, Herd 2: same feeding pattern as Herd 1, except for the contaminated rice straw. The percentages shown on the x- and y-axes represent the contribution to PC1 and PC2, respectively.

Fig. 2. Differences in each representative metabolite among the three herds, which were identified as significantly different by ANOVA ($p < 0.05$). Values on the *y*-axis were normalized to the peak area on the gas chromatography/mass spectroscopy chromatogram by the peak area of internal standard and then normalized by the measured values of urinary creatinine in individual cattle.

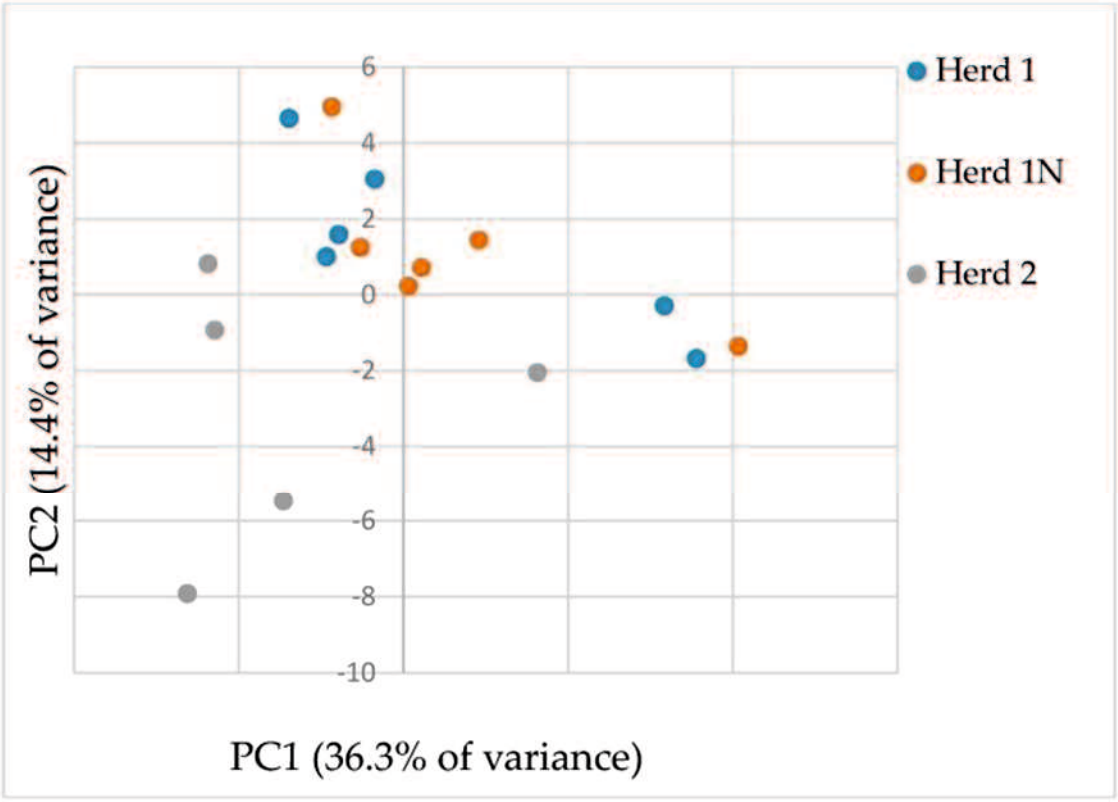
Herd 1
Herd 1N
Herd 2
PC2 (14.4% of variance)
PC1 (36.3% of variance)
6
4
2
0
-2
-4
-6
-8
-10

Fig.1

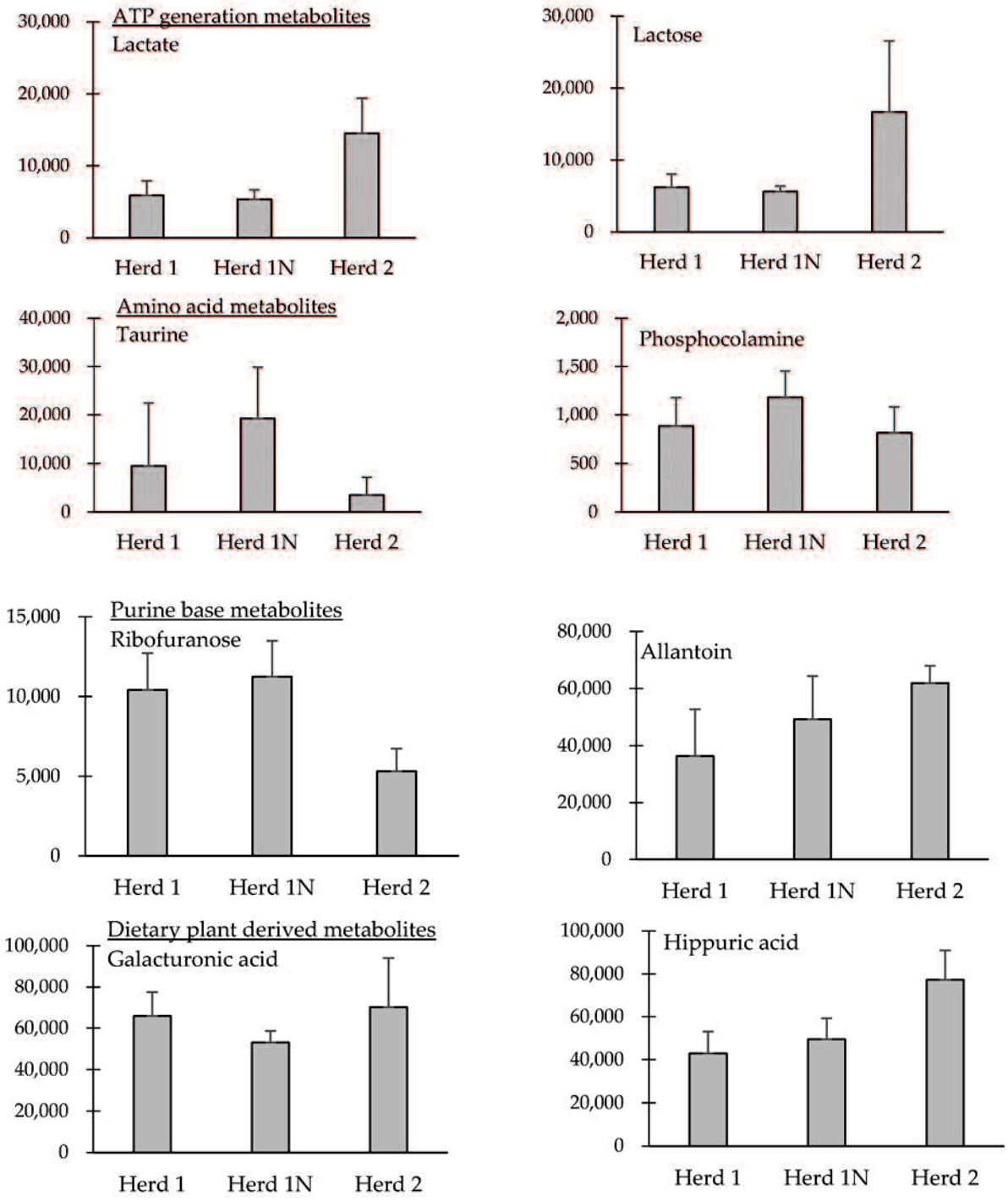
ATP generation metabolites
Lactate
30,000
20,000
10,000
0
Herd 1
Herd 1N
Herd 2
Lactose
Amino acid metabolites
Taurine
40,000
30,000
20,000
10,000
Phosphocolamine
2,000
1,500
1,000
500
Purine base metabolites
Ribofuranose
15,000
5,000
Allantoin
80,000
60,000
40,000
Dietary plant derived metabolites
Galacturonic acid
100,000
Hippuric acid

Fig 2

Table 1. Composition of feed provided to the 2 cattle herds kept for fattening purpose (as-fed basis)

	Forage Feed, kg	Formula Feed						
Herd		**Total, kg**	**Bran, %**	**Cereal, %**	**Oil Seed Meal, %**	**Other, %**	**TDN, %**	**CP, %**
Herd 1	Straw, 2	9	17	77	5	1	>75	>14
Herd 2	Straw, 4	9	17	77	5	1	>75	>14

These results have been reported previously (Hasunuma *et al.*, 2012)

Table 2. Zearalenone (ZEN) and sterigmatocystin (STC) concentrations in the dietary straw, and ZEN, α-Zearalenol (α-ZOL), and β-Zearalenol (β-ZOL) concentrations in urine samples of cattle derived from each experimental group collected on the same day.

Experimental Group	Straw (mg/kg)		Urine Samples (Mean ± SEM; pg/mg Creatinine)			
	ZEN	STC	ZEN (*n* = 6)	α-ZOL (*n* = 6)	β-ZOL (*n* = 6)	STC (*n* = 6)
Herd 1	7.6	0.24	3702 ± 747	859 ± 178	5503 ± 1130	569 ± 111
(- 42 days) *	7.5	0.17	2444 ± 394	759 ± 148	5496 ± 1197	209 ± 22
Herd 1N	ND	0.04	63 ± 12	20 ± 3	119 ± 18	33 ± 8
Herd 2	0.2	<0.01	50 ± 8	ND	82 ± 27	47 ± 19
(- 34 days) **	0.2	0.03	68 ± 10	21 ± 9	215 ± 51	147 ± 39

These concentrations in straw and urine samples have been partially reported previously (Hasunuma *et al.*, 2012 and Fushimi *et al.*, 2014a, b). *: Straw and urine samples collected 42 days before the sampling for metabolomics in Herd 1. **: Straw and urine samples collected 34 days before the sampling for metabolomics in Herd 2.

Table 3. Metabolites contributing to the clustering of each group along PC1 and PC2 on the PCA score plot, and *p* values showing significant differences by one-way ANOVA among the three cattle herds.

	Metabolite	**PC1**	**PC2**	***p* Value**
ATP generation	xylitol	0.88		0.12
	pantothenic acid	0.79		0.63
	galactose	0.59		0.79
	myo-inositol	0.56		0.51
	aconitic acid	0.54		0.55
	glucose	0.53		0.78
	isocitric acid	0.53		0.50
	lactate		−0.87	0.038
	lactose		−0.84	0.03
	lyxose		−0.59	0.47
	xylitol		−0.50	0.80
Organic acid	hydroxyisovaleric acid	0.64		0.48
	methyl succinic acid	0.66		0.32
	ethylhydracrylic acid		−0.69	0.19
	phenaceturic acid		−0.63	0.35
Amino acid	threonine	0.78		0.08
	lysine	0.64		0.31
	taurine	0.52		0.028
	tyrosine	0.86		0.36
	oxoproline (pyroglutamic acid)	0.70		0.38
	tryptophan	0.74		0.32
	phosphocolamine	0.73		0.0008
	serine	0.62	−0.61	0.09
Glycin-conjugate	butyrylglycine	0.76		0.70
	hippurate	0.75		0.66
	methylbutyrylglycine	0.62		0.82
Purine base	ribofuranose	0.76		0.003
	allantoin		−0.56	0.011
	β-pseudouridine	0.85		0.87
	uric acid	−0.60	−0.58	0.23
Dietary plant-derived	adonitol	0.88		0.87
	threitol	0.88		0.87
	cinnamate	0.82		0.30
	hydroxyphenyllactate	0.82		0.48
	glycolic acid	0.56	−0.48	0.75
	indol-3-acetic acid	0.54		0.54
	galacturonic acid		−0.66	0.002
	hippuric acid		−0.67	0.0002
	deoxytetronic acid	0.95		0.53
	threonic acid	0.90		0.68
	gluconic acid	0.88		0.77

Chapter 2

Fruct-oligosaccharide (DFA Ⅲ) feed supplementation for mitigation of mycotoxin exposure in cattle-clinical evaluation by a urinary zearalenone monitoring system

ABSTRACT

The potential effect of difructose anhydride III (DFA III) supplementation in cattle feed was evaluated using a previously developed urinary-zearalenone (ZEN) monitoring system. Japanese Black cattle from two beef herds aged 9–10 months were used. DFA III was supplemented for two weeks. ZEN concentrations in feed were similar in both herds (0.27 and 0.22 mg/kg in roughage and concentrates, respectively), and below the maximum allowance in Japan. ZEN, α-zearalenol (α-ZOL), and β-ZOL concentrations in urine were measured using LC/MS/MS the day before DFA III administration, 9 and 14 days thereafter, and 9 days after supplementation ceased. Significant differences in ZEN, α-ZOL, β-ZOL, and total ZEN were recorded on different sampling dates. The concentration of inorganic phosphate in DFA III-supplemented animals was significantly higher than in controls on day 23 (8.4 vs. 7.7 mg/dL), suggesting a possible role of DFA III in tight junction of intestinal epithelial cells. This is the first evidence that DFA III reduces mycotoxin levels reaching the systemic circulation and excreted in urine. This preventive effect may involve an improved tight-junction-dependent intestinal barrier function. Additionally, our practical approach confirmed that monitoring of urinary mycotoxin is useful for evaluating the effects of dietary supplements to prevent mycotoxin adsorption.

INTRODUCTION

Contamination of agricultural commodities with mycotoxins, which are secondary fungal metabolites, is a major worldwide problem in agriculture and livestock production (Fink-Gremmels, 2008). Consumption of mycotoxin-contaminated products is generally believed to cause acute and chronic effects in humans and animals; thus, contamination of food, feed, and ingredients by mycotoxins poses significant health risks (Fink-Gremmels, 2008; Liu *et al.*, 2013). Recently, we found mycotoxin contamination in the dietary feed (rice straw) of a cattle herd whose urine was shown to contain zearalenone (ZEN) produced by Fusarium spp., and its secondary metabolites, by means of our liquid chromatography-tandem mass spectrometry (LC-MS/MS) monitoring technique (Hasunuma *et al.*, 2012). Moreover, we suggested that monitoring ZEN levels in urine is a practical and useful way of evaluating the contamination status of a cattle herd and assessing the efficiency of the mycotoxin adsorbents (MAs) supplemented in dietary feed to impair intestinal adsorption of mycotoxins (Takagi *et al.*, 2011; Hasunuma *et al.*, 2012).

Several approaches for protecting animals from the toxic effects of natural mycotoxin contamination from both pre- and post-harvest products have been reported, such as appropriate field management and crop husbandry, the introduction of a non-toxigenic antagonistic fungal strain in the field prior to harvest, adequate storage management, and application of fungicidal agents and other protective silage additives at the post-harvest stage. In animal feed, the application of adsorbing agents, pro- and prebiotics, particularly yeast and yeast cell fractions, or mycotoxin-degrading enzymes has become common practice (Sabater-Vilar *et al.*, 2007; Kutz *et al.*, 2009; Award *et al.*, 2010; Wambacq *et al.*, 2016). MAs generally consist of a mixture containing a mineral

clay carrier, yeast cell wall preparations, and, in some cases, enzymes or living microorganisms (probiotics) that may adsorb and detoxify mycotoxins. We recently reported on the use of MAs and indicated their significant effects on the reduction in urinary ZEN concentration, concomitant with the reduction in the somatic cell count in a dairy cattle herd (Takagi *et al.*, 2011; Fushimi *et al.*, 2014).

Recently, there has been a growing interest in the health-promoting benefits of prebiotics and non-digestible oligosaccharides, such as mannan-oligosaccharides (Heinrichs *et al.*, 2003; Franklin *et al.*, 2005), fructo-oligosaccharides (FOS) (Donovan *et al.*, 2002), and lactulose (Fleige *et al.*, 2007), to reduce the incidence of diseases in animals (Fleige *et al.*, 2009). Difructose anhydride III (DFA III) is a naturally occurring, non-digestible disaccharide present in commercial roasted chicory that is manufactured from inulin by microbial fermentation (Yokota *et al.*,1991; Tamura *et al.*, 2004). DFA III promotes calcium absorption in rats (Mineo *et al.*, 2002; Shiga *et al.*, 2003), humans (Shigematsu *et al.*, 2004; Tomita *et al.*, 2007), and cattle (Sato *et al.*, 2007; Teramura *et al.*, 2015). Furthermore, Minamida et al. (Minamida *et al.*, 2005; Minamida *et al.*, 2006) reported that oral administration of DFA III in laboratory animals may help to maintain a healthy balance of intestinal microbiota; they suggested that DFA III is a novel candidate prebiotic. Additionally, we reported on the efficacy of DFA III supplementation as a prebiotic for the improvement in the health and intestinal microbiota of calves (Matsumoto *et al.*, 2009; Takagi *et al.*, 2011). Recently, direct interactions of these oligosaccharides with intestinal epithelial cells have been reported, which have indicated that these oligosaccharides improve and protect the intestinal barrier integrity and modulate the immune responses of epithelial cells (Akbari *et al.*, 2015; Akbari *et al.*, 2017a,b). Based on these findings regarding the possible efficacy of oligosaccharides as

prebiotics, we hypothesized that the etiotropic effects of DFA III could be applied to cattle herds in practice, and that the administration of DFA III would help to maintain good health, and improve and protect the intestinal barrier integrity in cattle as an alternative way to protect against the chronic low-dose mycotoxin contamination of cattle diets.

Therefore, the objectives of this field study were to evaluate the beneficial effects of supplementary DFA III on the intestinal barrier function in cattle, by monitoring urinary concentrations of ZEN and its metabolites as an indicator of the impact of DFA III on mycotoxin absorption. The concentrations of calcium (Ca), inorganic phosphate (Pi), and magnesium (Mg) were also measured in serum as indicators of optimal functioning of the intestinal epithelial cells.

MATERIALS AND METHODS

The experiments were conducted according to the regulations concerning the protection of experimental animals and the guidelines of Yamaguchi University, Japan (No.40, 1995, approval date 27 March 2017).

Chemicals and Solvents

DFA III was kindly donated from Nippon Beet Sugar Manufacturing Co. Ltd., Obihiro, Japan. ZEN was purchased from MP Biomedicals (Heidelberg, Germany). The metabolites α-ZOL and β-ZOL were purchased from Sigma (St. Louis, MO, USA). Stock solutions of ZEN, α-ZOL, and β-ZOL, each at a concentration of 1 μg/mL in methanol, were stored under light protection at 4 °C. High performance liquid chromatography (HPLC)-grade methanol was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). β-Glucuronidase/arylsulfatase solution was purchased from Merck (Darmstadt, Germany). Sodium acetate was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan) and Tris was purchased from Nakalai Tesque Inc. (Kyoto, Japan).

Japanese Black Cattle Herds and Sample Collection

Japanese Black heifers from two beef herds (Herd 1: n = 10, Herd 2: n = 20, 10 months old, 250−300 kg) raised in Kagoshima Prefecture, Japan, were included in this experiment. Herds 1 and 2 consisted of 370 and 500 beef cattle, respectively. Basically, Herds 1 and 2 were fed with purchased concentrate and rice straw. The detailed compositions of the dietary feed of the two herds are shown in Table 1.

In both herds, the roughages and concentrates were stored at ambient temperature,

in feed sheds and silos, respectively. The ZEN level in the dietary feed of both herds was provisionally measured before the beginning of the experiment, via LC-MS/MS, as previously reported (Fushimi *et al.*, 2014). The concentration of ZEN in the mixture of roughages and concentrates fed to heifers was 0.27 mg/kg in Herd 1 and 0.22 mg/kg in Herd 2. These findings confirmed that the contamination of dietary feed with ZEN was below the threshold levels allowed by Japanese regulations (<1.0 ppm), and were very similar in both herds.

Two groups of heifers were randomly selected from each experimental Herd and divided into two treatments that differed in feed supplementation as follows: DFA III group (Herd 1: n = 5, Herd 2: n = 10) was fed 40 g DFA III/day (20 g each feeding time) mixed with concentrate, and the control group (Herd 1: n = 5, Herd 2: n = 10) was fed with no DFA III supplementation. This dose of DFA III is the recommended dose for the prevention of hypocalcemia in dairy cows (Sato *et al.*, 2007; Teramura *et al.*, 2015), which may affect tight-junction functions.

Two hours after the morning feeding, urine samples were collected from the animals by massaging the pudendum, and blood samples were collected from the jugular vein in silicone-coated tubes. This sampling was performed at the start of DFA III supplementation (Day 0), 9 days (day 9), and 14 days (day 14, i.e., on the last day of DFA III supplementation) after treatment initiation, and on the last day of the experimental period, 23 days (day 23) after treatment initiation. In addition, samples of rice straw and feed concentrate (approximately 1 kg each) were obtained from both herds to measure ZEN concentration in the feed. The protocol for DFA III supplementation and all sampling procedures is summarized in Figure 1.

All samples were immediately placed in a cooler containing dry-ice for protection

from light and transported to the laboratory. The urine and blood samples were centrifuged at 1000× g and 2000× g, respectively, for 10 min at room temperature. The urine and serum samples were frozen at −30 °C until the analysis of ZEN and its metabolites, urine creatinine (as a reference for the correction of urine volume), and the serum ion concentrations of Ca, Mg, and Pi, using a Labospect 7180 autoanalyzer (Hitachi, Japan).

Methods of Urine Sample Analysis

All urine samples were analyzed by LC/MS/MS, as described in our previous report (Takagi *et al.*, 2011). Briefly, 0.5 mL of each urine sample was mixed with 3.0 mL of 50 mM ammonium acetate buffer (pH 4.8) and 8 μL of glucuronidase/arylsulfatase solution, and incubated for 12 h at 37 °C. The solution was loaded onto a C18 SPE column, which was preconditioned with 3 mL 100% methanol and 2 mL of Tris buffer, followed by the addition of 2 mL Tris buffer and 3 mL of 40% methanol. After washing the SPE column with approximately 1 mL of 80% methanol, the volume of the eluted solution was adjusted to exactly 1 mL. Then, 20 μL of the reconstituted solution was injected into the LC/MS/MS system. The LC/MS/MS analyses were performed on an API 2000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface and a 1200 Infinity Series HPLC system (Agilent Technologies, Santa Clara, CA, USA). The detection limits for ZEN, α-ZOL, and β-ZOL were 0.04 ng/mL, 0.05 ng/mL, and 0.05 ng/mL, respectively, while the mean recovery rates for ZEN, α-ZOL, and β-ZOL were 90%, 109%, and 90%, respectively. The urine creatinine concentrations were determined by using a commercial kit (Sikarikit-S CRE, Kanto Chemical, Tokyo, Japan), according to instructions by the manufacturer, and were

measured using the 7700 Clinical Analyzer (Hitachi High-Tech, Tokyo, Japan). All urine concentrations were expressed as a ratio of creatinine (pg/mg creatinine), as described previously (Takagi *et al.*, 2011).

Statistical analysis

The results for ZEN, α-ZOL, β-ZOL, and ΣZEN (ZEN + α-ZOL + β-ZOL) concentrations and serum ion concentrations are expressed as means ± SD. The urine and serum values of the DFA III and control groups were compared using Student's t-tests with Welch's correction when the variances differed. P values less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

No significant clinical differences were observed between both cattle herds in this experiment.

Concentration of ZEN and Its Metabolites with or without Supplementation of DFA III

Results for the analysis of urinary concentrations of ZEN, α-ZOL, β-ZOL, and total ZEN (ΣZEN; ZEN + α-ZOL + β-ZOL) during the experimental period, with and without DFA III supplementation, are shown in Figure 2.

Urinary ZEN concentrations on day 0 revealed that each herd had approximately the same level of contamination with ZEN. However, when comparing the DFA III-treated and control groups, significant differences ($p < 0.05$) were confirmed for ZEN on day 14 (11.0 ± 8.5 vs. 22.2 ± 12.2 pg/mg of Creatinine); for α-ZOL on day 23 (11.3 ± 16.7 vs. 25.2 ± 17.9 pg/mg of Creatinine), for β-ZOL on day 9 (8.6 ± 18.5 vs. 25.4 ± 9.3 pg/mg of Creatinine), on day 14 (12.7 ± 18.9 vs. 34.3 ± 21.5 pg/mg of Creatinine) and day 23 (16.5 ± 24.8 vs. 46.5 ± 19.9 pg/mg of Creatinine); and for ΣZEN (ZEN + α-ZOL + β-ZOL) on day 9 (11.6 ± 19.5 vs. 38.3 ± 28.0 pg/mg of Creatinine) and day 14 (31.0 ± 33.3 vs. 63.9 ± 38.9 pg/mg of Creatinine).

Ca, Mg, and Pi Concentrations with or without Supplementation of DFA III

The results for the concentration of serum Ca, Mg, and Pi during the experimental period are shown in Figure 3. Although no differences were observed in either the serum Ca or Mg, the Pi concentration in DFA III-supplemented animals was significantly higher than that in the control animals on day 23 (8.4 ± 0.5 vs. 7.7 ± 1.0 mg/dL), suggesting a

possible role of DFA III in the tight-junction functions of intestinal epithelial cells.

DISCUSSION

Recently, Akbari *et al*. (2015) reported that galacto-oligosaccharides (GOS) can prevent the typical adverse effects of the mycotoxin deoxynivalenol in a concentration-dependent manner. The effects of GOS, which facilitated tight-junction assembly and regulated claudine-3 gene expression, were of special interest and the authors further examined the microbiota-independent effects of oligosaccharides on intestinal epithelial cells and compared the effects of GOS and FOS. They reported that not only GOS, but also FOS showed a protective effect on deoxynivalenol-induced impairment of Caco-2 monolayer integrity, and they accelerated tight junction reassembly (Akbari *et al.*, 2017a,b). Based on these results and our previous reports regarding the clinical effects of DFA III (Matsumoto *et al.*, 2009; Takagi *et al.*, 2011), we hypothesized that DFA III has similar protective effects on the intestinal barrier functions and intestinal ion transport. We selected ZEN as a model compound/substance, again based on our previous results of mycotoxin exposure monitoring in cattle herds. The results reported herein indicated that, when comparing the DFA III and control groups, DFA III supplementation of dietary feed altered ZEN adsorption and, hence, excretion levels in cattle. Moreover, although no differences were observed in either serum Ca or Mg, the Pi concentration was significantly higher in the DFA III-supplemented animals than in the controls on day 23 (8.4 vs. 7.7 mg/dL), suggesting a role of DFA III in the tight junctions of intestinal epithelial cells.

Compared with the results of our previous report (Fushimi *et al.*, 2014), in the present study, there was a much clearer reduction in β-ZOL than in ZEN or α-ZOL in the DFA III group compared with that in the control group, and there were different ratios of

ZEN and its metabolites. Additionally, significant differences among ZEN, α-ZOL, and β-ZOL at the different sampling times were obtained. Malekinejad *et al.* (2006) reported differences between species in the hepatic biotransformation of ZEN and demonstrated that β-ZOL is the dominant hepatic metabolite in cattle. Therefore, our results may also reflect the alteration in ZEN (parent) adsorption in the DFA III group compared with that in the control group. Regarding the urinary concentrations of ZEN and its metabolites (α-ZOL and β-ZOL), and the differences obtained among the sampling times, although the reason for the differences between reports is obscure, we assume that age differences, variations in ZEN contamination levels, or both may have affected liver metabolism. Further research taking into account cattle age and ZEN contamination levels are warranted.

Strategies for the detoxification of mycotoxin-contaminated feed in a cost-effective way are still poorly developed, and the most promising approach for reduction in the risk of mycotoxin exposure remains the use of non-nutritive adsorptive materials in animal diets (El-Nekeety *et al*., 2017). It was previously stated that ruminating animals develop mycotoxicoses at low frequencies, as the rumen flora act as a first line of defense against mycotoxins at the usual levels of exposure in cattle herds. However, drastic changes in feed composition and a high percentage of protein in dairy diets have modified the detoxification capacity of rumen microorganisms. Therefore, MA supplementation is a widely used approach to reduce the risk of mycotoxicosis even in cattle (Fink-Gremmels *et al.*, 2008; Fushimi *et al.*, 2014). Moreover, accumulating evidence in the last decade suggests that one of the major organs suffering the adverse effects of mycotoxins is the gastrointestinal tract, and more specifically the intestinal barrier. Disturbance of intestinal barrier integrity results in the translocation of feed antigens and even pathogens into the

surrounding tissue, causing an intense and often systemic inflammatory response (Pinton *et al*., 2009; Gajecka *et al.*, 2017). It has been suggested that glutamine, L-arginine, various fatty acids, and particularly non-digestible oligosaccharides play a role in the regulation of intestinal barrier function, and may have potential applications for the prevention and treatment of diseases associated with intestinal barrier impairment (Suzuki *et al.*, 2011; Goossens *et al.*, 2012; Ferrer *et al*., 2015). We have previously demonstrated the usefulness of urine analysis for the objective evaluation of the effects of supplemental feed additives on the bioavailability of mycotoxins (Fushimi *et al.*, 2014).

In the present study, our in vivo results clearly indicated that DFA III (part of the FOS family) showed a capacity to protect intestinal epithelial cells against ZEN in cattle. In addition, the significantly higher Pi concentration in DFA III-supplemented animals than in control animals, after the DFA III supplemental period, suggests a possible role of DFA III in the tight-junction functions of intestinal epithelial cells. DFA III is an indigestible oligosaccharide that is enzymatically synthesized from inulin (Kikuchi *et al*., 2004). In vitro experiments conducted on the small intestines of rats (Suzuki *et al*., 1998; Mineo *et al*., 2002) and duodena of cows (Teramura *et al*., 2015) have shown that Ca absorption via the paracellular pathway can be accelerated by agents that act upon tight junctions. DFA III promotes paracellular transport by reducing transepithelial electrical resistance (TEER) and enhancing the transport of paracellular markers (Suzuki *et al.*, 2004; Suzuki *et al.*, 2006), with alterations to claudin-1, a component of tight junctions and actin filaments in Caco-2 cells (Suzuki *et al*., 2006). The tight junctions play a crucial role in paracellular nutrient transport, as well as barrier function in the intestines. The paracellular route largely contributes to the transport and absorption of certain minerals, such as calcium and magnesium (Mineo *et al*., 2006; Suzuki *et al.*, 2011). The

modification of tight-junction structure and function by DFA III may influence the paracellular absorption of these essential elements. Meanwhile, these minerals are also known to be absorbed by the active transcellular pathway in the intestine. The contribution of each pathway depends on the dietary level of the minerals, and transcellular transport is generally more tightly regulated than in paracellular transport (Suzuki *et al*., 2011). Therefore, transcellular transport may compensate for changes in the paracellular transport of ions. Further study is required to clarify the effects of DFA III in cattle, and some of the research priorities include the investigation of optimal DFA III supplemental volume and method of application in cattle herds.

In conclusion, supplementation of dietary feed with DFA III altered ZEN and differentially affected ion adsorption levels in cattle. Our results are the first clear indication that DFA III supplementation can reduce the levels of mycotoxins that reach the systemic circulation and are excreted in the urine. This preventive effect may be associated with an improved tight-junction-dependent intestinal barrier function. Furthermore, our practical approach confirmed that monitoring urinary mycotoxin is useful for the evaluation of the effects of dietary supplements that may prevent mycotoxin adsorption. Further field studies are in progress to create a database for the assessment of DFA III as a dietary supplement to reduce mycotoxin absorption in cattle and other animal species.

FIGURE LEGENDS

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

Fig. 2. Results for the analysis of urinary concentrations of ZEN, α-ZOL, β-ZOL, and total ZEN (ΣZEN; ZEN + α-ZOL + β-ZOL) during the experimental period, with (DFA III group) or without DFA III supplementation (control group). * Significant difference ($p < 0.05$) between DFA III group and control group on each day. ** Significant difference ($p < 0.01$) between DFA III group and control group on each day.

Fig. 3. Results for the concentration of serum Pi, Ca, and Mg during the experimental period with (DFA III group) and without DFA III supplementation (control group). ** Significant difference ($p < 0.05$) between DFA III group and control group.

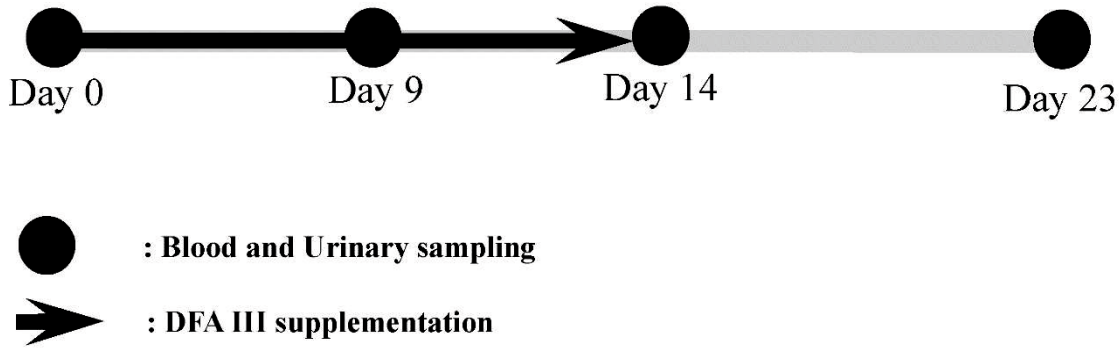
Day 0
Day 9
Day 14
Day 23
: Blood and Urinary sampling
: DFA III supplementation

Fig. 1

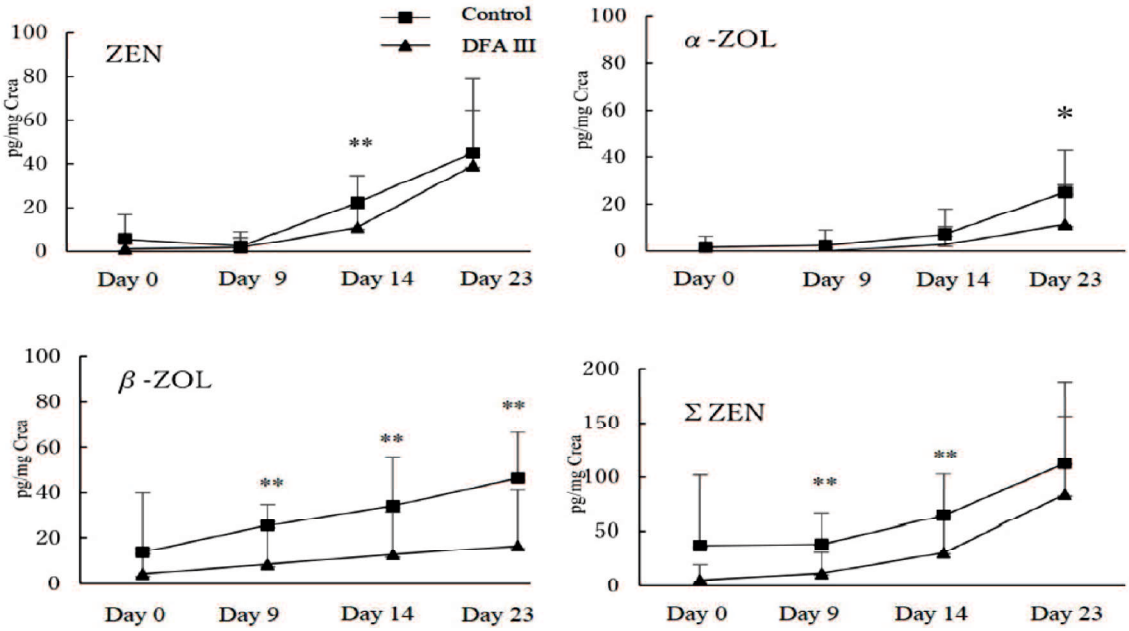
ZEN
Control
DFA III
pg/mg Crea
100
80
60
40
20
0
**
Day 0
Day 9
Day 14
Day 23
α -ZOL
*
β -ZOL
**
Σ ZEN
200
150
100
50

Fig. 2

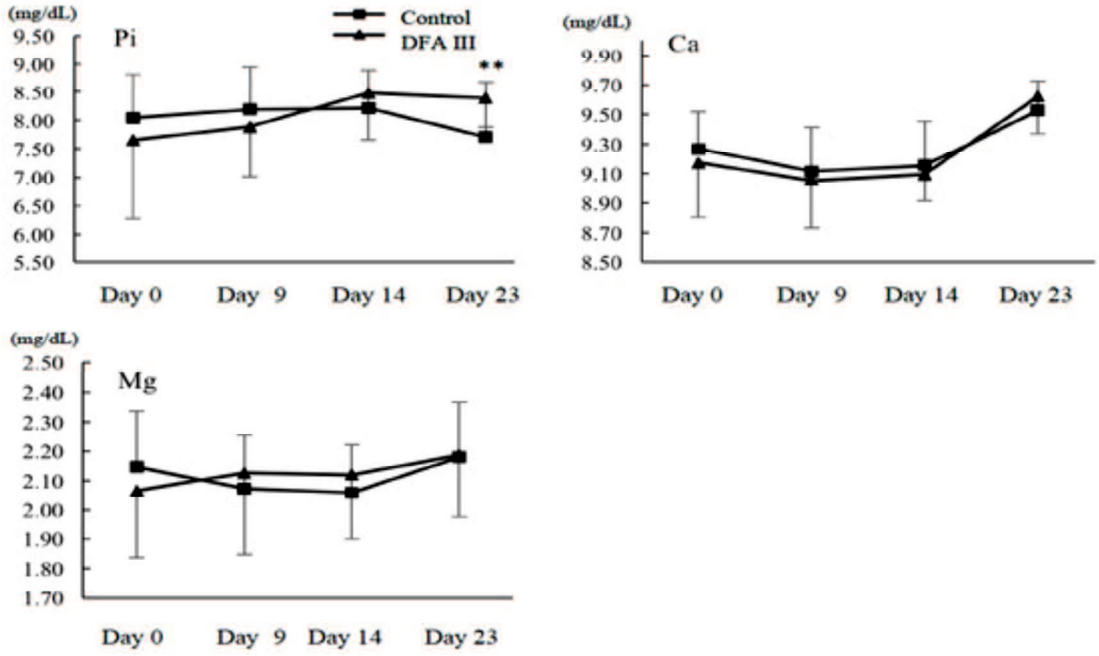
(mg/dL)
Pi
Control
DFA III
9.50
9.00
8.50
8.00
7.50
7.00
6.50
6.00
5.50
**
Day 0
Day 9
Day 14
Day 23
Ca
9.90
9.70
9.50
9.30
9.10
8.90
8.70
8.50
Mg
2.50
2.40
2.30
2.20
2.10
2.00
1.90
1.80
1.70

Fig. 3

Table 1. Composition of feed provided to the two herds 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.

OVERALL DISCUSSION

In Chapter 1, we revealed that feed contaminated with ZEN or STC has several effects on cattle metabolites. If the cattle feed was contaminated with multiple mycotoxins, systemic metabolic processes associated with ATP generation, amino acids, glycine conjugates, organic acids, and purine bases may be altered. In Chapter 2, we confirmed that the levels of ZEN and its metabolites differed significantly between the DFA III-added group and the control group, even at low mycotoxin concentrations. Furthermore, the serum Pi concentration was significantly higher in the DFA III-supplemented group than in the control group.

Many recent studies have focused on mycotoxin-contaminated feed, which may negatively impact livestock performance and productivity, even at low concentrations. However, it is difficult to extract mycotoxin from feed samples, because mycotoxin concentrations tend not to be even within a given batch of feed. For this reason, many studies have monitored mycotoxin levels *in vivo* using blood, feces, or urine samples. In particular, urine can be considered a more concentrated indicator of the blood contents, so it allows mycotoxin absorption by the intestine to be monitored, even at very low levels. For these reasons, our established urinary ZEN-monitoring system may be beneficial for evaluating cattle health.

In conclusion, we can confirm that our system for monitoring urinary ZEN and STC concentrations can be used to ascertain whether a herd is contaminated with mycotoxins, as well as to evaluate the efficacy of mycotoxin adsorbents in feed. We previously reported that DFA III supplementation acts as a prebiotic to improve the health and microbiota of calves. This was an important finding, because interest has been growing

in the health benefits of prebiotics and non-digestible oligosaccharides. Further field studies should be carried out to assess of effects of DFA III, elucidate its mechanism more thoroughly, and clarify how multi-mycotoxin contamination in feed is related to cattle performance and productivity.

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