Enzymatic hydrolysis of fumonisins in the gastrointestinal tract of broiler chickens

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ABSTRACT Fumonisins (FB) are among the most frequently detected mycotoxins in feedstuffs and finished feed, and recent data suggest that the functions of the gastrointestinal tract (GIT) in poultry species might be compromised at doses ranging from 10 to 20 mg/kg, close to field incidences and below the US and EU guidelines. Strategies are therefore necessary to reduce the exposure of poultry to FB. In the present study, we assessed the efficacy of fumonisin esterase FumD (EC 3.1.1.87, commercial name FUM*zyme*®) to cleave the tricarballylic acid side chains of FB, leading to the formation of non-toxic hydrolyzed fumonisins in the GIT of broiler chickens. Broiler chickens were fed for 14 d (7 to 21 d of age) 3 different diets (6 birds/cage, 6 cages/diet), i) control feed (negative control group), ii) feed contaminated with 10 mg FB/kg (FB group), and iii) feed contaminated with 10 mg FB/kg and supplemented with 100 units of FUM*zyme*®/kg (FB+FUM*zyme*® group). To determine the degree of reduction of FB in the GIT, 2 characteristics were analyzed. First, the sphinganine-to-sphingosine ratio in the serum and liver was determined as a biomarker of effect for exposure to FB. Second, the concentration of fumonisin B_1 and its hydrolyzed forms was evaluated in the gizzard, the proximal and distal parts of the small intestine, and the excreta. Significantly reduced sphinganine-to-sphingosine ratios in the serum and liver of the FB+FUM*zyme*® group (serum: 0.15 ± 0.01; liver: 0.17 ± 0.01) compared to the FB group (serum: 0.20 ± 0.01; liver: 0.29 ± 0.03) proved that supplementation of broiler feed with FUM*zyme*® was effective in partially counteracting the toxic effect of dietary FB. Likewise, FB concentrations in digesta and excreta were significantly reduced in the FB+FUM*zyme*® group compared to the FB group ($P < 0.05$; up to 75%). FUM*zyme*® furthermore partially counteracted FB-induced up-regulation of cytokine gene expression (IL-8 and IL-10) in the jejunum. The FB group showed significantly higher gene expression of IL-8 and IL-10 compared to the negative control group (IL-8: fold change = 2.9 ± 1.1, $P < 0.05$; IL-10: fold change = 3.6 ± 1.4, $P < 0.05$), whereas IL-8 and IL-10 mRNA levels were not significantly different in the FB+FUM*zyme*®® group compared to the other 2 groups. In conclusion, FUM*zyme*® is suitable to detoxify FB in chickens and maintain gut functions.

Key words: fumonisins, fumonisin esterase, sphinganine, sphingosine, gastrointestinal tract

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INTRODUCTION

Fumonisins (**FB**) are a group of mycotoxins produced by *Fusarium verticillioides*, a common fungal contaminant of corn and other cereals (Marasas, 2001). Fumonisin B_1 (**FB_1**) is the most abundant and toxic of this class and has been linked to various diseases in humans and animals. The role of FB_1 as an etiologic agent in diseases such as equine leukoencephalomalacia and porcine pulmonary edema has been established (reviewed by Voss et al., 2007). An association between dietary exposure to FB_1 and occurrences of human esophageal cancer in southern Africa and primary liver cancer in China was reported (reviewed by Soriano et al., 2005), and the toxin was classified as possibly carcinogenic to humans (group 2B carcinogen) by the International Agency for Research on Cancer (International Agency for Research on Cancer, 2002).

Most contamination of food and feed with FB occurs at concentrations lower than those causing overt

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clinical symptoms (Streit et al., 2013a). However, the ingestion of subclinical doses of FB has been reported to result in immunologic and metabolic disturbances that may lead to susceptibility to diseases and metabolic disorders. In pigs, FB doses of 3 to 30 mg/kg of feed (considered realistic to occasional doses according to the scale of Grenier and Applegate, 2013) may predispose the animals to lung inflammation (Halloy et al., 2005), intestinal colonization by opportunistic pathogenic bacteria (Oswald et al., 2003), and digestive disorders (including villus alterations and reduced peptidase activity; Piva et al., 2005; Lessard et al., 2009). According to published studies, it seems that poultry species are able to tolerate higher doses of FB, at least in terms of performance response, with no effect on growth of broiler chickens up to 75 to 100 mg FB/kg of feed (Ledoux et al., 1992; Henry et al., 2000; Broomhead et al., 2002). This relative tolerance in avian species to toxins from *Fusarium* in general might be explained either by their low sensitivity to the mechanisms of toxicity of fusariotoxins or by differences in toxicokinetic properties (Guerre et al., 2015). Regarding FB, it already has been demonstrated that the toxin absorption is very low in poultry compared to pigs (3- to 10-fold lower; Guerre et al., 2015; Grenier and Applegate, 2013). Nonetheless, the very poor absorption of FB (less than 5%) implies that a substantial part of FB remains in the gastrointestinal tract (**GIT**), and therefore intestinal epithelial cells are exposed to high FB concentrations. In agreement with that, new evidence has emerged that broiler chickens fed with 10 to 20 mg FB/kg of feed (EU and US guidance levels set at 20 and 50 mg/kg, respectively) were more susceptible to necrotic enteritis and coccidiosis (Antonissen et al., 2015a; Grenier et al., 2016), and underwent digestive and immune disturbances (Antonissen et al., 2015a; Grenier et al., 2015).

It is now well established that the initial mechanism leading to the toxicity and carcinogenicity of FB is related to the disruption of the sphingolipid metabolism that occurs as a result of the inhibition of ceramide synthase (Soriano et al., 2005; Voss et al., 2007). The inhibition of ceramide synthase is attributed to the structural similarity of FB to the 2 sphingoid bases, sphinganine (**Sa**) and sphingosine (**So**). This inhibition results in an increase of Sa, So, and their metabolites, all of which are implicated in cellular signals regulating cell growth, differentiation, survival, and apoptosis (Merrill et al., 2001). As Sa is an intermediate in the de novo biosynthesis of ceramides, and So an intermediate of ceramide turnover, ceramide synthase inhibition increases Sa concentration more than So concentration. This effect of FB on sphingolipid metabolism has been reported in a variety of animals, including avian species (with dietary FB as low as 2 mg/kg in ducks; Tardieu et al., 2006) even though growth is not affected at relatively high dosages. Therefore, exposure of animals to FB is widely assessed by measuring the concentrations of Sa and So in biological matrices (serum, liver, and kidney) and by determining the Sa-to-So (**Sa/So**) ratio.

The contamination of feed with mycotoxins is a continuing feed safety issue leading to economic losses in animal production (Bryden, 2012). Consequently, a variety of methods for decontamination of feed has been developed, with mycotoxin detoxifying agents supplemented as feed additives considered to be the most promising approach that is most commonly used (Kolosova and Stroka, 2011). Due to the extensive use of these feed additives, the European Union in 2009 introduced a new group of additives designated "substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption of mycotoxins (mycotoxin binders), or modify their mode of action (mycotoxin modifiers)" [Regulation (EC) No 386/2009]. The structural and chemical diversity of mycotoxins implies that a unique and specific approach has to be developed for each mycotoxin. For instance, mycotoxin binders, such as clays, are quite effective with respect to aflatoxins, but fail to prevent toxic effects of *Fusarium* mycotoxins, such as FB (Kolosova and Stroka, 2011). The conversion of FB to their hydrolyzed forms (**HFB**) can be achieved by enzymatic degradation using fumonisin esterase FumD (EC 3.1.1.87), an enzyme of bacterial FB catabolism (Heinl et al., 2010; Hartinger and Moll, 2011) that has recently been commercialized as FUM*zyme*® and authorized by the EU for the use in poultry and pigs. In addition to elimination of the parent toxin, a successful detoxification process facilitates the formation of nontoxic metabolites. This has been investigated in pigs that were orally treated with high doses of FB_1 and its hydrolyzed form HFB_1, and no sign of toxicity (i.e., with respect to effects on Sa/So ratio, mucosal immunity, and intestinal and hepatic lesions) was reported in HFB_1-treated pigs compared to FB_1-treated pigs (Grenier et al., 2012). Furthermore, HFB_1 showed a greatly reduced reproductive toxicity (Collins et al., 2006; Voss et al., 2009) and hepatotoxicity (Howard et al., 2002) in rodents. Likewise, the partially hydrolyzed fumonisin B_1 forms a and b (**pHFB$_{1a}$** and **pHFB$_{1b}$**), which are formed as intermediate products of FB degradation by FUM*zyme*®, did not affect the Sa/So ratio in rats (Hahn et al., 2015).

FUM*zyme*® was shown to be effective as a FB-degrading feed additive in pigs and turkeys. In both species, it was shown to facilitate gastrointestinal degradation of dietary FB_1 and to prevent an increase of the Sa/So ratio in serum (Masching et al., 2016). It furthermore neutralized toxic effects of dietary fumonisins on the liver, lung, jejunum, and immune response of pigs (Grenier et al., 2013). The aim of the present study was to assess the efficacy of FUM*zyme*® in broiler chickens that received a diet contaminated with 10 mg FB/kg. As FB remain in the GIT and may compromise intestinal functions, we evaluated the concentration of FB and their metabolites in the GIT (gizzard and proximal and distal parts of the small intestine) and in excreta of the

birds after the enzymatic treatment as a biomarker of exposure. Concentrations of Sa and So were measured in the serum and liver as biomarkers of effect. Furthermore, as 10 mg FB/kg of feed was recently reported to affect the gene expression of cytokines related to intestinal inflammation in broiler chickens (Grenier et al., 2015), the effect of FB and FUM*zyme*® on cytokine gene expression in the jejunum was evaluated in the present study.

MATERIALS AND METHODS

Experimental Birds and Housing, Diet Formulation, Study Design, and Sampling

All animal care and use procedures for the experiment were approved by the Purdue University Animal Care and Use Committee. A 14-day feeding study was conducted with 7-day-old male broilers (Ross 708). For artificial contamination of feed with FB, culture material of *Fusarium verticillioides* (M-3125; Desjardins et al., 1992) was grown on rice, homogenized, freeze-dried, and mixed into the basal diet. The culture material contained 15.4 mg/g FB_1 + fumonisin B_2 (**FB_2**). A batch of FUM*zyme*® was produced by fermentation of a recombinant *Pichia pastoris* strain, which secretes fumonisin esterase FumD into the fermentation medium (Heinl *et al.*, 2010). The fermentation medium containing FumD was separated from biomass by centrifugation and microfiltration and spray-dried with maltodextrin as carrier to achieve a FumD concentration of 3.500 units (**U**) per gram (one U is the enzymatic activity that releases 1 μmol tricarballylic acid per min from 100 μM FB_1 in 20 mM Tris-Cl buffer pH 8.0 with 0.1 mg/mL BSA at 30°C).

Six replicate cages (6 birds per cage) were fed experimental diets formulated to contain i) basal feed (negative control), ii) feed artificially contaminated with 10 mg/kg FB_1 + FB_2, or iii) feed artificially contaminated with 10 mg/kg FB_1 + FB_2 and supplemented with 100 U/kg FUM*zyme*® (analysis of the FUM*zyme*® activity in the final diet was 78.5 U/kg ± 2.0). FUM*zyme*® is intended to be used at a dosage of 15 to 300 U/kg feed (EFSA FEEDAP Panel, 2016). In this study, we applied a dosage of 100 U/kg, which is within this range. FB_1 and FB_2 levels in all diets were analyzed by Romer Labs GmbH (Romer Labs, Union, MO) via multitoxin LC-MS/MS. Presence of other major mycotoxins in the diets was evaluated (IFA, Tulln, Austria) as described by Streit et al. (2013b). Deoxynivalenol and zearalenone were found to be naturally present in all final diets, and concentrations measured in feed for individual groups ranged from 0.590 to 0.846 mg/kg, and 0.033 to 0.049 mg/kg, respectively. Ingredient formulation, nutrient composition, and FB content of the diets are reported in Tables 1 and 2. Birds were housed in stainless-steel battery cages equipped with nipple-type waterers and thermostatically controlled heaters. The mortality of birds was recorded daily. Body weight and feed intake were measured per cage at the beginning and at the end of the trial (7 and 21 d of age), and feed conversion ratio was calculated as the feed intake during this period divided by the body weight gain over the same period.

Table 1. Diet formulation and nutrient specification

Item	Starter diet
Ingredient (% of diet)	
Corn	53.98
Soybean meal (48% CP)	38.05
Soy oil	3.52
Sodium chloride	0.48
DL-Methionine	0.25
Threonine	0.07
L-Lysine HCl	0.10
Limestone	1.68
Monocalcium phosphate	1.52
Vitamin and mineral premix[1]	0.35
Nutrient composition (calculated)	
ME, kcal/kg	3066
CP, %	22.43
Ca, %	1.01
Nonphytate P, %	0.43
Met, %	0.59
Thr, %	0.92
Lys, %	1.34

[1]Supplied the following per kilogram of diet: iron, 50.2 mg; copper, 7.7 mg; manganese, 125.1 mg; zinc, 125.1 mg; iodine, 2.1 mg; selenium, 0.3 mg; vitamin A, 13,233 IU; vitamin D_3, 6,636 IU; vitamin E, 44.1 IU; vitamin K activity, 4.5 mg; thiamine, 2.2 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.3 mg; folic acid, 1.1 mg; biotin, 0.3 mg; vitamin B_{12}, 24.8 mcg; choline, 669.8 mg. ME, Metabolizable Energy; CP, Crude Protein.

Table 2. Experimental treatments and fumonisin content of diets

Treatment group	FB_1 (mg/kg feed)[1]	FB_2 (mg/kg feed)[1]	Total FB (mg/kg feed)[1]	FUM*zyme*® (U/kg feed)
Negative control	0.02	/	0.02	/
FB	8.2	2.8	11.0	/
FB+FUM*zyme*®	7.8	2.4	10.2	78.5

[1]**FB_1**, fumonisin B_1; **FB_2**, fumonisin B_2; **FB**, fumonisins.

All the birds were euthanized by an overdose of carbon dioxide. Blood was collected from the jugular veins of broilers (6 replicates/diet, pool of 2 birds/replicate), incubated at room temperature for 30 min for coagulation, and centrifuged at 1,000 × g for 20 min at 4°C. The serum samples were stored at -80°C until analysis. Livers were removed from 2 birds per cage, flash frozen in liquid nitrogen, and stored at -80°C prior to sphingolipid analyses. A small piece of mid-jejunum was taken from one bird per cage and placed in a cryovial containing RNAlater (Ambion Inc., Austin, TX) for subsequent RNA isolation. All the birds were sampled for determination of FB_1 and its metabolites in digesta. Digesta contents of 6 birds (from the same cage) were pooled and considered as one replicate per diet. The digesta contents from the gizzard, the proximal part of the small intestine (**SI**) (i.e., content from the duodenum to the mid jejunum), and the distal part of the SI (i.e., content from the mid jejunum to the

ileocecal junction) were collected by gentle squeezing, which were then stored at -20°C until analysis. Similarly, fresh excreta from each cage were collected and pooled to represent one replicate.

High-performance Liquid Chromatography Tandem Mass Spectrometry Based Analysis of Sphingolipids

Determination of So and Sa in serum was performed as described by Antonissen et al. (2015b). Liver samples were homogenized and extracted as described by Grenier et al. (2015). The obtained extracts were diluted 7.5-fold before injection of 1 μl. High-performance liquid chromatography tandem mass spectrometry (**HPLC-MS/MS**) analysis of So and Sa was done according to Masching et al. (2016). Briefly, a 1290 Infinity series HPLC system (Agilent Technologies, Waldbronn, Germany) was equipped with a Kinetex C18 column (150 × 2.1 mm, 2.6 μm; Phenomenex, Torrace, CA) and coupled to a Triple Quad 5500 mass spectrometer (AB Sciex, Framingham, MA). The instrument was operated in selected reaction monitoring mode, using the following transitions for quantification: m/z 300.3 to 252.3 for So and m/z 302.3 to 60.1 for Sa. Concentrations of So and Sa were determined based on external standard calibration functions (So and Sa standards were purchased from Avanti Polar Lipids, Inc., Alabaster, AL). Results were corrected for recovery (So 64%, Sa 59%), which was determined by spiking of 6 different concentration levels (in triplicates) into blank liver homogenates. Recovery was consistent over the range of concentrations studied. Limit of Quantification (**LOQ**) of the method is 0.071 μg/g liver for Sa and 0.056 μg/g liver for So; Limit of Detection (**LOD**) is 0.024 μg/g liver for Sa and 0.019 μg/g liver for So. The concentration of Sa and So in the samples of this trial were well within the calibrated range, between 0.375 and 4.5 μg Sa/g liver and between 3.6 and 8.7 μg So/g liver.

Gene Expression Analysis by Quantitative Reverse Transcription PCR

Intestinal tissues (mid-jejunum) were processed in lysing bead tubes containing guanidine-thiocyanate acid phenol (QIAzol reagent, Qiagen, Valencia, CA) for use with the FastPrep-24 (MP Biomedicals). Concentrations, integrity, and quality of RNA were determined spectrophotometrically using Nanodrop ND1000 (Fisher Scientific, St. Louis, MO). Two micrograms of total RNA were treated with DNase I (Sigma Aldrich, Saint Louis, MO), as some genes were lacking introns. RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI). Quantitative real-time PCR was performed using the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) with the iQ SYBR Green Supermix (Bio-Rad). Thermal cycling conditions for the PCR reactions were 95°C for 5 min followed by 40 cycles of 95°C for 10 s, 55°C for 20 s, and 72°C for 20 seconds. To verify the absence of genomic DNA in the RNA preparations, quantitative PCR was performed on non-reverse-transcribed RNA preparations. Each sample was assessed in duplicate on 2 separate plates (in triplicate in case of a high coefficient of variation). The specificity of the PCR products was checked at the end of the reaction by analyzing the curve of dissociation. In addition, the amplicon size was verified by electrophoresis. The genes studied were IL-1β, IL-6, IL-8, IL-10, SOCS1, IL-17, IL-21, and IFN-γ, and the sequences of the primers used are detailed in Table 3. Amplification efficiency and initial fluorescence were determined by using the Data Analysis for Real-Time PCR method (Peirson et al., 2003). Then, values obtained were normalized to both housekeeping genes encoding glyceraldehyde 3-phosphate dehydrogenase and β2-μglobuline. Finally, gene expression was expressed relative to the control group.

HPLC-MS/MS Based Analysis of FB_1, $pHFB_1a$, $pHFB_1b$, and HFB_1 in Digesta and Excreta

The sample preparation method for determination of FB_1, $pHFB_1a$, $pHFB_1b$, and HFB_1 in digesta and feces included 3-fold extraction of 1.00 ± 0.01 g of homogenized sample (thorough stirring with a spoon) with 10 mL, 10 mL, and 5 mL of ACN/water/formic acid (74/25/1, v/v/v) by shaking for 30 min, 20 min, and 10 min, respectively. Shaking was performed on a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) at 200 rpm. Supernatants obtained by centrifugation (2,655 × g, 10 min) were combined, vortexed, diluted 1 + 3 with extraction solvent, and centrifuged at 14,000 × g for 10 min prior to LC-MS/MS analysis as described by Hahn et al. (2015). LOD (LOQ) values in fresh GIT samples were 0.05 (0.12) μg/g for HFB_1, 0.08 (0.23) μg/g for $pHFB_1$a and b, and 0.12 (0.38) μg/g for FB_1.

Statistics

Data were subjected to analysis of variance using IBM SPSS statistics software (Version 19.0, IBM corp., Armonk, New York 2010). The data were first analyzed as a completely randomized design with the experimental unit as a cage of birds (one bird/cage or a pool of birds/cage) to examine the overall effects of diets. The Fisher's Least Significance Difference test was then used as a post hoc test or the Games-Howell test when equal variances could not be assumed (Levene statistic). Statements of significance were $P \leq 0.05$ unless noted otherwise. In addition, the gene expression was considered "biologically" significant across treatments when both P-value ≤ 0.05 and cut-off value of > 2 or < -2 for

Table 3. Nucleotide sequence of primers for quantitative real-time PCR

GENE[1]	PRIMER SEQUENCE	AMPLICON Size	AMPLICON Intron[2]	ENSEMBL ACCESS #	REFERENCES
Housekeeping genes					
GAPDH	F (300 nM) TCCTAGGATACACAGAGGACCA R (300 nM) CGGTTGCTATATCCAAACTCA	151 bp	2 (499)	ENSGALG00000014442	Grenier et al., 2015
β2-μglob.	F (300 nM) CCACCCAAGATCTCCATCAC R (300 nM) AACGTCCAGTCGTCGTTGA	92 bp	0	ENSGALG00000002160	Present study
Pro-inflammatory cytokines					
IL-1β	F (300 nM) GCATCAAGGGCTACAAGCTC R (300 nM) CAGGCGGTAGAAGATGAAGC	131 bp	1 (87)	ENSGALG00000000534	Adedokun et al., 2012
IL-6	F (300 nM) GAATGTTTTAGTTCGGGCACA R (300 nM) TTCCTAGAAGGAAATGAGAATGC	130 bp	0	ENSGALG00000010915	Grenier et al., 2015
IL-8	F (300 nM) GCGGCCCCCACTGCAAGAAT R (300 nM) TCACAGTGGTGCATCAGAATTGAGC	146 bp	2 (1210)	ENSGALG00000011670	Grenier et al., 2015
Treg signature					
IL-10	F (300 nM) GCTGAGGGTGAAGTTTGAGG R (300 nM) AGACTGGCAGCCAAAGGTC	121 bp	2 (1127)	ENSGALG00000000892	Grenier et al., 2015
SOCS1	F (300 nM) CAAGCGGATTTCAGTAGCATC R (300 nM) GGCTCAGACTTCAGCTTCTCA	110 bp	no intron	ENSGALG00000007158	Grenier et al., 2015
Th17 and Th1 signature					
IL-17	F (300 nM) TATCAGCAAACGCTCACTGG R (300 nM) AGTTCACGCACCTGGAATG	110 bp	1 (666)	ENSGALG00000016678	Grenier et al., 2015
IL-21	F (300 nM) GCTTTCAAAGACAATTGACCATC R (300 nM) TACAGCTGTGAGCAGGCATC	106 bp	2 (3765)	ENSGALG00000011844	Grenier et al., 2015
IFN-γ	F (300 nM) AGCTGACGGTGGACCTATTATT R (300 nM) GGCTTTGCGCTGGATTC	259 bp	2 (998)	ENSGALG00000009903	Hong et al., 2006

[1]**β2-μglob.**, β2-μglobuline; **GAPDH**, Glyceraldehyde 3-phosphate dehydrogenase; **IFN-γ**, Interferon-γ; **IL**, Interleukin; **SOCS1**, Suppressor of cytokine signaling 1.
[2]Number of introns spanned in the design of primers; the brackets report the total size of introns in bp.

fold change were seen, as commonly reported in transcriptomics studies.

RESULTS

A feeding trial was performed to evaluate the efficacy of FUM*zyme*® in chickens. Groups of animals (Table 2) received either basal feed (negative control group), FB-contaminated feed (10 mg/kg FB_1 + FB_2; FB group), or FB-contaminated feed supplemented with FUM*zyme*® (10 mg/kg FB_1 + FB_2, 100 U/kg FUM*zyme*®; FB+FUM*zyme*® group) for 14 days. As expected, none of the experimental diets had an effect on body weight gain, feed intake, or feed conversion ratio of birds during the 2-week feeding period (data not shown, but body weight average at 21 d was about 735 g).

Effect of Treatments on The Sphingolipid Metabolism of Birds – biomarker of Effect of FB

As expected, the analysis of the sphingoid base content showed an increase of the Sa/So ratio in the serum and liver of birds fed a FB-contaminated diet as compared to birds fed an uncontaminated diet (Figure 1). Addition of FUM*zyme*® to the FB-containing diet significantly reduced this specific increase of the Sa/So ratios in the serum and liver (Figure 1).

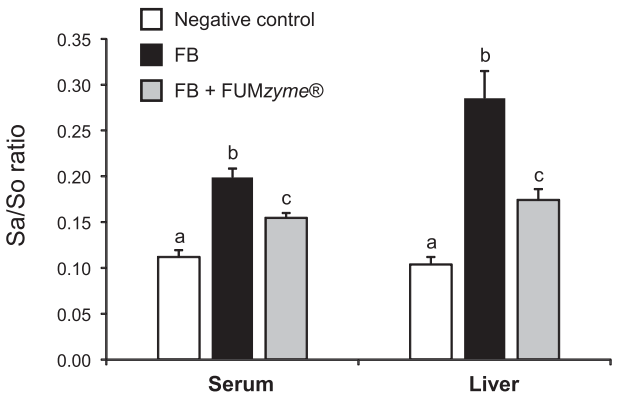
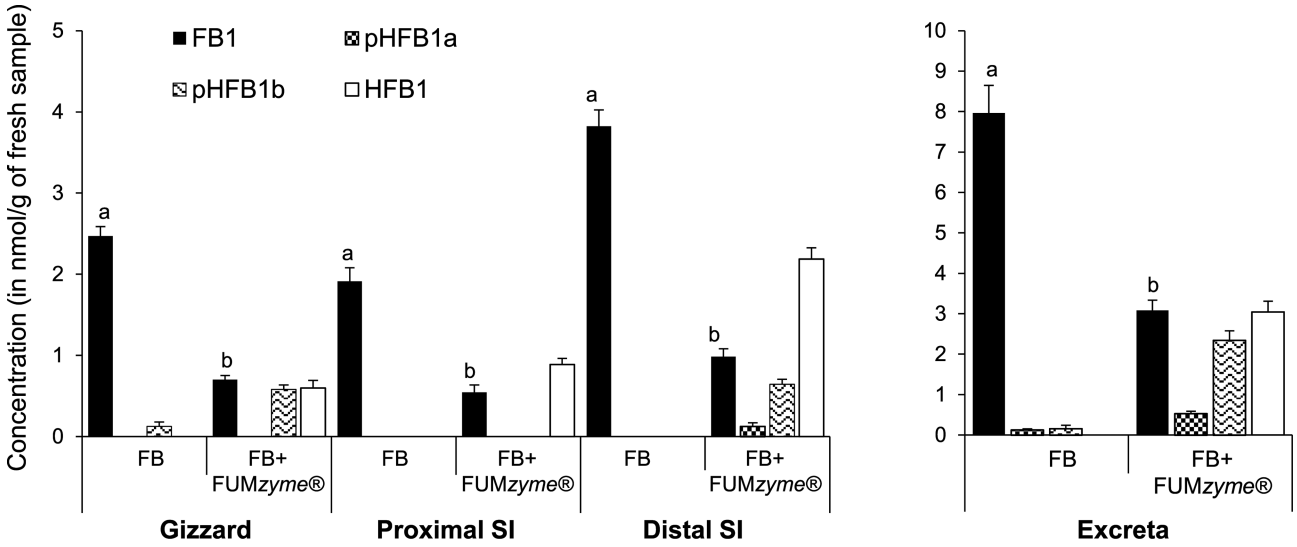
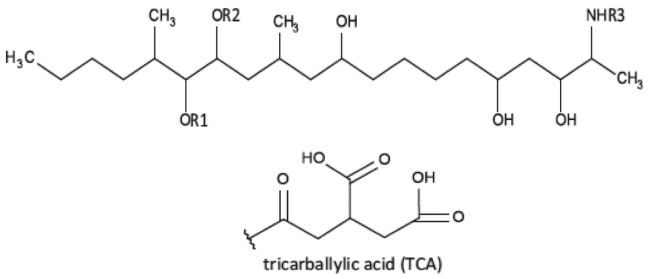


Figure 1. Sphinganine (**Sa**)/sphingosine (**So**) ratio in serum and liver. Values are means ± SEM for 6 replicates (pool of 2 birds/replicate) for each treatment. Means with no common superscript are significantly different ($P < 0.05$).

Effect of Treatments on The Content of FB_1 and its Partially and Fully Hydrolyzed Forms in the Digestive Tract and The Excreta of Birds – biomarker of Exposure to FB

FB_1 was recovered in high concentrations from the different parts of the intestinal tract and the excreta of birds fed a FB-contaminated diet without FUM*zyme*® addition (Figure 2). This is in line with a low intestinal absorption of FB in poultry (reviewed by Guerre, 2015) and other animals. The recovery of low concentrations of $pHFB_1a$ and $pHFB_1b$ indicates that FB are hydrolyzed in the digestive tract of chickens, as also





Analyte	R1	R2	R3
FB_1	TCA	TCA	H
$pHFB_1a$	TCA	H	H
$pHFB_1b$	H	TCA	H
HFB_1	H	H	H

Adapted from Hahn et al. (2015)

Figure 2. Fumonisin B1 (**FB_1**), partially hydrolyzed fumonisin B_1a (**$pHFB_1a$**), partially hydrolyzed fumonisin B_1b (**$pHFB_1b$**), and hydrolyzed fumonisin B_1 (**HFB_1**) in digestive tract and excreta. The figure does not display the negative control group, as neither FB_1 nor its metabolites were above the limit of quantification in samples of these birds. Values are means ± SEM for 6 replicates (pool of 6 birds/replicate) for each treatment. For FB_1, means with no common superscript are significantly different ($P < 0.05$). Also displayed are the chemical structures of fumonisin B_1 (FB_1), partially hydrolyzed fumonisin B_1 ($pHFB_1a$, $pHFB_1b$), and hydrolyzed fumonisin B_1 (HFB_1) – adapted from Hahn et al. (2015).

has been observed for turkeys (Masching et al., 2016) and different mammalian species (Shephard et al., 1994; Fodor et al., 2008; Hahn et al., 2015), and has been ascribed to the gut microbiota (rather than to the natural hydrolysis of FB in the culture material or in the feed). The difference between $pHFB_1a$ and $pHFB_1b$ is the carbon position of the tricarballylic acid chain left after partial hydrolysis. If the remaining tricarballylic acid chain is attached to C14 of the FB backbone, the compound is called $pHFB_1b$. If it is attached to C15, the compound is called $pHFB_1a$ (as shown in Figure 2, adapted from Hahn et al., 2015). The inclusion of 100 U FUM*zyme*®/kg in the FB-containing diet strongly reduced the exposure of birds to FB in the intestinal tract (Figure 2). FUM*zyme*® decreased the FB content by 3.5 times in the gizzard, by 3.5 times in the proximal part of the SI, by 3.9 times in the distal part of the SI, and by 2.6 times in the excreta. HFB_1 and $pHFB_1$ concentrations increased accordingly. Data from the control birds are not presented in Figure 2, as only traces of FB_1 (less than 0.1 μg/g and 0.3 μg/g FB_1 in the intestinal tract and excreta, respectively) and no HFB_1 or $pHFB_1$ were detected.

Effect of Treatments on The Expression of Genes Related to Inflammatory and Immune Responses in The Jejunum of Birds

The expression of several genes related to inflammation and T cell responses (Th_1, Th_{17}, T_{reg}) was assessed in the jejunum of birds following the 2 wk of exposure to the experimental diets. The ingestion of FB without FUM*zyme*® addition (FB group) resulted in significant up-regulation of mRNAs encoding for interleukin (**IL**)-8 and IL-10 (Figure 3; $p = 0.048$ and 0.029, respectively, compared to the negative control group). Also, higher levels of IL-17 and suppressor of cytokine signaling 1 (**SOCS1**) mRNAs were detected in the FB group compared to the negative control group (Figure 3; *P*-values = 0.194 and 0.045, respectively), but the fold changes were below the cutoff values set at > 2 or < -2. The addition of FUM*zyme*® to the feed (FB+FUM*zyme*®

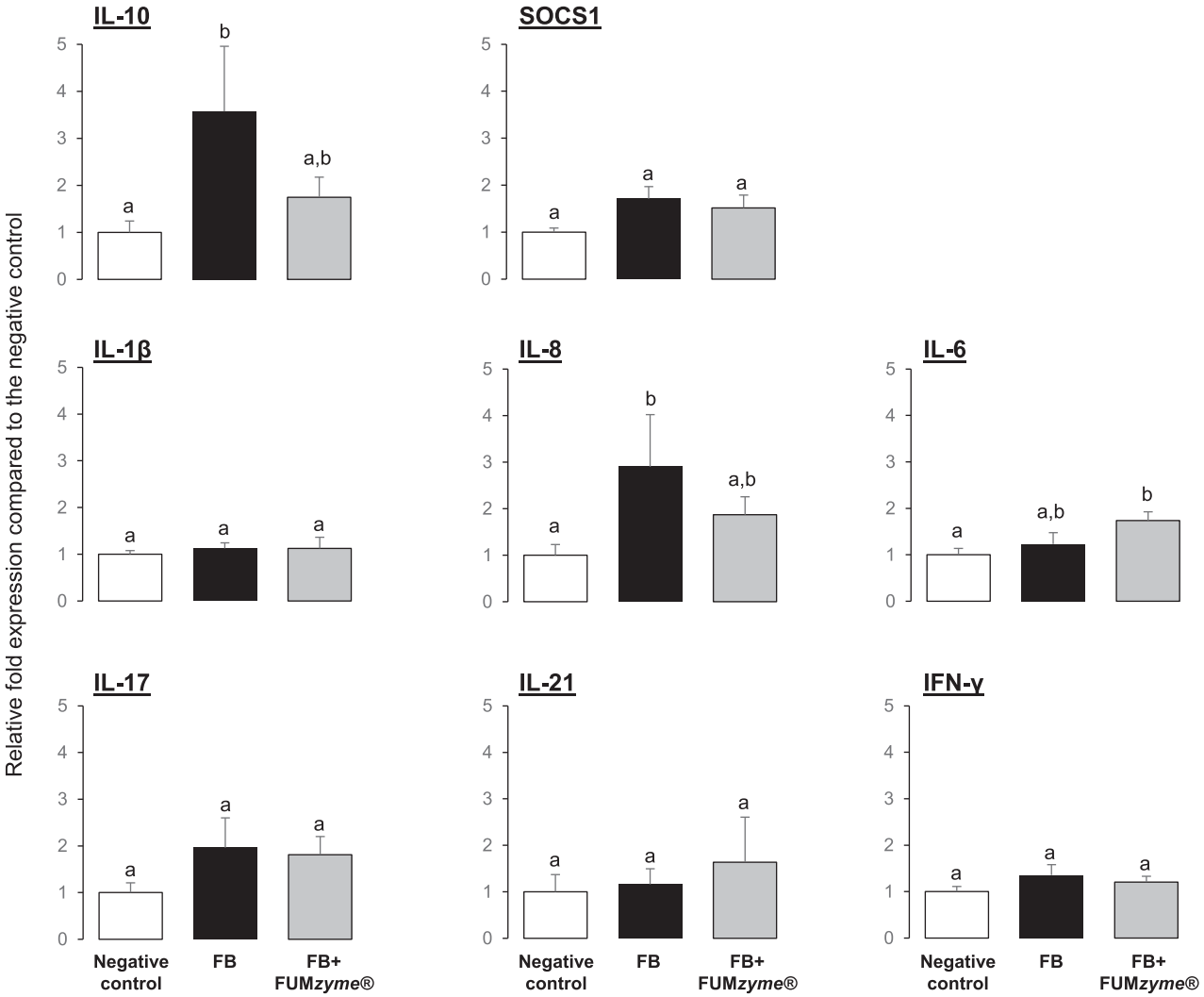


Figure 3. Cytokine gene expression in the jejunum. Values are means ± SEM for 6 replicates (one bird/replicate) for each treatment. Means with no common superscript are significantly different ($P < 0.05$). **IFN-γ**, Interferon-γ; **IL**, Interleukin; **SOCS1**, Suppressor of cytokine signaling 1.

group) enabled a numerical reduction in IL-8 and IL-10 expression compared to the FB group (Figure 3). However, expression of IL-6, and to a lesser extent IL-21, was found slightly increased in the jejunum of birds fed FB+FUM*zyme*® compared to birds of the negative control group (Figure 3; $P = 0.024$ for IL-6 but below the cut off > 2; non-significant for IL-21).

DISCUSSION

The main mechanism of FB toxicity is via disruption of sphingolipid metabolism due to the structural similarity between the 2 sphingoid bases, Sa and So, and FB. The inhibition of ceramide synthase by FB results in accumulation of Sa and to a lesser extent of So. Accumulation of sphingoid bases and the accompanying increase in the Sa/So ratio in tissues following FB exposure has been demonstrated to be a useful biomarker in a variety of mammalian, avian, and piscine species (Voss et al., 2007). Although the Sa/So ratio is considered a biomarker of an effect, it seems to be a more suitable biomarker to evaluate the exposure of animals to FB than the analysis of FB concentrations in biological matrices such as blood, liver, or kidney. This is especially true in avian species in which the very low absorption of FB hampers the detection of the toxins in these matrices (Grenier et al., 2015). As expected, the ingestion of FB in our experiment resulted in a significant increase of the Sa/So ratio in both the serum and liver (Figure 1). This effect was significantly reduced by the addition of FUM*zyme*® to contaminated feed (Figure 1). The decrease of the Sa/So ratio upon FUM*zyme*® addition suggests that the enzyme reduced the exposure to FB. This was confirmed by measuring concentrations of FB and its metabolites in the GIT and in excreta. Although FB_2 is known to be equally or less toxic compared to FB_1 in vivo, we restricted our analysis of FB degradation products to FB_1, which is more prevalent than FB_2 (here 3x as high as FB_2 concentration in the experimental diets), and the most thoroughly studied from a toxicological standpoint (Voss et al., 2007). The detection of only very low

concentrations of $pHFB_1a$ and $pHFB_1b$, and no detection of HFB_1 in the FB group (Figure 2) indicated that FB_1 was hydrolyzed in the GIT with very low efficiency. Therefore, most of the FB_1 hydrolysis that occurred in the GIT of the FB+FUM*zyme*® group animals (Figure 2) can be attributed to the activity of FUM*zyme*®. The proportion of FB_1 left in the GIT after ingestion of the mycotoxin and the enzyme in the FB+FUM*zyme*® group was 37% in the gizzard, 38% in the proximal SI, 25% in the distal SI, and 34% in the excreta compared to the total metabolites (FB_1 + $pHFB_1$ + HFB_1). It appeared that the hydrolysis of FB_1 started already in the gizzard with significant presence of HFB_1 but also of $pHFB_1b$, which is the first metabolite produced by FUM*zyme*®. Full hydrolysis to HFB_1 seemed to follow downstream the gizzard, although partially hydrolyzed forms of FB_1 (re)appeared in the distal SI. This would deserve further investigations on the pharmacokinetic of these metabolites.

The strong reduction of FB exposure in the GIT is of major interest, as this toxin is poorly absorbed and therefore remains in the intestinal tract of birds. Poultry have been long considered very resistant to FB, with concentrations up to 75 to 100 mg/kg not affecting performance in chickens (Ledoux et al., 1992; Henry et al., 2000; Broomhead et al., 2002), which is in accordance with our results on bird performance. However, the effect of FB in the GIT of chickens had never been investigated until very recently (Rauber et al., 2012; Antonissen et al., 2015a; Antonissen et al., 2015b; Grenier et al., 2015; Grenier et al., 2016). The present study shows that even at a dose as low as 10 mg FB/kg, the mucosal immunity of the jejunum might be affected. This is in accordance with our previous report showing local modulation of the intestinal immune response when chickens were exposed to increasing concentrations of FB (Grenier et al., 2015). In the present study, the exposure of the jejunal epithelium to the toxin resulted in higher expression levels of IL-8 and IL-10 mRNAs (Figure 3). By reducing exposure of the gut to FB, FUM*zyme*® reduced the FB induced up-regulation of IL-10 and IL-8, and thereby counteracted a negative effect of dietary FB on gut immunity. However, a slight increase in IL-6 expression (significant but below typical fold change cutoff > 2) also has been observed in this group. These cytokines are characterized by both pro- and anti-inflammatory properties. In pathological conditions, such as chronic intestinal inflammation, these immune mediators along with other cytokines have been shown to be increased. This increase may be related to intestinal permeability impairment, possibly leading to translocation of bacteria. An effect on cytokine production already has been demonstrated for another mycotoxin produced by *Fusarium* species, deoxynivalenol, especially in the pig model in in vitro, ex vivo, and in vivo studies (Cano et al., 2013; Grenier and Applegate, 2013; Pinton and Oswald, 2014). Mycotoxins rarely occur individually in feedstuffs (Streit et al., 2013a; Streit et al., 2013b), and co-occurring mycotoxins might amplify the effect of FB as previously demonstrated in pigs (reviewed by Grenier and Oswald, 2011). Although the toxicity of FB in poultry is still a matter of debate, recent data (including this study) pointed to the susceptibility of birds to metabolic and immunologic disturbances at subclinical doses of FB. Therefore, further investigations are necessary to characterize these effects of FB in more detail. Also, lower dietary FB concentrations should be tested. The level of contamination investigated here is most likely to be encountered in the case of unfavorable weather conditions during crop production, whereas lower contamination levels could be expected in the case of more favorable conditions.

Our experimental design did not include a set of birds on control feed and supplemented with FUM*zyme*®, as safety of FUM*zyme*® already had been established in previous trials. As no adverse effects were observed upon administration of doses up to 30,000 U/kg, the European Food Safety Authority (**EFSA**) concluded that the enzyme is safe for use at a maximum recommended dose of 300 U/kg (EFSA FEEDAP Panel, 2016). Although not reported in poultry, an effect on the Sa/So ratio of the enzyme alone is not expected, as already demonstrated in pigs (Masching et al., 2016). Therefore, the current study provides new information of FUM*zyme*® in poultry, and at dosage below the EFSA recommendation.

In conclusion, the present experiment provides clear evidence that the enzyme used is able to hydrolyze FB in the GIT of chickens, and therefore reduces the exposure to the toxin in both systemic and intestinal tissues. Until now, very few processes have been reported to successfully decontaminate FB-containing feed or detoxify FB within the GIT. Based on the presented results, as well as previous studies in pigs and turkeys (Grenier et al., 2013; Masching et al., 2016), the use of FUM*zyme*® as a feed additive is a promising approach.

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