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Modulation of Porcine β-Defensins 1 and 2 upon Individual and Combined *Fusarium* Toxin Exposure in a Swine Jejunal Epithelial Cell Line

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Defensins are small antimicrobial peptides (AMPs) that play an important role in the innate immune system of mammals. Since the effect of mycotoxin contamination of food and feed on the secretion of intestinal AMPs is poorly understood, the aim of this study was to elucidate the individual and combined effects of four common *Fusarium* toxins, deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), and fumonisin B1 (FB1), on the mRNA expression, protein secretion, and corresponding antimicrobial effects of porcine β-defensins 1 and 2 (pBD-1 and pBD-2) using a porcine jejunal epithelial cell line, IPEC-J2. In general, upregulation of pBD-1 and pBD-2 mRNA expression occurred following exposure to *Fusarium* toxins, individually and in mixtures ($P < 0.05$). However, no significant increase in secreted pBD-1 and pBD-2 protein levels was observed, as measured by enzyme-linked immunosorbent assay (ELISA). Supernatants from IPEC-J2 cells exposed to toxins, singly or in combination, however, possessed significantly less antimicrobial activity against *Escherichia coli* than untreated supernatants. When single toxins and two-toxin combinations were assessed, toxicity effects were shown to be nonadditive (including synergism, potentiation, and antagonism), suggesting interactive toxin effects when cells are exposed to mycotoxin combinations. The results show that *Fusarium* toxins, individually and in mixtures, activate distinct antimicrobial defense mechanisms possessing the potential to alter the intestinal microbiota through diminished antimicrobial effects. Moreover, by evaluating toxin mixtures, this improved understanding of toxin effects will enable more effective risk assessments for common mycotoxin combinations observed in contaminated food and feed.

Antimicrobial peptides (AMPs) are effector molecules of innate immunity with direct antimicrobial activity (1). AMPs can also be mediators of inflammation, influencing a variety of processes, such as proliferation, adaptive immune system regulation, wound repair, cytokine and histamine release, chemotaxis, and protease and antiprotease balance (1–8). To date, hundreds of AMPs identified from vertebrates, invertebrates, plants, and fungi have been characterized, with sequence data deposited in the Antimicrobial Sequences Database (AMSDb), which is publically available (http://www.bbcm.univ.trieste.it/~tossi/amsdb.html) (9).

One of the major AMP subclasses includes the β-defensin family. The members are mainly expressed in epithelial cells of organs exposed to the external environment, such as the skin, gastrointestinal tract, and respiratory tract (10). Dynamic regulation of β-defensins has been shown in various models of gastrointestinal illnesses and inflammation. For example, *Campylobacter jejuni* was shown to induce bactericidal human β-defensins 2 and 3 (hBD-2 and hBD-3) in intestinal epithelial cells (IECs) (11). Reports have also demonstrated that *Salmonella* infection differentially affected the expression of porcine β-defensin 1 (pBD-1) and pBD-2 mRNA transcript levels in porcine ileum and jejunum epithelial cells (10, 12).

Fusarium spp. are commonly recovered from cereals grown in temperate areas of America, Europe, and Asia (13). *Fusarium* toxins elicit a wide spectrum of toxic effects, including the capacity to modify normal immune functions in both humans and animals (14). The most important *Fusarium* toxins potentially affecting mammalian health are zearalenone (ZEA), fumonisin B1 (FB1), and trichothecenes, such as deoxynivalenol (DON) and nivalenol (NIV). In crops and associated food products, the presence of multiple mycotoxin types is commonly observed around the world, making *Fusarium* toxins a serious public health concern. However, investigations on the combined effects of mycotoxins observed in the food supply are scarce. The few studies performed have been based on endpoints that include inhibition of protein and DNA synthesis, DNA methylation and fragmentation, cell viability, and proliferation (15–20). However, the combined effects of mycotoxins on intestinal immunity are currently poorly understood. Previous experiments have shown that exposure to several mycotoxins may increase susceptibility to experimental or natural mucosal infections by inducing bacterial translocation and colonization across the intestinal epithelium (21, 22), but no data are available examining the role mycotoxin combinations may play in intestinal infections. Reports have been done demonstrating that the antimicrobial activity of AMPs, such as β-defensins, may be associated with bacterial populations in the intestine, which naturally present a barrier limiting undesirable gut mucosal infections (23–25). Accordingly, we hypothesized that a mixture of naturally cooccurring *Fusarium* toxins that can be observed in food exert significant effects on the synthesis and secre-

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TABLE 1 Design matrix for four *Fusarium* toxins

Treatment combination no.	DON (0.5 or 2 μM)	NIV (0.5 or 2 μM)	ZEA (10 or 40 μM)	FB1 (20 or 40 μM)
1	0[a]	0	0	0
2	1[b]	0	0	0
3	0	1	0	0
4	0	0	1	0
5	0	0	0	1
6	1	1	0	0
7	1	0	1	0
8	1	0	0	1
9	0	1	1	0
10	0	1	0	1
11	0	0	1	1
12	1	1	1	0
13	1	1	0	1
14	1	0	1	1
15	0	1	1	1
16	1	1	1	1

[a] 1, mycotoxin treatment.
[b] 0, no mycotoxin treatment.

tion of AMPs compared to their effects as individual toxins. Such effects could have a significant influence on microbial survival in the gut. This study used a porcine intestinal epithelial cell line, IPEC-J2 (25), to investigate the individual and interactive effects of DON, NIV, ZEA, and FB1 on the mRNA expression and secretion of pBD-1 and pBD-2 and examined antimicrobial effects observed in toxin-exposed cell supernatants.

MATERIALS AND METHODS

Chemicals and reagents. All mycotoxins (DON, NIV, ZEA, and FB1), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), Luria-Bertani (LB) broth, and agar were obtained from Sigma Chemical Company (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM)–Ham's F-12 (1:1) and fetal bovine serum (FBS) were provided by Gibco-Life Technology (Eggenstein, Germany). RNAiso Plus was purchased from TaKaRa (Dalin, China). SuperScript III First-Strand Synthesis SuperMix was supplied by Invitrogen Life Technologies (Carlsbad, CA). Fast SYBR green master mix was obtained from Applied Biosystems (Foster City, CA). Commercial enzyme-linked immunosorbent assay (ELISA) kits for pBD-1 and pBD-2 were purchased from Uscn Life Science Inc. (Wuhan, China).

Cell line and culture conditions. IPEC-J2 is a nontransformed intestinal cell line originally derived from the jejunums of neonatal, unsuckled piglets (26) and was a kind gift of Per Torp Sangild (Department of Human Nutrition/Clinical Nutrition, Faculty of Life Science, University of Copenhagen, Copenhagen, Denmark). Cells (passages 80 to 92) were maintained in DMEM–Ham's F-12 (1:1) containing high glucose (4.5 g/liter) supplemented with 10% FBS and incubated at 37°C in an atmosphere of 5% CO_2-95% air mixture. All cells were screened for mycoplasma contamination prior to use (Lonza, Basel, Switzerland).

Preliminary mycotoxin concentration response experiment. *Fusarium* toxin (DON, NIV, ZEA, and FB1) concentrations were optimized previously by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (L. Y. M. Wan, P. C. Turner, and E. El-Nezami, submitted for publication). Identified concentrations for toxins, individually and in combination, were used to examine the impact on mRNA expression, secreted-protein levels, and associated antimicrobial effects, as described below.

Identification of mycotoxin combination treatments. To minimize the number of possible toxin combinations (i.e., all possible combinations for every concentration of the respective toxin), an inscribed central composite design was used (27). It incorporated a fractional factorial approach with four factors (i.e., DON, NIV, ZEA, and FB1) and has been used similarly previously (Wan et al. submitted). Through this approach, the number of toxin combination treatments was reduced from 4^4 to 16. The design matrix is presented in Table 1.

qPCR. IPEC-J2 cells were seeded at a density of 5×10^5 CFU/well in 6-well culture plates (Costar, Corning, NY) and allowed to adhere for 24 h. Typically, confluence was achieved after 3 to 4 days, with media being replaced three times weekly; for all experiments, cells were used within 14 days from seeding (26). The cells were washed with PBS and treated with *Fusarium* toxins in serum-free medium for 48 h. Total RNA was extracted using RNAiso Plus according to the manufacturer's instructions. RNA was resuspended in 30 μl of nuclease-free water and stored at −80°C. RNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, Wilmington, DE). Prior to use in quantitative PCR (qPCR), RNA quality was determined by ensuring a value of >1.8 for the A_{260}/A_{280} ratio. cDNA was prepared from 1 μg of total RNA using SuperScript III First-Strand Synthesis SuperMix according to the manufacturer's instructions. qPCR was performed to quantify mRNA transcript levels for pBD-1 and pBD-2 relative to the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous housekeeping control gene. All samples were run on a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) using 1 μl of cDNA and Fast SYBR green master mix, with final primer concentrations of 0.5 μM per primer in a final volume of 20 μl. Porcine specific cytokine primers (Table 2) were generated from published GenBank sequences using Primer Express software (Applied Biosystems). Samples were centrifuged briefly and thermocycled using the default fast program (40 cycles of 95°C for 3 s, 60°C for 30 s). Relative changes in gene expression levels of pBD-1 and pBD-2 in cultured jejunal enterocytes resulting from mycotoxin treatments were normalized against GAPDH using the $2^{-\Delta\Delta CT}$ method as described previously (28). Experiments were repeated four times independently, with each treatment performed in triplicate.

ELISA analysis of pBD-1 and pBD-2. Protein levels of pBD-1 and pBD-2 in cell supernatants were analyzed using a commercial ELISA kit for pBD-1 and pBD-2 according to the manufacturer's instructions. All samples were run in duplicate, with experiments repeated three times independently for each treatment.

Analysis of antibacterial activity. To examine the antibacterial effects of toxin treatments on exposed IPEC-J2 cells, a clinical *Escherichia coli* strain, ATCC 25922, kindly provided by W. C. Yam, Department of Microbiology, University of Hong Kong, was employed. In short, all tests were performed with *E. coli* grown at 37°C in LB broth to an optical density at 600 nm of 0.4. Cells were centrifuged at 2,060 × *g* (Beckman

TABLE 2 Primer sequences for quantification of pBD-1, pBD-2, and GAPDH by qPCR

Primer set	Product length (bp[a])	Primer sequence (5′–3′)		Accession no.
		Forward	Reverse	
pBD-1	141	CTCCTCCTTGTATTCCTCCT	GGTGCCGATCTGTTTCAT	NM_213838
pBD-2	148	GACTGTCTGCCTCCTCTC	GGTCCCTTCAATCTGTTG	NM_214442
GAPDH	120	ATGGTGAAGGTC GGAGTG	GTAGTGGAGGTCAATGAAGG	NM_001206359

[a] bp, base pairs.

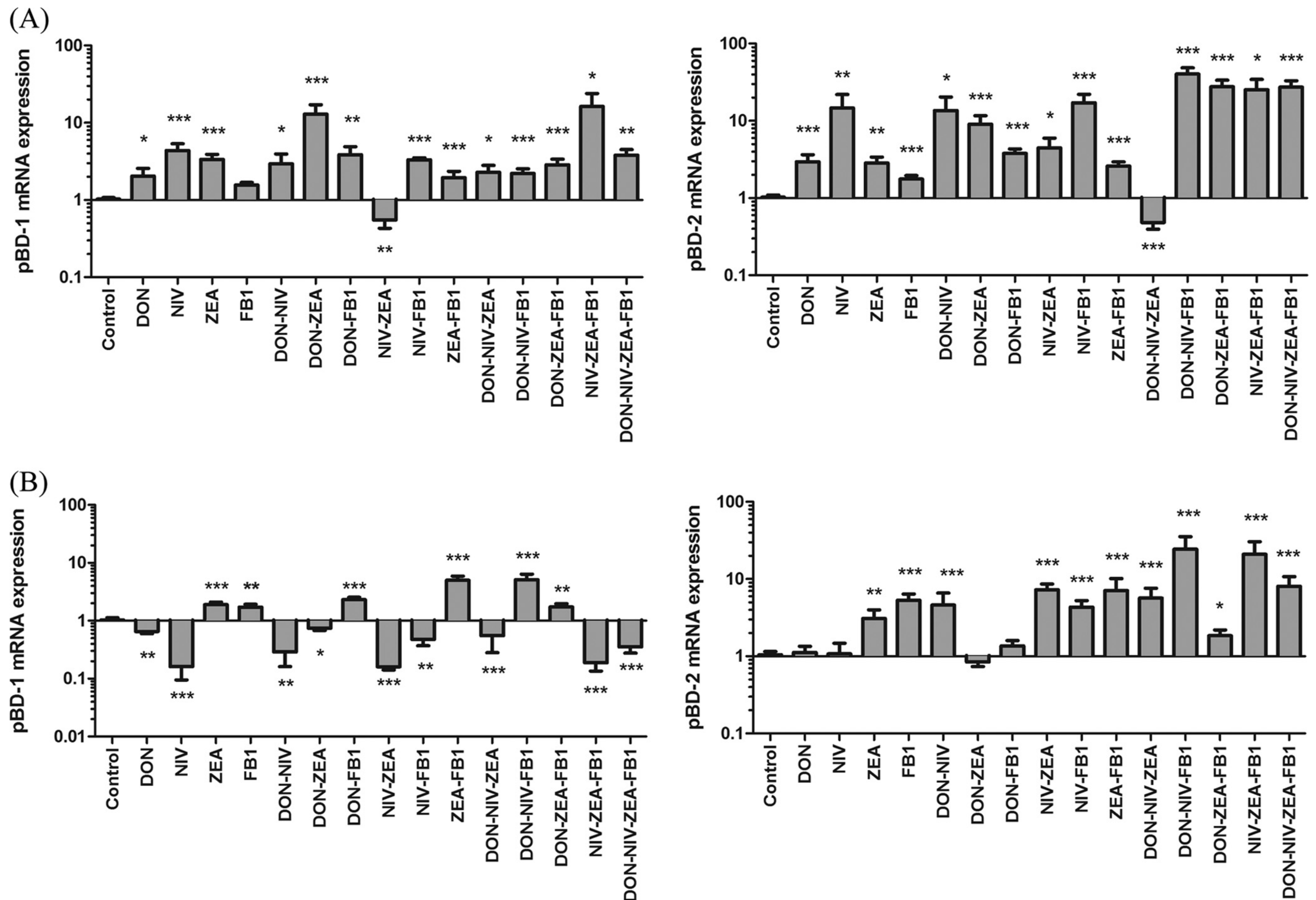

FIG 1 Relative abundances of pBD-1 and pBD-2 mRNAs from porcine IPEC-J2 cells isolated 48 h following treatment, individually or in combination, with cytotoxic (A) and noncytotoxic (B) concentrations of DON, NIV, ZEA, and FB1. The results are expressed as means (±standard errors of the mean [SEM]; $n = 4$) relative to the control; *, **, and ***, $P < 0.05$, 0.01, and 0.001, respectively, compared to the control.

Coulter, Fullerton, CA; GS-6R centrifuge) for 15 min and resuspended in sterile phosphate-buffered saline (PBS) at a final concentration of 1.0×10^7 CFU/ml. A 0.5-ml aliquot was mixed with 0.5 ml of supernatant obtained from IPEC-J2 cells exposed or unexposed to the respective toxin treatments. Following incubation for 2 h at 37°C with shaking (200 rpm), serial dilutions were plated on LB agar, with CFU counted after 18 h at 37°C. Relative changes in CFU following treatment were calculated as (CFU after mycotoxin-treated cell supernatant incubation)/(CFU after control cell supernatant incubation).

Statistical analyses. Results of qPCR and antimicrobial effects were expressed as the mean ± standard error of the mean (SEM) of four individual experiments. All data analyses were performed using the SPSS statistical package (SPSS version 20.0 for Windows; SPSS Inc., Chicago, IL). Data were first evaluated for normality with the Shapiro-Wilk and Levene's variance homogeneity test. The data were not normally distributed for pBD-1 and pBD-2 mRNA and protein levels but were normally distributed for CFU counts. One-way analysis of variance (ANOVA) with the Kruskal-Wallis test, followed by the Mann-Whitney U test, was used to identify significant differences for nonparametric data. One-way ANOVA with Dunnett's multiple-comparison test was used for analyzing parametric data. Differences were considered to be statistically significant when P values were less than 0.05. Differences in pBD-1 and pBD-2 protein levels for three independent experiments were analyzed using a scatter plot.

Univariate analyses of variance were performed to determine if there were any associations between different toxin treatments and bacterial survival. Effects with P values of less than 0.05 at the 95% confidence interval were regarded as significant, suggesting potential interactive effects (either synergistic or less than additive) of different *Fusarium* toxins detected in the bacterial-survival assay, whereas effects with P values of >0.05 at the 95% confidence interval were considered nonsignificant. A lack of interaction indicates the effects are additive (i.e., combined effects would be the sum of their individual effects) (29). Correlations between pBD-1 and pBD-2 gene expression, supernatant protein levels, and antibacterial effects of supernatants of IPEC-J2 cells treated with cytotoxic and noncytotoxic concentrations of DON, NIV, ZEA, and FB1, individually and in mixtures, were assessed by Pearson's (parametric) and Spearman's (nonparametric) correlations.

RESULTS

Individual and combined effects of mycotoxins on pBD-1 and pBD-2 mRNA expression in IPEC-J2 epithelial cells. Levels of mRNA expression of pBD-1 and pBD-2 in IPEC-J2 cells following exposure to DON, NIV, ZEA, or FB1 are presented in Fig. 1. Relative levels of pBD-1 were upregulated in IPEC-J2 cells upon treatment with cytotoxic concentrations (expressed as toxin and concentration in μM, e.g., DON 2 represents DON at 2 μM) of *Fusarium* toxins alone (DON 2, NIV 2, or ZEA 40) or in any combination of DON 2, NIV 2, ZEA 40, and FB1 40, except NIV 2-ZEA 40. When cells were treated with noncytotoxic concentrations of DON 0.5, NIV 0.5, ZEA 10, and FB1 20, relative levels of pBD-1 mRNA were significantly ($P < 0.05$) downregulated in

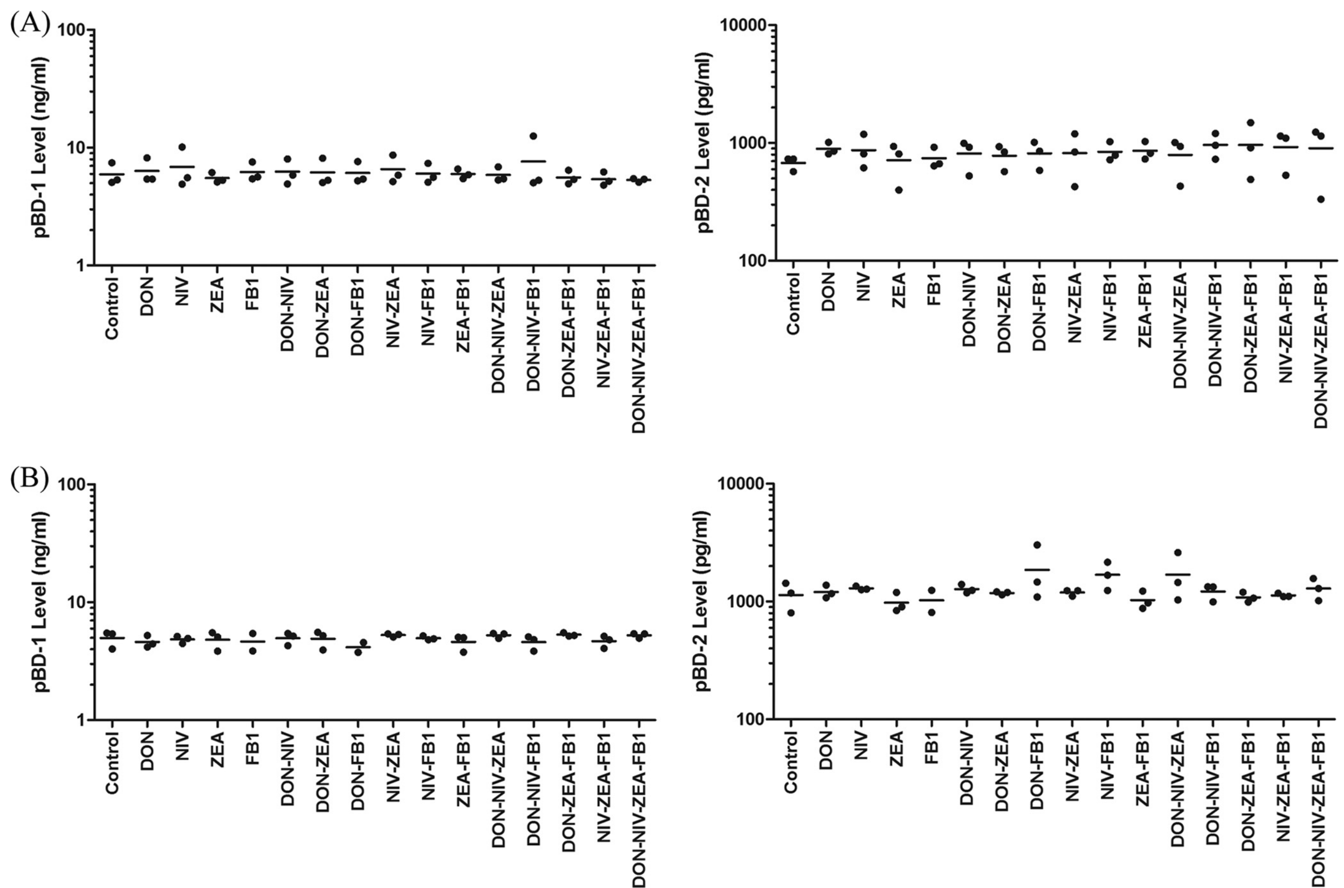

FIG 2 Scatter plots illustrating the cytotoxic (A) and noncytotoxic (B) effects of DON, NIV, ZEA, and FB1, individually and in mixtures, on pBD-1 and pBD-2 levels in cell supernatants as analyzed by ELISA. Means are indicated by the horizontal lines ($n = 3$).

DON 0.5 and NIV 0.5 alone and in mixtures of DON 0.5-NIV 0.5, DON 0.5-ZEA 10, NIV 0.5-ZEA 10, NIV 0.5-FB1 20, DON 0.5-NIV 0.5-ZEA 10, NIV 0.5-ZEA 10-FB1 20, and DON 0.5-NIV 0.5-ZEA 10-FB1 20 but were upregulated in ZEA 10 and FB1 20 alone and mixtures of DON 0.5-FB1 20, ZEA 10-FB1 20, DON 0.5-NIV 0.5-FB1 20, and DON 0.5-ZEA 10-FB1 20.

Similarly, pBD-2 mRNA expression was significantly ($P < 0.05$) upregulated in most of the cytotoxic treatment groups, except for DON 2-NIV 2-ZEA 40 treatment. However, when cells were treated with noncytotoxic concentrations of DON 0.5, NIV 0.5, ZEA 10, and FB1 20, significant upregulation of pBD-2 mRNA expression was observed only in ZEA 10 and FB1 20 alone and in mixtures of DON 0.5-NIV 0.5, NIV 0.5-ZEA 10, NIV 0.5-FB1 20, ZEA 10-FB1 20, DON 0.5-NIV 0.5-ZEA 10, DON 0.5-NIV 0.5-FB1 20, DON 0.5-ZEA 10-FB1 20, NIV 0.5-ZEA 10-FB1 20, and DON 0.5-NIV 0.5-ZEA 10-FB1 20.

ELISA analysis of pBD-1 and pBD-2 protein levels in IPEC-J2 cell culture supernatants. Since the qPCR data revealed changes in mRNA expression of pBD-1 and pBD-2 following exposure to cytotoxic and noncytotoxic concentrations of DON, NIV, ZEA, and FB1, individually and in mixtures, cell supernatants were also measured for their respective protein levels by ELISA. Scatter plots showing the effects of mycotoxins, individually and in mixtures, on pBD-1 and pBD-2 secretion levels are presented in Fig. 2. Differences were not statistically significant for any treatment.

Antibacterial activities of cell culture supernatants. The antibacterial activities of pBD-1 and pBD-2 in cell culture supernatants were evaluated by plate counting. When IPEC-J2 cells were treated with cytotoxic concentrations of DON 2, NIV 2, ZEA 40, and FB1 40, concentrations of *E. coli* were significantly increased ($P < 0.05$) in DON 2, NIV 2, and FB1 40 alone and in mixtures of DON 2-NIV 2, NIV 2-FB1 40, ZEA 40-FB1 40, DON 2-NIV 2-ZEA 40, DON 2-NIV 2-FB1 40, DON 2-ZEA 40-FB1 40, NIV 2-ZEA 40-FB1 40, and DON 2-NIV 2-ZEA 40-FB1 40 (Fig. 3A). However, when cells were treated with noncytotoxic concentrations of DON 0.5, NIV 0.5, ZEA 10, and FB1 20, significant increases in the concentrations of *E. coli* were observed only for NIV alone, NIV 0.5-ZEA 10, DON 0.5-NIV 0.5-FB1 20, NIV 0.5-ZEA 10-FB1 20, and DON 0.5-NIV 0.5-ZEA 10-FB1 20 (Fig. 3B). Such increases in *E. coli* concentrations demonstrated that treated cell supernatants possessed reduced antibacterial activity.

In order to determine if there were any interactions among DON, NIV, ZEA, and FB1 affecting *E. coli* survival, univariate ANOVA was conducted (Table 3). ANOVA provides a powerful statistical tool for tests involving multiple factors and their interactions (30). The results reveal nonadditive interactions in mixtures of NIV 2-FB1 40 ($F_{1,151} = 31.383$; $P < 0.001$), DON 2-NIV 2-FB1 40 ($F_{1,151} = 17.788$; $P < 0.001$), and DON 2-NIV 2-ZEA 40-FB1 40 ($F_{1,151} = 12.432$; $P = 0.001$) (Table 3). No interactions

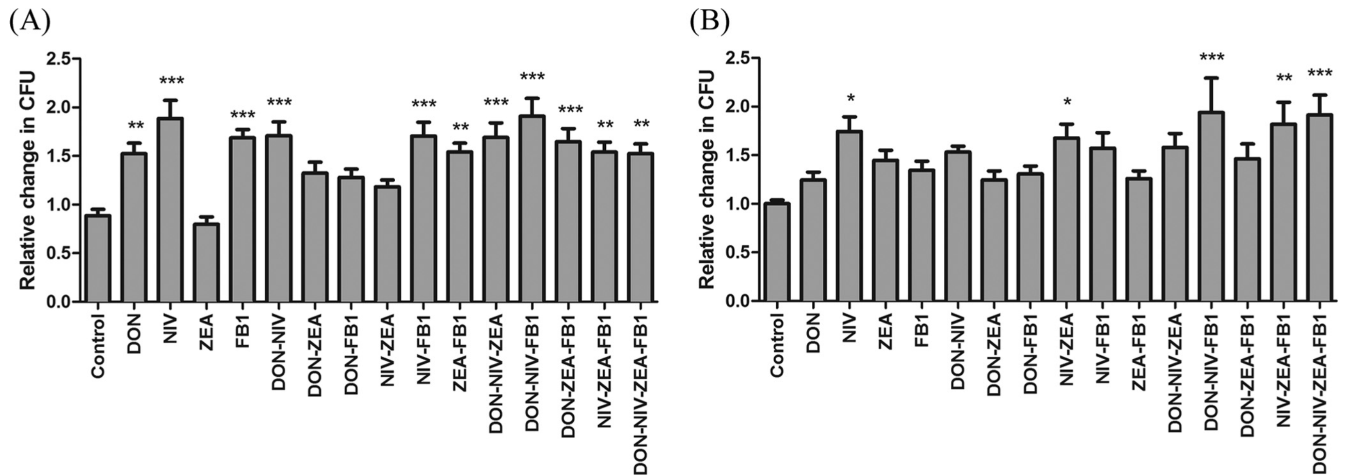

FIG 3 Antibacterial activities of IPEC-J2 cell culture supernatants as determined by numbers of CFU following exposure to cytotoxic (A) and noncytotoxic (B) concentrations of DON, NIV, ZEA, and FB1, individually and in mixtures. The number of *E. coli* CFU in the control was set at 1. The results are expressed as the relative change in the CFU count (±SEM) compared to the control ($n = 4$). *, **, and ***, $P < 0.05$, 0.01, and 0.001, respectively, compared to the control.

were observed for noncytotoxic concentrations of DON, NIV, ZEA, and FB1 (data not shown).

Correlations among pBD-1 and pBD-2 mRNA expression, protein levels, and antibacterial activity. Upon exposure to cytotoxic concentrations of DON, NIV, ZEA, and FB1, significant positive correlations were observed between pBD-1 and pBD-2 mRNA expression levels ($P < 0.001$) as determined by qPCR. Significant negative correlations were revealed between pBD-1 and pBD-2 protein levels ($P < 0.001$). The antibacterial activities of the cell culture supernatants correlated significantly ($P = 0.031$) with pBD-2 mRNA expression. However, when cells were treated with noncytotoxic concentrations, the only significant correlation was between pBD-1 mRNA expression and pBD-2 levels ($P = 0.038$), though the reasons for this are not clear (Table 4).

DISCUSSION

This is the first study to demonstrate the individual and combined effects of four commonly occurring *Fusarium* toxins, DON, NIV, ZEA, and FB1, on the mRNA expression and protein secretion of AMPs pBD-1 and pBD-2. Following treatment with most toxins, mRNA expression was significantly affected for both genes, though cell supernatant levels of pBD-1 and pBD-2 were not significantly changed. In this study, the expression of pBD-1 and pBD-2 was shown to be low and is similar to what has been reported for other cells (10, 24, 31). It has previously been suggested that this basal level of expression may reflect a potential for upregulation of these defensins upon bacterial infection, and upregulation of these defensins has been reported following *Salmonella* infection (10, 24).

TABLE 3 Results of univariate analyses of multiple-*Fusarium*-toxin exposure (cytotoxic) on IPEC-J2 cells as analyzed by surviving CFU

	CFU	
Toxin combination	*F* value	*P* value[a]
DON-NIV	0.506	0.478
DON-ZEA	0.939	0.334
DON-FB1	0.977	0.325
NIV-ZEA	0.029	0.865
NIV-FB1	31.383	**<0.001**
ZEA-FB1	1.874	0.173
DON-NIV-ZEA	3.415	0.067
DON-NIV-FB1	17.788	**<0.001**
DON-ZEA-FB1	0.35	0.555
NIV-ZEA-FB1	1.235	0.268
DON-NIV-ZEA-FB1	12.432	**0.001**

[a] Boldface indicates $P < 0.05$.

pBD-1 is a well-known porcine homologue of hBD-2 (32). Zhang et al. (8) and Elahi et al. (33) previously showed pBD-1 to be constitutively expressed in different tissues (8). However, induction of pBD-1 mRNA expression may also be stimulated upon exposure to food contaminants or enteric pathogens (10, 33, 34) and is in line with our data, which show pBD-1 mRNA expression was upregulated following exposure to DON, NIV, ZEA, and FB1, individually and in mixtures. Elevated expression of pBD-1 in, for example, IECs may play a potential role in surveillance and maintenance of a homeostatic state of microflora on the mucosal epithelium and may be significant in preventing the development or progression of diseases (33, 35, 36).

pBD-2, on the other hand, is a recently discovered and described porcine defensin found in the intestines of pig species and is speculated to be the porcine orthologue of hBD-1 (24, 37). Results show that mRNA expression of pBD-2 was upregulated in IPEC-J2 cells following exposure to DON, NIV, ZEA, and FB1, individually and in mixtures. It has been reported that upregulation of defensins contributes to the early response to bacterial infections, tissue injury, and inflammation (23). Any disturbance of defensin production may lead to the disruption of microbial homeostasis, potentially contributing to chronic enteric diseases, such as Crohn's disease (11, 38) and inflammatory bowel disease (39).

To date, regulation of pBD-1 and pBD-2 levels has been described at the transcriptional level (10, 12, 24, 31), with little data on posttranscriptional regulation available (40). Thus, we investigated the protein levels of pBD-1 and pBD-2 present in cell supernatants and compared them with our transcriptional data obtained by qPCR. For both proteins, no significant increases in

TABLE 4 Correlations among pBD-1 and pBD-2 mRNA expression, protein levels, and antibacterial activity for cytotoxic and noncytotoxic concentrations of *Fusarium* toxins

Parameter at *Fusarium* toxin concn:	Value[a]			
	pBD-1		pBD-2	
	mRNA	Secretion	mRNA	Secretion
Cytotoxic				
pBD-1 secretion	−0.041 (0.787)			
pBD-2 mRNA	0.344 (**<0.001**)	0.130 (0.465)		
pBD-2 secretion	0.003 (0.986)	−0.666 (**<0.001**)	−0.104 (0. 557)	
CFU	0.015 (0.859)	0.138 (0.395)	0.186 (**0.031**)	0.086 (0.597)
Noncytotoxic				
pBD-1 secretion	−0.191 (0.204)			
pBD-2 mRNA	0.003 (0.969)	−0.006 (0. 972)		
pBD-2 secretion	−0.304 (**0.038**)	−0.289 (0.051)	−0.198 (0.202)	
CFU	0.088 (0.259)	0.054 (0.720)	−0.054 (0.489)	0.052 (0.728)

[a] Values are expressed as correlation coefficients, with P values in parentheses (boldface indicates $P < 0.05$).

protein levels were observed under our experimental conditions, which were inconsistent with the significant changes observed with mRNA levels. Discrepancies between mRNA expression, protein abundance, and biological effects have been reported in previous studies (41–43). These differences may be explained, at least partly, by posttranscriptional or posttranslational regulatory mechanisms linked to defensin molecule secretion and are likely influenced by protein degradation pathways (44), though this remains to be shown. Moreover, differences between defensin mRNA and protein secretion may also reveal issues associated with detection thresholds for either mRNA or protein, or secretion of the proteins. Despite advances in the technologies for measuring protein abundance in recent decades, the experimental techniques for protein identification and quantification still lag considerably behind the highly sensitive methods available for quantifying mRNA transcript levels (45). However, while mRNA expression values are useful in various applications, such as classification, identification, and prediction of drug-induced toxicities or cancers (46, 47), the results are correlative rather than causative. It is generally recognized that changes in protein levels, even when subtle (i.e., less than what is deemed statistically significant), may have significant biological effects, and this is currently an area in which analytical techniques with increased sensitivity are required (45).

The results of the antibacterial assay indicate the relevance of defensin expression in gastrointestinal immunity (1). Interestingly, exposure of cells to mycotoxins, individually and/or in combination, resulted in significant reductions in antibacterial activity against *E. coli*, though significant changes in protein levels of pBD-1 and pBD-2 were not observed. It is possible that mycotoxin exposure leads to the production of other antimicrobial metabolites or proteins in cell supernatants that may indirectly contribute to the observed results. Nevertheless, although no significant increases in protein levels for either pBD-1 or pBD-2 were observed, subtle changes may still contribute to and/or reflect dysregulated immunity in the IECs (48), which is supported by the apparent and significant reductions in antibacterial effects associated with toxin-treated cells reported in the current study.

Additionally, in order to determine whether changes in mRNA expression, protein secretion, and/or antibacterial activity may be attributed to cytotoxic effects, noncytotoxic concentrations of DON (0.5 μM), NIV (0.5 μM), ZEA (10 μM), and FB1 (20 μM) were investigated (Wan et al., submitted). The results showed that noncytotoxic concentrations of DON, NIV, ZEA, and FB1 alone or in combination also affected mRNA expression and protein secretion of pBD-1 and pBD-2, as well as antibacterial activity. However, significant upregulation of pBD-1 and pBD-2 mRNA expression and reductions in antibacterial effects were primarily observed in toxin mixtures found to cause cytotoxicity in the MTT assay (Wan et al., submitted). Therefore, it is possible that cytotoxic effects may contribute to the antibacterial activity of IPEC-J2 cells. However, it is currently not clear whether this may have occurred, and if so, what the mechanism(s) may be. Further studies are required to characterize the effects of individual and combined *Fusarium* toxins on the regulation of pBD-1 and pBD-2 protein levels and the associated antibacterial activities.

There is no information in the literature regarding the combined effect of *Fusarium* toxins on antibacterial activity *in vitro*. In this study, significant interactions between treatments in the present analysis would indicate that the addition of more than one mycotoxin has a nonadditive effect, whereas an observed lack of interaction would indicate that effects were additive (29). The results showed that additive effects were noted in most of the toxin mixtures, leading to increases in bacterial survival compared with single treatments of *Fusarium* toxin. A similar phenomenon was reported by Groten et al. (49) and Tajima et al. (51), who observed additive effects on the inhibition of DNA synthesis when L929 fibroblasts were exposed to multiple *Fusarium* toxins (DON, NIV, T-2 toxin, ZEA, and FB1), though some synergistic interactions were also reported (49, 51). Additive effects may occur when there is cooccurrence of more than one mycotoxin causing toxicity through the same mechanism of action (52). In our study, interactive effects, which have not been reported previously, were observed in mixtures containing NIV and FB1, though the reason for such interaction remains unknown. This may indicate that these commonly occurring *Fusarium* toxins, when combined, may interact with each other so that the magnitude of the resulting toxic effects generated may be potentiated or reduced by actions of other toxins (51). Synergistic actions may occur when mycotoxin mixtures act at different stages of the same toxicity pathway or when the presence of one mycotoxin increases the absorption or decreases the metabolic degradation of another (52). Antagonism,

on the other hand, may occur when mycotoxins compete with one another for the same target/receptor site (53). However, the present results with multiple *Fusarium* toxins suggest that *Fusarium* toxins alone may not predict their effects in natural environments, where combinations of toxins are frequently observed, meaning that investigations of commonly observed toxin mixtures are necessary and would enable more accurate and effective risk assessments (54). To understand the nature of these interactive effects, a molecular-level understanding of mycotoxin-mycotoxin interaction is required in the future in order to develop more effective detoxification and remediation strategies aimed at understanding mycotoxin impacts on animal and human health (55).

In summary, gene expression, protein secretion for pBD-1 and pBD-2, and the consequent antimicrobial impact of cell supernatants of IPEC-J2 cells following exposure to DON, NIV, ZEA, and FB1, individually and in mixtures, were determined. A consequent induction of mRNA for both defensins was observed, though this significant transcriptional induction did not correlate with significant changes in the respective secreted protein levels. Regardless, toxin exposure resulted in reduced antibacterial effects observed in cell supernatants, suggesting exposure to mycotoxins may contribute to microbiome changes in the gastrointestinal environment. Based on our data, *Fusarium* toxins, either alone or in combination, potentially activate distinct antimicrobial defense mechanisms, which may alter the intestinal microbiota, potentially leading to imbalances impacting the overall health and well-being of animals and humans (56). However, the precise mechanisms by which mycotoxins elicit these effects remain unclear. Further studies using molecular approaches, such as high-throughput mRNA sequencing and proteomics, will be useful to elucidate the mechanistic pathways involved in understanding the dynamic interplay that occurs between *Fusarium* toxins and IECs (57).

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