A Thesis

for the Degree of Doctor of Philosophy

Hydrolyzed Fumonisin B_1 induces less inflammatory responses than Fumonisin B_1 in the co-culture model of porcine intestinal epithelial and immune cells

장 상피세포와 면역세포 공동 배양 모델에서 Fumonisin B_1과 그 가수 분해 산물에 의한 염증 반응 비교

February 2019

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농학 박사 학위 논문

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이 논문을 농학 박사 학위 논문으로 제출함

2019 년 02 월

서울대학교 대학원

농생명공학부

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한승은의 박사 학위 논문을 인준함

2019 년 02 월

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Summary

Many fungi are able to produce several types of mycotoxins. Among them, deoxynivalenol (DON) is the most prevalent trichothecene mycotoxin, which is produced by *Fusarium* species globally. Fumonisin B_1 (FB_1), mainly produced by *Fusarium verticillioides* and *Fusarium proliferatum*, is also abundant in agricultural commodities. Therefore, both humans and animals are often exposed to the toxic effects of multiple mycotoxins, resulting in subclinical symptoms in the gastrointestinal tract. In the present study, the toxic effects of combined DON and FB_1 exposure in the intestinal epithelium were examined with a co-culture model using a porcine intestinal epithelial cell line (IPEC-J2) and peripheral blood mononuclear cells (PBMCs) from pig blood, with a focus on gut integrity and immune modulation, respectively. First, we confirmed that DON, in the presence of an endotoxin (lipopolysaccharide: LPS), disrupted gut permeability and induced IL-8 production. Furthermore, FB_1 induced additional damage to gut barrier function and promoted pro-inflammatory responses in the presence of LPS and DON compared to only LPS/DON treatment. In the co-culture system, FB_1/LPS/DON induced increased cell death of PBMCs and pro-inflammatory cytokines than LPS/DON treatment. In contrast, the application of hydrolyzed FB_1 (HFB_1), the product of enzyme-degraded FB_1, resulted in reduced levels of chemokines and pro-inflammatory cytokines together with marginal immune cell death compared to FB_1/LPS/DON in the IPEC-J2–PBMC co-culture system. These findings suggest that FB_1 aggravates LPS/DON-induced intestinal inflammation, and hydrolyzation of FB_1 may be considered as an effective strategy to reduce intestinal inflammation in pigs.

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List of Abbreviations

AGPs	Antibiotic growth promoters
Afla	Aflatoxins
APC	Antigen presenting cell
BALT	Bronchus-associated lymphoid tissue
BrdU	5-bromo-2′-deoxyuridine
BMDC	Bone marrow derived-dendritic cell
BW	Body weight
ConA	Concanavalin A
CCL-20	Chemokine (C-C motif) ligand 20
DMEM	Eagle's minimal essential medium
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DOM-1	Deoxynivalenol metabolite
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal regulated kinase
ETEC	Enterotoxigenic *Escherichia coli*
FB_1	Fumonisin B_1
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract

HFB_1	Hydrolyzed fumonisin B_1
IEC	Intestinal epithelial cell
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
LPA	Lymphocyte proliferation assay
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MHC-II	Major histocompatibility complex II
MIP-1β	Macrophage inflammatory protein-1β
MoDC	Monocyte-derived dendritic cells
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIV	Nivalenol
OTA	Ochratoxin A
PBL	Porcine blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PKC	Protein kinase C
PMN	Polymorphonuclear leukocytes
SRBC	Sheep red blood cell
TCA	Tricarballylic acid
TEER	Transepithelial electrical resistance
TGF	Transforming growth factor

TJ	Tight junction
TNF-α	Tumor necrosis factor-α
ZEN	Zearalenone
ZO-1	Zonula occludens-1

I. Literature Review

1. Mycotoxins

1.1. What are mycotoxins?

Mycotoxins are secondary metabolites produced by certain molds as a result of their natural biological metabolism. In general, they are of low molecular weight with low immunogenic capacity (Mallmann and Dilkin, 2007). Even though medically and nutritionally beneficial fungi exist, along with their metabolites such as penicillin, mycotoxins are known to be toxic to crops, and contaminate animal feeds and animal products. Thus, mycotoxin cause poorly identified, yet substantial economic losses, requiring increased awareness and regular analyses.

Among more than 600 mycotoxins that have been fully identified and their toxicity levels tested in livestock, the mycotoxins most relevant to the animal production industry are aflatoxins, trichothecenes (represented by deoxynivalenol (DON) and T-2 toxin), zearalenone, ochratoxin A, fumonisin B_1 (FB_1), and ergot alkaloids. Mycotoxins are invisible, odorless, and tasteless (Task Force Report, Council for Agricultural Science and Technology, 2003). It is important to note that molds do not always produce toxins; they do only under stress and as part of their natural defense mechanisms (Task Force Report, Council for Agricultural Science and Technology, 2003). Most mycotoxins are chemically stable and remain generally intact under high temperature and they can endure for long periods under normal storage conditions (Task Force Report, Council for Agricultural Science and Technology, 2003).

1.2. Mycotoxins and their immune modulation in pigs

Mycotoxins can impair the immune system at several levels. The lymphocyte proliferation assay is a classic method for assessing lymphocyte activity or responsiveness. In general, a mitogen for the induction of mitosis and lymphocyte transformation is used (Mosmann, 1983). Numerous studies have measured cellular immunity and examined the impact of mycotoxins using the lymphocyte proliferation assay. For example, the effect of DON on the proliferation of concanavalin A (ConA)-stimulated porcine blood lymphocytes was evaluated *in vitro* and *in vivo* (Goyarts et al., 2006). A dose-dependent decrease of IC_{50} values based on MTT and BrdU assays in ConA-stimulated porcine blood lymphocytes was observed when pure DON was added to a lymphocyte culture. Monocyte-derived dendritic cells from pigs receiving a control diet or a DON-contaminated diet were treated with DON *ex vitro*, which revealed interrupted phenotypic maturation of the cells (Bimczok et al., 2007).

In piglets fed diets containing purified nivalenol, the number of splenocytes decreased in a dose-dependent manner. Flow cytometric analysis further revealed decreased numbers of both $CD4^+$ and $CD8^+$ T cells in the spleen (Hedman et al., 1997). Furthermore, piglets orally administered FB_1 showed a decrease in the expression and synthesis of interleukin (IL)-8 in the ileum (Bouhet et al., 2006). Consumption of feed contaminated with FB_1 led to a prolonged *Escherichia coli* infection in piglets compared to the control group (Devriendt et al., 2009). Furthermore, enterocytes, neutrophils, and macrophages produced more inflammatory cytokines as a response to damage by FB_1. Interestingly, the production of IL-12p40 (in the ileal Peyer's patches) and IL-6 (in the jejunal lamina propria) was reduced, probably due to the inhibitory effect of FB_1 on dendritic cell maturation. The incubation of FB_1 induced a decrease in the proliferation of swine peripheral blood mononuclear cells (PBMCs) and a significant decrease in IL-2 production in the supernatant of ConA-stimulated PBMCs treated with FB_1 (Marin et al., 2007).

Louis Pasteur was the first to demonstrate that protective immunity against infectious agents could be produced by vaccination. A satisfying vaccination response in pigs is characterized by the production of a certain level of neutralizing antibodies specific to the pathogen. For example, pigs exposed to the T-2 toxin via inhalation showed a reduced antibody titer to sheep red blood cells (Pang et al., 1988). Weaned piglets fed with FB_1 contaminated-feed showed a significant decrease in antigen-specific antibody titer after vaccination against *Mycoplasma agalactiae* (Marin et al., 2006). A reduction in cytokines, including IL-10, IL-6, and IL-4, was anticipated to reduce the antigen-specific B cell responses, since they are important soluble regulators in different processes of the immune system, including activation and differentiation of B lymphocytes and synthesis of antibodies.

1.3. Mycotoxins and their toxic effects in the intestine

Not all mycotoxins are absorbed in the gastrointestinal tract (GIT) to the same degree due to their differing molecular weights and chemical structures (Grenier and Applegate, 2013). For example, aflatoxins, ochratoxin A, and DON (55–85%) are absorbed much more rapidly in the GIT than fumonisins (FUMs) (3–6%) in the proximal section of the GIT in pigs. However, due to enterohepatic circulation, there is a high possibility that FUMs remain in circulation along the GIT much longer than other mycotoxins (Grenier et al., 2013). When intestinal epithelium is exposed to mycotoxins, mycotoxin-induced damage to intestinal epithelial cells (IECs) prevents the normal cross-talk between IECs and the underlying immune cells. Eubiosis, describing the state of a well-balanced gut microbiome, is instrumental to intestinal health, and mycotoxins can directly impair the proliferation of commensal bacteria or modulate gut-associated lymphoid tissue (GALT) and its immune responses, triggering an imbalance in eubiosis (Rescigno, 2011). For instance, T-2 toxin increased the aerobic intestinal bacteria count in *in vivo* trials in piglets (Tenk et al., 1982),

and low doses of DON also increased the number of aerobic intestinal bacteria in pigs (Wache et al., 2009). It has been suggested that FB_1 is also involved in the T-helper 1/T-helper 2 (Th1/Th2) cytokine balance in pigs as a mediator of mucosal immunity (Marin et al., 2006; Taranu et al., 2005). *Ex vivo* experiments conducted by Liu et al. (2002) demonstrated that FB_1 reduced viable cell counts and induced cell death in swine alveolar macrophages. Taken together, this shows that DON and FB_1 can influence intestinal health by modulating consequent immune responses.

1.4. Mycotoxins and toxicological interactions

Molds can produce several mycotoxins simultaneously and in the animal feed industry, multiple raw feed materials are often mixed into compound feeds. In many cases, as many as ten mycotoxins have been detected in crop samples, depending on the analysis methods and case (Streit et al., 2012). The importance of understanding the toxicological interactions among co-occurring mycotoxins in raw feed materials and compound feeds has been highlighted from a practical viewpoint. Two experiments, performed in pigs, investigated the combined effects of DON and FB_1, the most frequently detected *Fusarium* mycotoxins (Grenier et al., 2011; Harvey et al., 1996). However, the effects on body weight gain differed among the experiments, where a synergistic interaction was reported by Harvey et al. (1996), but no change was observed by Grenier et al. (2011) regardless of the toxins present in the diet. The immune response was also evaluated following exposure to both DON and FB_1, and the effect of FB_1 together with DON resulted in an increased reduction in lymphocyte proliferation upon mitogenic stimulation compared to FB_1 alone (Harvey et al., 1996).

2. Host response to mycotoxins

2.1. Intestinal epithelial cells and mycotoxins

The primary function of the GIT is nutrient absorption, yet it also acts as a barrier against harmful materials and microbes, preventing them from entering the bloodstream (Celi et al., 2017). The GIT mucosa is made up of the epithelium, lamina propria, and muscular mucosae, of which the epithelial layer is crucial to the barrier function of the GIT. However, the functions of the intestinal epithelium are contradictory, as it must be able to discriminate between nutrients and harmful agents such as mycotoxins, endotoxins, and pathogenic bacteria (Celi et al., 2017)

Trichothecenes activate MAPK p44/42, given that DON binds to the ribosome and subsequently transduces the signals for a series of protein kinases leading to translational disruption of protein synthesis (Springler et al., 2016). Trichothecenes, acting as strong protein inhibitors, target high-turnover cells, represented by the intestinal epithelial cells and intestinal immune cells in the lamina propria, through oxidative stress-induced DNA damage and apoptosis (Pestka et al., 2004; Wu et al., 2014; Yang et al., 2017). Typically, villi became shorter when pigs were fed with feed contaminated with trichothecenes (Alizadeh et al., 2015). Furthermore, the hepatic and intestinal mRNA expression of proinflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor (TNF)-α was elevated in DON-fed broiler chickens (Osselaere et al., 2013), indicating that inflammation plays a crucial role in altering the integrity of the gut. The production of mucin, which is an important element of gut protection, was also reduced upon stimulation with DON (Pinton et al., 2014).

Sphingolipids are possibly related to neurological and immunological diseases and cancers. The enzymes sphingomyelinase and ceramidase are required to degrade sphingolipids into

ceramide, which is essential for lipid metabolism (Boini et al., 2017). FB_1 is known to interrupt sphingolipid metabolism by inhibiting synthesis of ceramide synthase and as a result, sphingosine accumulates in cells (Mashing et al., 2016). FB_1 is responsible for higher sphingosine levels in the cells found in urine, serum, kidney, liver and the small intestine, and it possibly induces cytotoxicity and cell death (Hahn et al., 2015). For instance, FB_1 induced cell death and inhibited cell proliferation in a concentration-dependent manner in intestinal porcine epithelial cell (IPEC)-1, Caco-2, and HT29 cell lines (Minervini et al., 2014). The accumulation of sphinganine in cells upon FB_1 exposure likely underlies its cytotoxicity. It has also been suggested that sphinganine accumulation in IECs blocks the G0/G1 phase, resulting in apoptosis (Espaillat et al., 2015).

2.2. Effect of mycotoxins on intestinal immune responses

The gut represents the largest immune organ in the body, as ~70% of immune-related cells reside in the GIT. Fully developed GALT in poultry consists of the Peyer's patches, mesenteric lymph nodes, and cecal tonsils. Immune cells are in a constant state of readiness to generate efficient, rapid, and appropriate immune responses. Moreover, IECs can form part of the defense mechanism with their barrier function preventing harmful agents and antigens from entering the bloodstream. The GIT is a common route for vaccinations in pigs because both infections resulting from pathogens and delivery of vaccines can activate immune cells in the GIT. It has been suggested that chronic contamination of feed by multiple mycotoxins, but especially *Fusarium* mycotoxins, poses a great risk in terms of a higher translocation of pathogens and harmful toxins, thus increasing susceptibility to enteric diseases (Grenier and Applegate, 2013).

2.3. Mycotoxins act on the integrity of the intestinal barrier

The barrier function of IECs is vital to maintaining gut health and protecting against food- or feed-borne diseases. There are two major pathways in IECs that facilitate the transport of molecules; the transcellular pathway for nutrient absorption and the paracellular pathway that maintains the barrier function of the IEC (**Figure 1**). The apical surface consists of microvilli and the basolateral surface faces the lamina propria. Between IECs and along the paracellular pathway, there exist tight junctions (TJs) that act like glue between cells, and any damage to the TJs can lead to compromised gut integrity. After differentiation, the IEC monolayer becomes a vital part of the gut integrity, playing a crucial role as the first line of defense against antigens, toxins, and pathogens. Mycotoxins can damage the gut integrity by inhibiting cell proliferation of IECs and TJ proteins (**Table 1**).

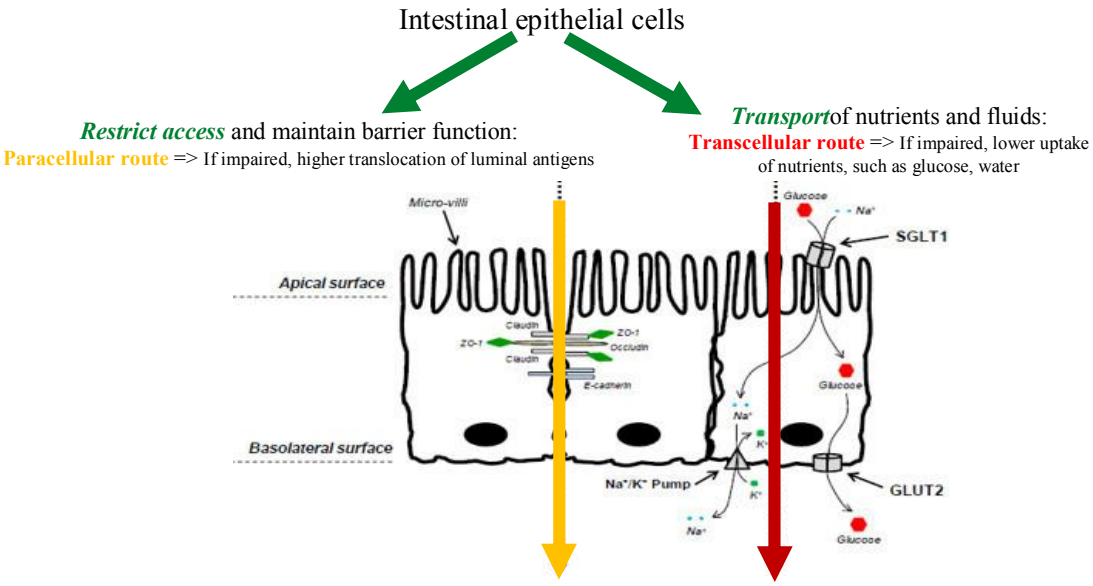


Figure 1. IECs – transcellular and paracellular routes in IECs.

Table 1. Mycotoxin-induced damage in intestinal barrier function in *in vitro* and *ex vivo* experiments.

	Transepithelial electrical resistance	Tight Junction Proteins
DON	***IPEC-1***: reduced TEER (Pinton et al., 2009; Pinton et al., 2010; Pinton et al., 2012)	***IPEC-J2***: reduced expression of ZO-1 and claudin 3 (Diesing et al., 2011; Gu et al., 2016)
	IPEC-2: reduced TEER (Diesing et al., 2011)	***IPEC-1***: reduced expression of ZO-1 (Diesing et al., 2011)
	Caco-2: reduced TEER (Van de Walle et al., 2010)	***Caco-2***: reduced expression of claudin 4 but not occludin (Van de Walle et al., 2010)
		IPEC-1: reduced expression of claudin 4 (Pinton et al., 2009; Pinton et al., 2010; Pinton et al., 2012) & but not ZO-1 and occludin (Pinton et al., 2009)
		Ex-vivo out of pig: reduced expression of claudin 4 in jejunum (Pinton et al., 2009), occludin & E-cadherin in ileum (Bracarense et al., 2012***)***
FB	***IPEC-1***: reduced TEER (Bouhet et al., 2004)	***Ex-vivo from Pig***: reduced expression of occludin & E-cadherin in ileum (Bracarense et al., 2012)

The ZO-1, occludin, and claudin families of proteins are representative TJ proteins. TJ proteins connect the luminal ends of cells and screen transport via the paracellular pathway. ZO-1 is a platform that organizes porous TJ proteins and recruits various signaling molecules to TJ proteins. Occludin regulates permeability through the TJ system after it binds to ZO-1 and the actin cytoskeleton (McLaughlin at al., 2004). The molecular mode of action underlying the cytotoxicity of DON is the inhibition of protein synthesis in actively dividing cells via MAPK signaling (Van de Walle at al., 2010); therefore, the reduced expression of claudin 4 must be related to protein inhibition by DON. Van de Walle et al. (2010) experimented using an inhibitor of protein degradation to confirm whether protein degradation was the cause of the reduction in claudin 4 expression. The results showed that the reduction in claudin 4 expression did not stem from protein degradation, as claudin 4

expression was not recovered through the use of the protein degradation inhibitor. Gu et al. (2016) examined changes in the expression of TJ proteins in IPEC-J2 cells treated with DON, which resulted in decreased claudin 3 and ZO-1 expression.

IPEC-1 cells treated with FB_1 exhibited reduced transepithelial electrical resistance (Bouhet at al., 2004). Another study conducted in piglets challenged with a low dose of FB_1 indicated that a dose of 10 μM of FB_1 reduced expression of E-cadherins and occludin (Bracarense et al., 2011). It has been speculated that FB_1 acts as a mediator of gut integrity because it is closely related to the metabolism of sphingolipids (Loiseau et al., 2007). However, the exact mechanisms underlying the role of FB_1 in weakening gut integrity remain unclear. When FB_1 inhibits the synthesis of ceramide synthase, free sphinganine increases and complex sphingolipids and ceramide decrease, which subsequently impacts collagen formation in the TJs.

It was speculated that mycotoxin-induced increases in intestinal permeability and inhibition of sodium ion-dependent glucose transport may have caused diarrhea in pigs fed with mycotoxins (Pinton et al., 2010). The percentage of absorption of FB_1 in the upper part of the GIT is only 3–6% in pigs (Pinton et al., 2012). However, driven by enterohepatic circulation, FB_1 is circulated within the GIT much longer than other mycotoxins, and if the gut integrity is damaged by mycotoxins, the chance of FB_1 entering the bloodstream under the lamina may increase. Supporting this, the cytotoxicity of FB_1 in the GIT was greater in co-contamination with DON, showing synergistic effects on several parameters, such as the necrotic enteritis lesion score in the gut and other enteric disease-related symptoms *in vivo* (Bracarense et al., 2011; Grenier et al., 2011).

3. Detoxification of mycotoxins

3.1. Prevention methods

Many *Fusarium* species can infect cereal grains. Among them, *Fusarium graminearum* is the major pathogenic fungi responsible for red ear disease in maize. *Fusarium* species infect cereals in two ways. First, the spores infect the silks during emergence and second, under stress (e.g., dramatic weather changes, extreme high or low humidity, and attack from birds or insects), spores already present can damage the grain kernels before maturation (Reid et al., 1999). However, there are methods to prevent some of the damage, including crop rotation, plant variety, tillage procedure, reducing plant stress, and timely harvesting, which are based on addressing predisposing factors of infection (Jouany, 2007). However, even if all preventive measures are taken, there is no way to completely avoid mycotoxin infection, since many are also produced during storage and processing after harvest. Thus, during storage, precautions are taken to avoid fungal growth and further mycotoxin production (**Table 2**).

Table 2. Factors which influence mycotoxin occurrence during storage and preventive measures.

Factor	Mode of Interaction	Preventive measures
Moisture and temperature	The interaction between moisture level and temperature is the most important physico-chemical factor affecting preservation of commodities and feeds during storage	The facilities should be dry and clean. The temperature difference shouldn't be too much
Aeration	Mold growth in grains usually occurs heterogeneously, therefore, the development of "hot spots" (areas in which the concentration of mycotoxins in higher) is common	To aerate the area properly and try to maintain the uniform temperature
Sanitation	Fungal development is likely to occur at several points of storage to feeding pathway	Cleaning of equipment on a regular basis

Pests	Metabolic activity of insect and arthropods casues an increase of both moisture content and temperature of infested material	To use good housekeeping measures

3.2. Elimination of mycotoxins

In general, prevention methods employed during crop growth, harvesting, and storage can only decrease the potential risk of mycotoxin contamination to a certain degree. Therefore, detoxification procedures after harvest are a key area of focus. The elimination of mycotoxins in grains and feed occurs via three methods: physical, chemical, and biological processes in the grains, in the feed, and inside the bodies of animals. However, it is important that such measures do not compromise the nutritional value and acceptability of the feed (Rodrigues et al., 2009). Furthermore, any byproducts and metabolites resulting from any processes must be safe enough to be included in animal diets. Many of the physical processes described below have been used to decrease mycotoxin contamination of commodities. The efficacies of these processes depend on the levels of mycotoxin contamination and distribution in the grains and feed. However, these methods are typically expensive and as such, are often not economically feasible (**Table 3**).

Table 3. Physical processes are limited to eliminate mycotoxins.

Method	Description
Cleaning	The broken kernels should be removed in a timely manner
Sorting and separation	The clean product should be separated from mycotoxin-contaminated grains
Washing	Washing with water or sodium carbonate solution can wash off some mycotoxins
Heat treatment	Heat treatment works for elimination of fungi only up to a certain degree
Irradiation	Some experiments have been carried out (Ritieni et al., 1999; Kottapalli et al., 2003; Aziz and Moussa, 2004)

Various types of acids, aldehydes, bisulfites, and oxidizing agents have been tested for their abilities to eliminate mycotoxins. However, only a few chemical methods are effective and many have disadvantages, such as toxic byproducts or effects on the nutritional value and flavor of grains and feed (Rodrigues et al., 2009). For instance, ammonia has been used to treat contaminated feed, but many countries have banned its use in feed treatment, because it can produce toxic compounds during the procedure. Therefore, only a limited number of countries allow its use in treating aflatoxin-contaminated feed and food (Dakovic et al., 2005).

3.3. Biological methods

3.3.1. Adsorption

Adsorbent materials are commonly used in feed as additives to counteract mycotoxins by binding them inside the animals, which then excrete them in feces. Among the major mycotoxins, aflatoxins have a polar functional group with a planar structure that is well suited for adsorption. However, mycotoxins of a larger molecular size, with fewer polar functional groups and a three-dimensional structure are not readily bound by adsorbents. *Fusarium* mycotoxins mostly fall into the latter category, and binding or adsorption methods are not the optimal solutions to eliminate them from the body of animals (Dakovic et al., 2005). Substances scientifically investigated as potential mycotoxin-binding agents include bentonites, zeolites, organophilic clays, activated charcoal, and yeast cell walls. The most well-established adsorbents are clay minerals, organophilic clays, yeast cell walls, and activated charcoals (Dakovic et al., 2005).

3.3.2. Biotransformation

Many studies have noted that adsorption strategies are only effective against aflatoxins and *Fusarium* mycotoxins and cannot be used to effectively manage trichothecenes (e.g., DON and T-2 toxin) or FUMs (Avantaggiato et al., 2005; Huwig et al., 2001). Therefore, research is underway to develop alternative methods to eliminate feed-borne mycotoxins, especially *Fusarium* mycotoxins.

Biotransformation may represent the best alternative to adsorption for non-adsorbable mycotoxins, where the toxic component of a molecule is biodegraded using microbes or enzymes to form non-toxic metabolites. As with adsorption, biotransformation can take place in the GIT of animals (Rodrigues et al., 2009). The concept of biotransformation dates to the 1960s, when the first aflatoxin-degrading bacterial strain was identified (Ciegler et al., 1966). Since then, many other microorganisms with detoxification capabilities have been discovered (Varga et al., 2000; Wegst and Lingens, 1983; Yoshizawa at al., 1983).

The first experiments showing the degradation of DON into de-epoxy-deoxynivalenol (DOM-1) were performed by Yoshizawa et al. (1983). Since then, many researchers have conducted *in vitro* trichothecene transformation experiments with gut microflora, biodegrading DON into DOM-1 (He at al., 1992; King et al., 1984; Kollarczik et al., 1994; Swanson et al., 1987). Nonetheless, the culture of a pure DON-biotransforming strain has not been successful. It proved particularly difficult because of the variation of the medium, which consists of different energy sources, minerals, and antibiotics, and subsequent sub-cultivation in active enriched cultures. Schatzmayr et al. (2006) were the first to isolate a pure eubacterium, BBSH797, able to degrade DON into DOM-1 (**Figure 2**). The toxicity of the

compound formed by the biotransformation of DON into DOM-1 was tested using a chicken lymphocyte proliferation assay. At a concentration of 0.15 µg DON/mL the proliferation of lymphocytes was lower compared to the control. After adding 0.3 µg DON/mL to the cells, only one third was able to proliferate, whereas at a concentration of 0.63 µg DON/mL, lymphocyte growth was completely inhibited. In the case of DOM-1, 116 µg DOM-1/mL was required to inhibit proliferation of lymphocytes completely (Schatzmayr et al., 2006).

For the biotransformation of FUMs, *Sphingopyxis* MTA 144 was isolated from soil, which biodegraded FB_1 into the non-toxic metabolite 2-keto-HFB_1 (Hartinger et al., 2011). Two genes, *fumD* and *fumI*, were first isolated from *Sphingopyxis* MTA 144 by Heinl et al. (2010) to produce a purified enzyme to biotransform FB_1 into non-toxic metabolites in food and animals.

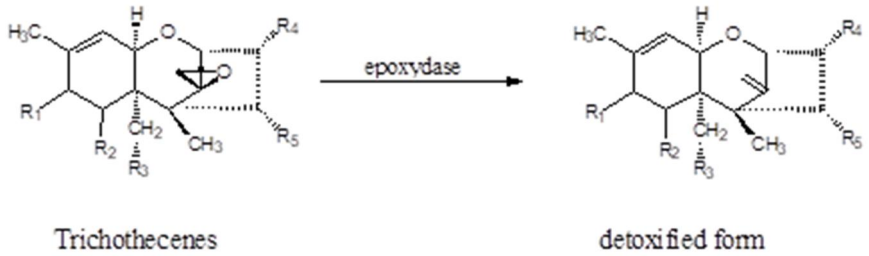


Figure 2. Trichothecenes molecular scheme before (left) and after being detoxification (right).

Subsequently, Hartinger and Moll (2011) produced the purified esterase FUM*zyme*®, encoded from MTA144, which enabled the bacterial strain to degrade FB_1 into the hydrolyzed FB_1 metabolite (HFB_1) (**Figure 3**). HFB_1 does not seem to cause intestinal or hepatic toxicity in animal models. The sphinganine to sphingosine ratio measured in serum, liver, and fecal samples indicated reduced disruption of sphingolipid metabolism when

FUM*zyme*® was used in the treatment diet (Grenier at al., 2012). To conclude, the use of fumonisin esterase in feed can be considered as an effective strategy to eliminate the toxic effects of FB_1 in animals.

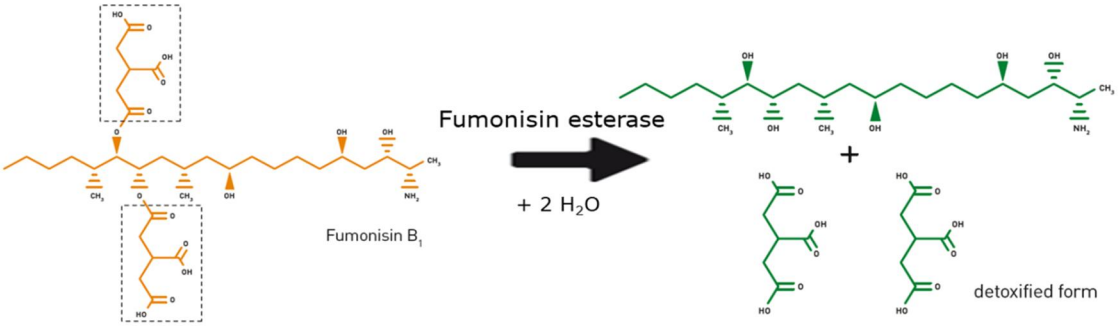


Figure 3. Microbial degradation of Fumonisin B_1.

II. Introduction

Mycotoxins are secondary metabolites of fungi that contaminate food and animal feed (Mallmann and Dilkin, 2007). *Fusarium* toxins are one of the most prevalent mycotoxins and they are harmful to animal productivity resulting in great economic damage for animal production (Grenier et al., 2013; Reddy et al., 2018). The most important *Fusarium* mycotoxins, in a toxicological sense, are trichothecenes (represented by DON and T-2 toxin), ZEN and FB_1 (Nesic et al., 2014). Trichothecenes especially DON bind to ribosome to inhibit translation of high turn-over cells such as IECs, TJ proteins and immune cells (Ehrlich et al., 1987). FB_1 has been studied for its adverse effects on the function of the gastrointestinal tract in animals. Ingestion of FB_1 induced an increase of heat shock proteins in the gastrointestinal tract (Lalles et al., 2010) and modulated intestinal microbial homeostasis (Antonissen et al., 2015; Dang et al., 2017). It has been demonstrated that FB_1 disrupts barrier function (Lalles et al., 2009; Bouhet et al., 2004) and chemokine expression (Bouhet et al, 2004) in intestinal epithelial cells and gut tissues. Fumonisin carboxylesterases catalyze the conversion of FB_1 to hydrolyzed fumonisin B_1 (HFB_1), a less toxic metabolite, which is one of the strategies to reduce fumonisin exposure in the animal feed industry (Masching et al., 2016). Dietary HFB_1 in animal feed showed a low intestinal toxicity with a minimal impairment of intestinal morphology and low inflammatory cytokines when compared to FB_1 in piglets and broilers (Grenier et al., 2012; Grenier et al., 2017). However,

the effect of HFB_1 on IECs and the gut immune system has not been investigated yet.

The gastrointestinal tract is chronically exposed to foreign antigens including microorganisms and toxic molecules. IECs provide the first line of host defense in the intestine. As a barrier function, paracellular and transcellular transit of molecules in the intestine is modulated by a complex network of TJ and gap junction proteins, linking IECs (Suzuki et al., 2013). The increased epithelial permeability of TJs can initiate and often maintain persistent inflammation in intestinal inflammatory diseases. Moreover, IECs keep close communication with immune cells in the lamina propria that regulate the gastrointestinal immune responses (Rescigno et al., 2011). IECs act as modulators of the mucosal immune response by recruiting and activating underlying immune cells via chemokine production (Iliev et al., 2006; Oswald et al., 2006). The dysfunction of epithelium can disrupt the homeostasis of intestinal immune system, leading to acute and chronic gut inflammation. Therefore, impact of mycotoxins on IECs and gut immune system must be further defined for its action mechanism at the cellular and molecular level.

In current study, a trans-well co-culture system using porcine intestinal epithelial cells and peripheral blood mononuclear cells was established to investigate the effect of damaged epithelium on intestinal immunity at basolateral side (Gu et al., 2016). It is well known that feed contamination of FB_1 often co-occurs with other toxins such as DON and T-2 (Streit et al., 2012). Furthermore, LPS, an endotoxin that is a major cell wall component of gram-negative bacteria including *E. Coli* and *Salmonella*, is present in the animal farm environment and often contaminated in the animal feed. Co-exposure to LPS and DON induced an additive or synergistic effect on porcine gut immune system through altering the barrier function and intestinal immune responses (Klunker et al., 2013; Halawa et al., 2012). Thus, it was hypothesized that co-exposure to FB_1 might amplify the toxic effect on epithelial cells and HFB_1 may be less toxic to the intestinal immune system. In the present study, it was

aimed to evaluate whether FB_1 and HFB_1 exhibit different effects on barrier function on IPEC-J2 in the presence of LPS and DON and also intestinal immune response in the presence of LPS and DON using the co-culture system with IPEC-J2 and PBMC.

III. Materials and method

Cell culture

Non-transformed IPEC-J2 cell line (ACC701; DSMZ, Braunschweig, Germany) was cultured in Dulbecco's modified Eagle medium (DMEM:Ham's F-12 [1:1]) (Gibco Life Technologies, Grand Island, USA) supplemented with 5% fetal bovine serum (FBS), 1% insulin-transferrin-selenium-X (ITS-X) and 1% antibiotics (all from Invitrogen, Grand Island, USA) in an incubator with atmosphere of 5% CO_2 at 39 °C. During growth and differentiation of the cells, the medium was replaced every three days.

Mycotoxin and endotoxin treatment

DON and LPS (from *E. coli* O55:B5) were purchased from Sigma-Aldrich (Missouri, USA). FB_1 and HFB_1 were provided from Biopure (Romer Labs®, Tulln, Austria). IPEC-J2 were treated with various concentrations (0-100 μM) of FB_1 or HFB_1 in the absence or presence of LPS (10 μg/mL) and DON (5 μM).

Measurement of transepithelial electrical resistance

5 x 10^4 cells/ml of IPEC-J2 were seeded in 500 μl of DMEM media in 1.12 cm^2 polyester membrane inserts with 0.4 μm pores (Corning, New York, USA) and the basolateral side was filled with 1 ml of DMEM media. During the cell growth and differentiation, the medium in both compartments was replaced three times per week. After FB_1 or HFB_1 treatment, the transepithelial electrical resistance (TEER) was measured with epithelial volt ohm meter (EVOM2; World Precision Instruments, Sarasora, USA) for 1-10 days.

Western Blot Analysis

IPEC-J2 were lysed in lysis buffer (20 mM Tris-HCl, 150 mMNaCl, 1mM EDTA, 1% Triton X-100) followed by a quantitation of protein using Micro BCA kit (Thermo, Rockford, USA). The same amount of protein extracts was loaded in 10% Tris-glycine polyacrylamide gels and electrophoresed. Then, the protein was transferred onto a polyvinylidene difluoride (PVDF) microporous membrane for 2 h at 4°C and blocked with 5% skim milk in TBS-T (20mMTrisHCl, 100mMNaCl, 0.05% Tween 20) for 90 min. The blot was incubated with rabbit anti-claudin-3, -occludin and -ZO-1 antibodies (Invitrogen) or mouse anti-β-actin monoclonal IgG1 antibody (Santa Cruz Biotechnology, Grand Island, USA) overnight. Subsequently, the membrane was washed and incubated with goat anti-rabbit and -mouse IgG-HRP (Santa Cruz Biotechnology) (diluted at 1: 10,000) for 1h. The target protein was visualized by enhanced chemiluminescence (ECL) system (GE Healthcare, Waukesha, USA) followed by analysis using ChemiDoc XRS (Bio-Rad, Hercules, USA). Intensity of the blotting was quantified using Multi Gauge software (Fujifilm).

Porcine peripheral blood cell isolation

Porcine blood samples were obtained from 4- to 6-month old pigs (Landrace–Yorkshire–Duroc) supplied by the Hyupsin Food Co., Ltd (Anyang, Korea). The use of porcine blood was approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. SNU-150327-2). Porcine whole blood was diluted with PBS at a ratio of 1:1, and porcine peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (400 x g for 20 min without brake) using Ficoll-paque Plus (Amersham Bioscience, Buckinghamshire, UK). PBMCs were suspended in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (hereafter referred to as RPMI media).

Co-culture IPEC-J2/PBMC

1 x 10^5 cells/ml of IPEC-J2 were seeded in 500 µl of DMEM media as described above in 1.12 cm^2 polyester membrane inserts with 0.4 µm pore size and the basolateral side was filled with 1 ml of DMEM. During the cell growth and differentiation, the medium in both compartments was replaced three times per week for 7-9 days. Then, 2 x 10^6 cells/ml of PBMCs were seeded in the basolateral compartment of the trans-well plate with 1 ml RPMI media, and toxins were added to the apical compartment.

Real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and reverse-transcribed to generate complementary DNA (cDNA) using oligo-dT primers (Bioneer, Daejeon, Korea). The real-time quantitative PCR was carried out using a Step One Plus real-time PCR system (Applied Biosystems, Foster City, USA). SYBR® Green PCR Master Mix was used according to the manufacturer's specification (Applied Biosystems). The PCR reaction was carried out in a 96-well reaction plate with 10 µl SYBR® green PCR master mix, 0.5 µl primers, 1 µl cDNA template and 8 µl nuclease-free H_2O. The 40 thermal cycles of 2 min at 50 ºC, 10 min at 95 ºC, 15 sec at 95 ºC, 30 sec at 60 ºC, and 30 sec at 72 ºC were utilized according to the manufacturer's recommendation. Relative quantification of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Target gene expression was normalized to GAPDH mRNA level. The nucleotide sequences of porcine specific primers are shown in **Table 4**.

Enzyme-linked immunosorbent assay

The cell culture supernatants were collected from the bottom well of the trans-well plate and

release of IL-8, TNF-α, IFN-γ was determined by commercial ELISA Duoset kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instruction. In brief, the cytokine capture antibody was coated on 96-well immuno-plate purchased from Nalgene Nunc International (Rochester, NY, USA) and incubated overnight at 4 °C. The plates were then washed with washing buffer (0.05% Tween 20 in PBS) for three times and blocked with blocking buffer (1% bovine serum albumin in PBS) for 1 h. After washing, the culture supernatants and respective standard proteins were added and incubated for 2 h followed by 2 h incubation with detection antibody conjugated with biotin. Specific binding was examined using streptavidin–HRP (R&D Systems) followed by the addition of the TMB substrate (Sigma-Aldrich). The reaction was stopped with 50 µl of 2 N H_2SO_4. The amount of cytokines was measured at absorbance of 450 nm by using a microplate reader purchased from Molecular Device (Molecular devices, Sunnyvale, USA). Porcine IL-17 was measured using the swine IL-17A VetSet™ ELISA Development kits (Kingfisher biotech, MN, USA) according to the manufacturer's instruction.

Flow cytometry

PBMCs were harvested from the basolateral side of IPEC-J2-PBMC co-culture system. 1 x 10^6 cells were stained with the following mAb at optimal concentrations; anti-porcine CD3e (clone PPT3; Southern Biotech, Birmingham USA), CD4 (clone 74-12-4; BD Biosciences), CD8a (clone 76-2-11; BD Biosciences), CD172a (clone 74-22-15; BD Biosciences), for 20 min at 4 °C in the dark. The cells were washed and the surface marker expression was measured using a FACS Canto II (BD Biosciences). All the flow cytometric data were analyzed with FlowJo software (Tree Star, California, USA).

Annexin V/7AAD analysis

First, floating cells were collected and thereafter attached cells were washed with PBS and trypsinized for 5 min. Finally, trypsinized cells and floating cells were united and stained with Annexin V-FITC (BD Biosciences) and 7-AAD (BD Biosciences). The intensity of the markers was examined by flow cytometry (FACS Canto II, BD Biosciences). All flow cytometric data was analyzed by using FlowJo software (Tree Star, California, USA).

MTT assay

1 x 10^5 cells/ml of IPEC-J2 were seeded in DMEM media in 96-well plates. 10 µl of MTT solution (5 mg/ml in PBS; Sigma) was added to each well and two hours later the media was discarded. One hundred microliter of DMSO (Sigma) was added to each well and shaken for 5 min to solubilize the formazan formed in the viable cells. Absorbance was measured at 595nm using a microplate reader, VersaMax (Molecular devices, Sunnyvale, USA).

Statistical analysis

Statistical analysis (one-way ANOVA with Tukey posttest or two-way ANOVA with Bonferroni posttest) was performed using the GraphPad Prism (version 7.03, GraphPad Software, San Diego, USA). Differences were considered significant if $P < 0.05$.

Table 4. The primer sequences for real time-PCR

Gene	Primer sequence
IL-8	Forward: 5'-GCTCTCTGTGAGGCTGCAGTT-3'
	Reversed:5'-AAGGTGTGGAATGCGTATTTATGC-3'
MCP-1	Forward: 5'-AAGTGGGCACACCCGTTTC-3'
	Reversed:5'-CGCCATTATGCGTGATTGTT-3'
CCL20	Forward: 5'-AGGATATTCACGGCTTGTTTCAC-3'
	Reversed: 5'-CACACAGCAGCTCGCCAAT-3'
TNF-α	Forward: 5'-ACGGCGTGAAGCTGAAAGAC-3'
	Reversed: 5'-TGTGAGTGAGGAAAACGTTGGT-3'
IL-6	Forward: 5'-CAGGAACGAAAGAGAGCTCCAT-3'
	Reversed: 5'-AAGGCAGTAGCCATCACCAGAA-3'
GAPDH	Forward: 5'-TGGGCGTGAACCATGAGAA-3'
	Reversed: 5'-CCTCCACGATGCCGAAGT-3'

IV. Results

1) FB_1 increased intestinal permeability of porcine intestinal epithelial cells

To examine effect of FB_1 on intestinal cell integrity, differentiated IPEC-J2 cells were treated with different doses (0, 10, or 100) FB_1 in the presence or absence of LPS for 10 days. The results showed that FB_1 treatment at 10 and 100 μM for longer than 6 days induced the disruption of TEER **(Figure 4A)**. However, co-treatment with FB_1 and LPS did not show synergic effect on the reduction of TEER **(Figure 4B)**. When 100 μM of FB_1 was treated together with LPS and DON, significant decrease in TEER was observed within 3 days compared to that at 0 and 50 μM FB_1 **(Figure 5A)**, which was consistent with decreased expression of tight junction proteins on IPEC-J2 (**Figure 5B**). In conclusion, FB_1 accelerated the reduction of gut integrity in the presence LPS and DON.

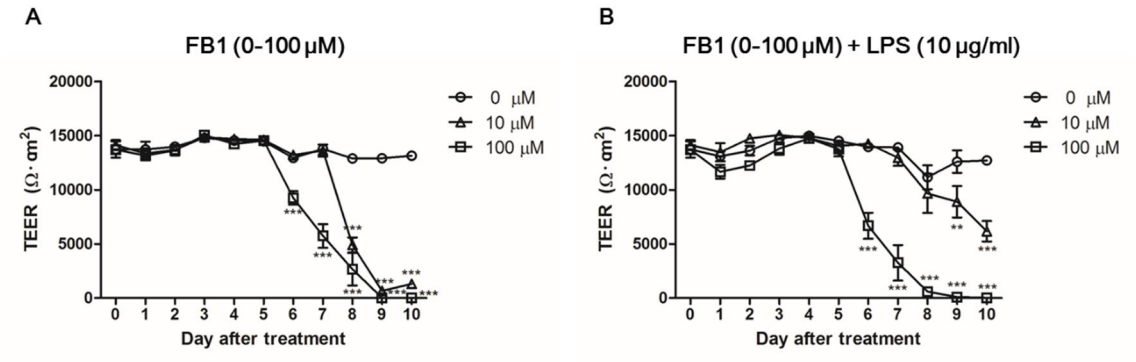


Figure 4. Increase in permeability on porcine epithelial cells exposed to FB_1 only or FB_1 with LPS. IPEC-J2 was seeded onto 1.12 cm^2 trans-well polyester membrane inserts and treated with (A) various concentration of FB_1 (0-100 μM) only, or (B) together with LPS (10 μg/ml). TEER values were measured using epithelial volt ohm meter for 10 days. Data represent mean ± SD of TEER (n=4). ***P<0.01, ****P<0.001* compared to the control (0 μM).

A

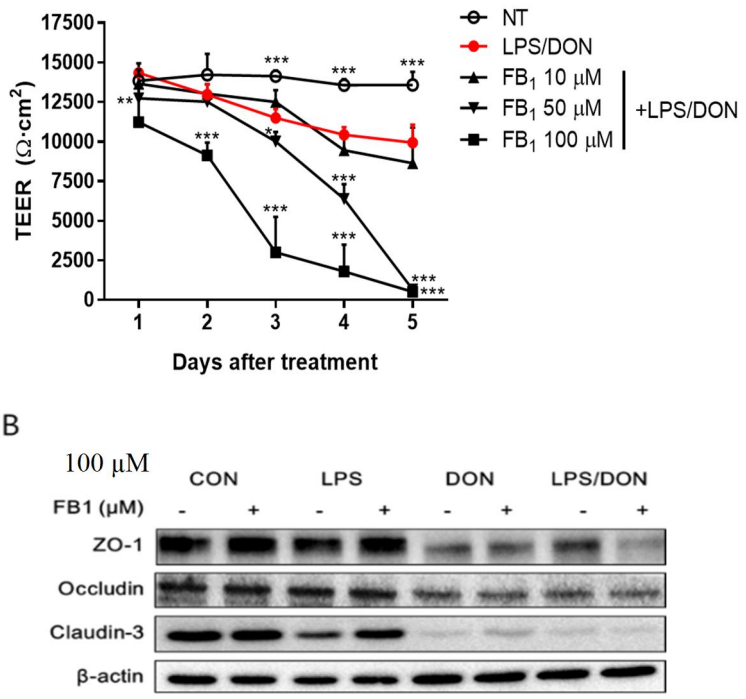


Figure 5. TEER value on IPEC-J2 exposed to FB_1 together with LPS and DON and tight junction protein expressions on IPEC-J2. IPEC-J2 was treated with FB_1 (0, 10, 50, 100 μM) in the presence of LPS (10 μg/ml) and DON (5 μM) for 5 days. TEER values were measured using epithelial volt ohm meter for 5 days. Data represent mean ± SD of TEER (n=5). ***P<0.01, ***P<0.001* compared to LPS/DON. NT; no treatment. (B) On day 3, whole-cell lysates from 100 μM FB_1 treatment group were analyzed for the expression of ZO-1, occludin, claudin-3, and β-actin by using Western blot assay.

2) Treatment of both FB_1 and HFB_1 exhibited comparable damage on barrier integrity

Next, the effect of FB_1 and HFB_1 on the barrier integrity of IPEC-J2 was monitored. TEER value started to decrease 6 days after the treatment with FB_1 **(Figure 6A)**. To investigate whether both toxins have a negative effect on permeability in the presence of LPS and DON, IPEC-J2 was exposed to FB_1 or HFB_1 together with LPS and DON. A significant decrease of the TEER value was found on IPEC-J2 treated with LPS/DON for 5 days, which was aggravated by either FB_1 or HFB_1 **(Figure 6B)**. The negative effect of HFB_1 on permeability (**Figure 6B**) and cell viability **(Figure 7)** was comparable to that of FB_1 in the presence or absence of LPS/DON. Furthermore, it was confirmed that dysregulation of permeability was not caused by solvents used (**Supplementary Figure 1**).

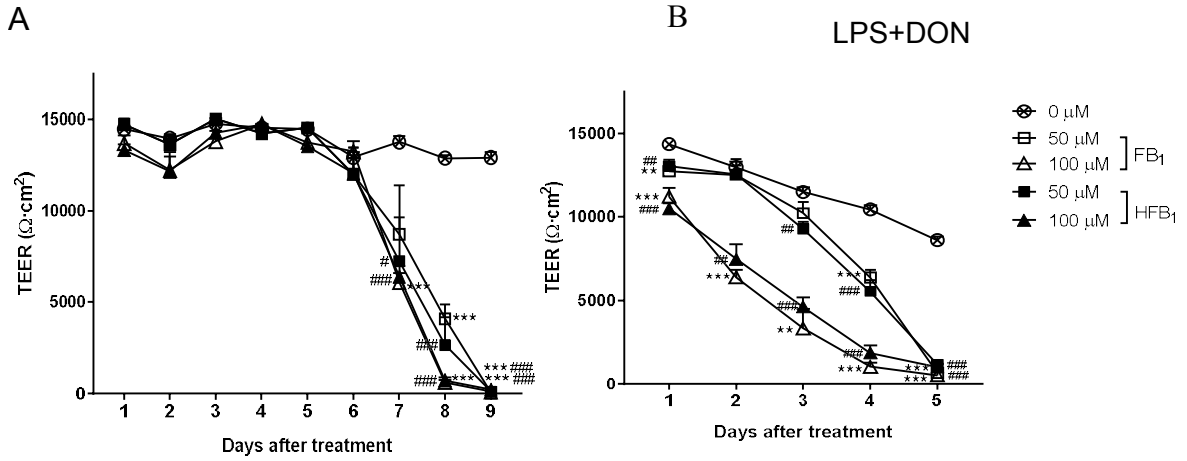


Figure 6. Permeability of porcine epithelial cells exposed to FB_1 or HFB_1. IPEC-J2 were seeded onto trans-well inserts and treated with different concentration (50 and 100 μM) of FB_1 or HFB_1 (A) without or (B) with LPS (10 μg/mL) and DON (5 μM). Data represent mean ± SD of TEER (n=4). ***P<0.01, ***P<0.001* FB_1 compared to the control (0 μM). *##P<0.01, ###P<0.001* HFB_1 compared to the control (0 μM).

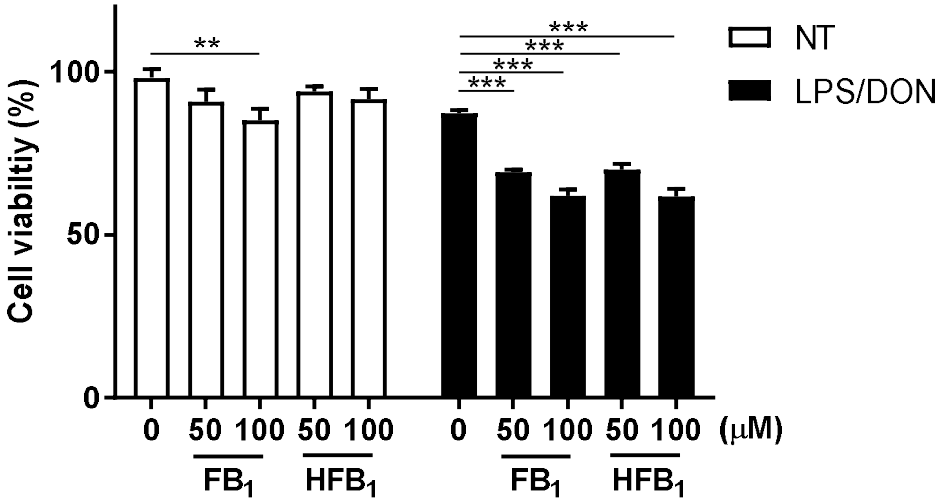


Figure 7. Cell viability of IPEC-J2 treated with FB_1 or HFB_1 in absence or presence of LPS/DON. IPEC-J2 were seeded onto trans-well membrane inserts and treated with different concentration of FB_1 or HFB_1 (50 and 100 μM) in the presence or absence of LPS (10 μg/mL) and DON (5 μM). Cell viability was examined by MTT assay 3 days after the treatment. Data represent mean ± SD of TEER (n=4). ***P<0.01, ****P<0.001* compared to the control (0 μM) within NT and LPS/DON group respectively.

3) HFB_1 induced less chemokine expression than FB_1 in porcine epithelial cells

The intestinal tract is likely the first target for mycotoxins following ingestion of contaminated feed. When exposed to harmful toxins or pathogens, IECs are known to chemokines such as interleukin (IL)-8, CC-chemokine-ligand (CCL)-20 and monocyte chemoattractant protein-1 (MCP-1) that are important for the recruitment of immune cells in the lamina propria (Oswald et al, 2006). To investigate whether FB_1 and HFB_1 affect chemokine production in the presence of LPS and DON, their effect on mRNA levels of IL-8, CCL20 and MCP-1 in IPEC-J2 was examined. Interestingly, the exposure to 50 and 100 μM

of FB_1 induced an increase in IL-8 and CCL20 in presence of LPS/DON, while 100 μM of HFB_1 showed a low mRNA expression of those chemokines. Though MCP-1 mRNA expression was upregulated when treated with either FB_1 or HFB_1 in the absence of LPS/DON, it was significantly increased when 100 μM of FB_1, but not HFB_1, was treated together with LPS/DON **(Figure 8)**.

IECs maintain a close and active communication with immune cells in the lamina propria (Rescigno et al, 2011). As shown in supplementary Figure 2A, IPEC-J2/PBMC co-culture model by culturing IPEC-J2 in the insert of trans-well plates and PBMCs at the bottom well was established and cultured for 7-10 days (Gu et al, 2016). To examine whether FB_1 induced the chemokine production in the gut immune system, the supernatants from basolateral side was collected and chemokine production was analyzed. LPS/DON was found to induce IL-8 production, which was further enhanced by treatment with 100 μM of FB_1. However, IL-8 production was significantly reduced when the cells were treated with 100 μM of HFB_1 **(Figure 9)**. In conclusion, together with LPS/DON, FB_1 upregulated the expression of IL-8, CCL-20 and MCP-1 whereas HFB_1 downregulated.

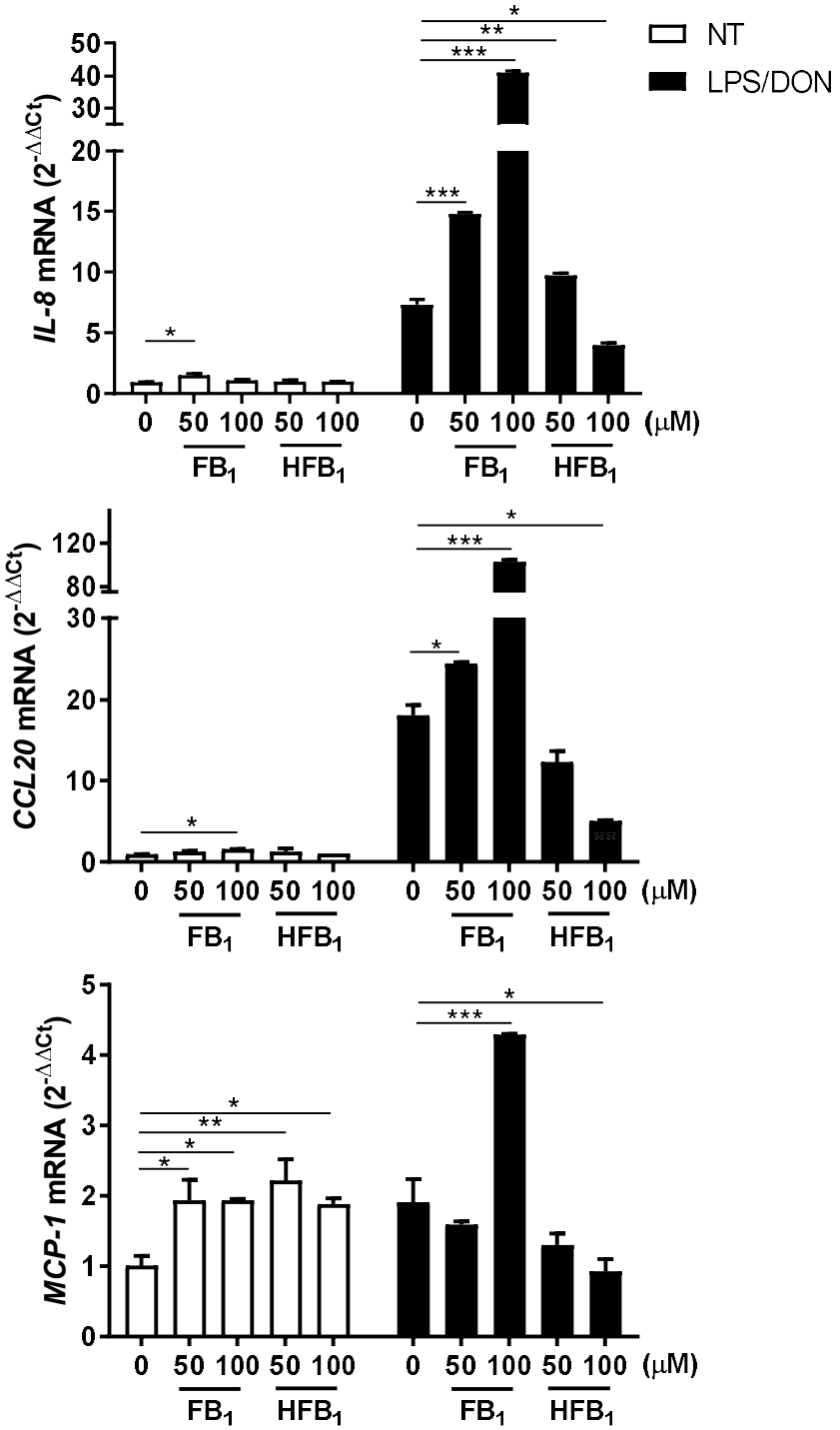

NT
LPS/DON
IL-8 mRNA (2^-ΔΔCt)
CCL20 mRNA (2^-ΔΔCt)
MCP-1 mRNA (2^-ΔΔCt)
0 50 100 50 100 0 50 100 50 100 (μM)
FB1 HFB1 FB1 HFB1

Figure 8. IL-8, CCL-20, and MCP-1 mRNA expression in porcine epithelial cells treated with FB_1 or HFB_1 in the absence or the presence of LPS/DON. IPEC-J2 was seeded onto trans-well inserts and treated with FB_1 or HFB_1 (0-100 μM) in the presence of LPS (10 μg/mL) and DON (5 μM). The bar graph showed mRNA levels of porcine IL-8, CCL-20, MCP-1 measured using real time-PCR at 4 h after the treatment. The expression was normalized to *gapdh* mRNA level (n=4). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to 0 μM within NT and LPS/DON group respectively.

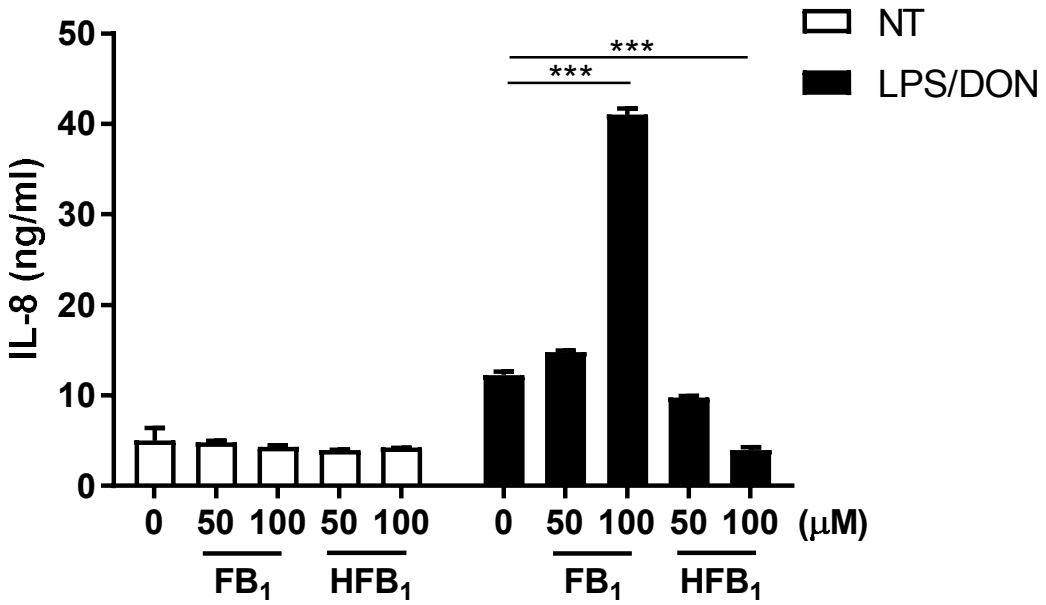
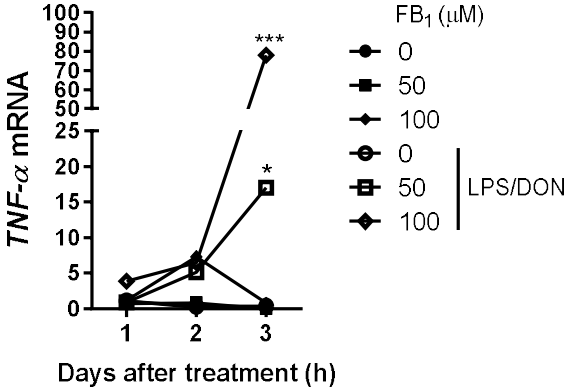


Figure 9. IL-8 production in the IPEC-J2/PBMC co-culture model after treated with FB_1 or HFB_1. Using the co-culture model, IPEC-J2 in the inserts were treated with FB_1 or HFB_1 (50 and 100 μM) in the presence of LPS/DON for 3 days. Then, the supernatant from PBMCs in the bottom well was examined for IL-8 production by ELISA. Data represent mean ± SD (n=4). ***$P<0.001$ compared to 0 μM within LPS/DON group. NT; no treatment.

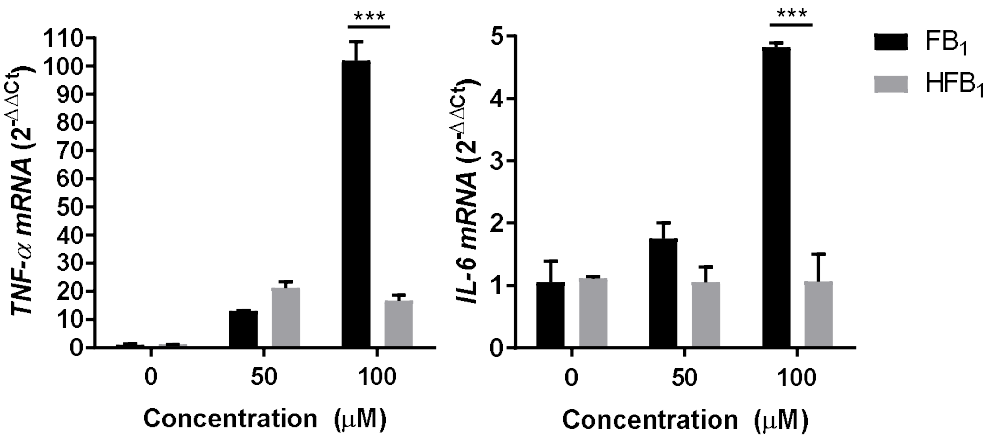
4) HFB$_1$ resulted in less TNF-α production than FB$_1$ in the IPEC-J2/PBMC co-culture model

Next, the production of pro-inflammatory cytokine, TNF-α in the co-culture model was investigated. TNF-α mRNA expression was not induced in the PBMCs of co-culture system when apically treated with FB$_1$, while it was significantly increased under the existence of LPS/DON at 3 days after the treatment (**Figure 10A**). At that time, 100 μM of HFB$_1$ did not lead to such a high TNF-α and IL-6 expression as did 100 μM of FB$_1$ (**Figure 10B**). Consistent with this, TNF-α was significantly elevated at protein level in the supernatant after treated with 100 μM of FB$_1$ in the presence of LPS/DON, while 100 μM of HFB$_1$ did not show such changes (**Figures 10C**). In conclusion, it was confirmed that HFB$_1$ induced weaker inflammatory cytokine expressions than those of FB$_1$ in the IPEC-J2/PBMC co-culture model.

A



B



C

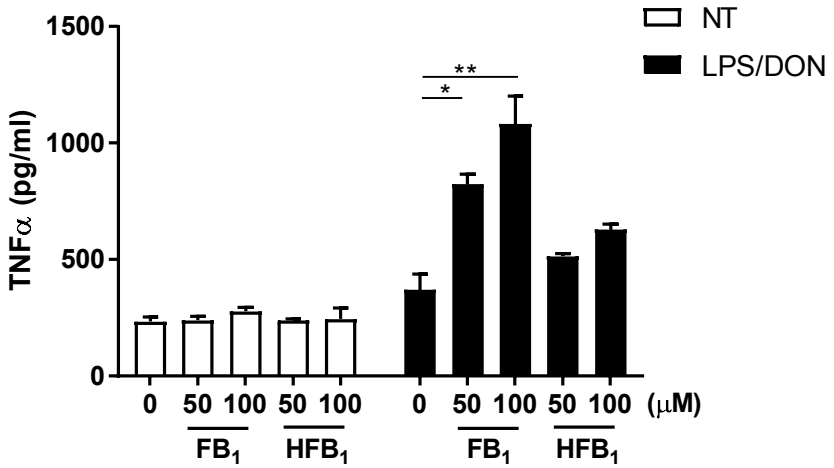


Figure 10. TNF-α production in the IPEC-J2/PBMC co-culture model after treated with FB_1 or HFB_1. Using the co-culture model, IPEC-J2 in the inserts were treated with FB_1 or HFB_1 (50 and 100 μM) in the presence or absence of LPS (10 μg/mL) and DON (5 μM). (A) PBMCs were collected and analyzed for TNF-α mRNA by real time-PCR at 1, 2 and 3 days after the treatment with FB_1. Expression was normalized to gapdh mRNA level and data

represent mean of $2^{-\Delta\Delta Ct}$ (n=4). *$P<0.05$, ***$P<0.001$ compared to 0 μM within LPS/DON group (B) PBMCs at the bottom well were collected and analyzed for TNF-α and IL-6 mRNA by real time-PCR at 3 days after the treatment with FB_1 or HFB_1 (50 and 100 μM) in the presence of LPS/DON. Expression was normalized to GAPDH mRNA level (n=4). ***$P<0.001$ compared to 0 μM (C) At the same time, supernatant from the basolateral side was examined for TNF-α production by ELISA at 3 days after the treatment. (n=4). ***$P<0.001$ compared to 0 μM within LPS/DON group.

5) HFB_1 caused lower immune cell death than FB_1 in the IPEC-J2/PBMC co-culture model

The effect of FB_1 and HFB_1 on immune cell survival in the co-culture model was compared. On the day 5 after the treatment, LPS/DON significantly reduced survival of PBMCs and addition of 100 μM of FB_1 further increased cell death. However, in PBMCs treated with LPS/DON in the presence of 50 or 100 μM of HFB_1, the number of dead cells was lower and comparable to those of PBMCs exposed to LPS/DON only **(Figure 11)**. Furthermore, the subpopulation changes of PBMCs after the treatment was examined. While LPS/DON reduced the number of $CD172^+$ myeloid cells and CD4+ T cells, no differences on the number were observed when the cells were treated with FB_1 in the presence of LPS/DON compared to LPS/DON only (Supplementary Figure 3). Thus, FB_1, not HFB_1, aggravated the death of immune cells when co-cultured with IPEC-J2 and PBMCs in the presence of LPS/DON suggesting FB_1 caused more toxicity to immune cells than HFB_1.

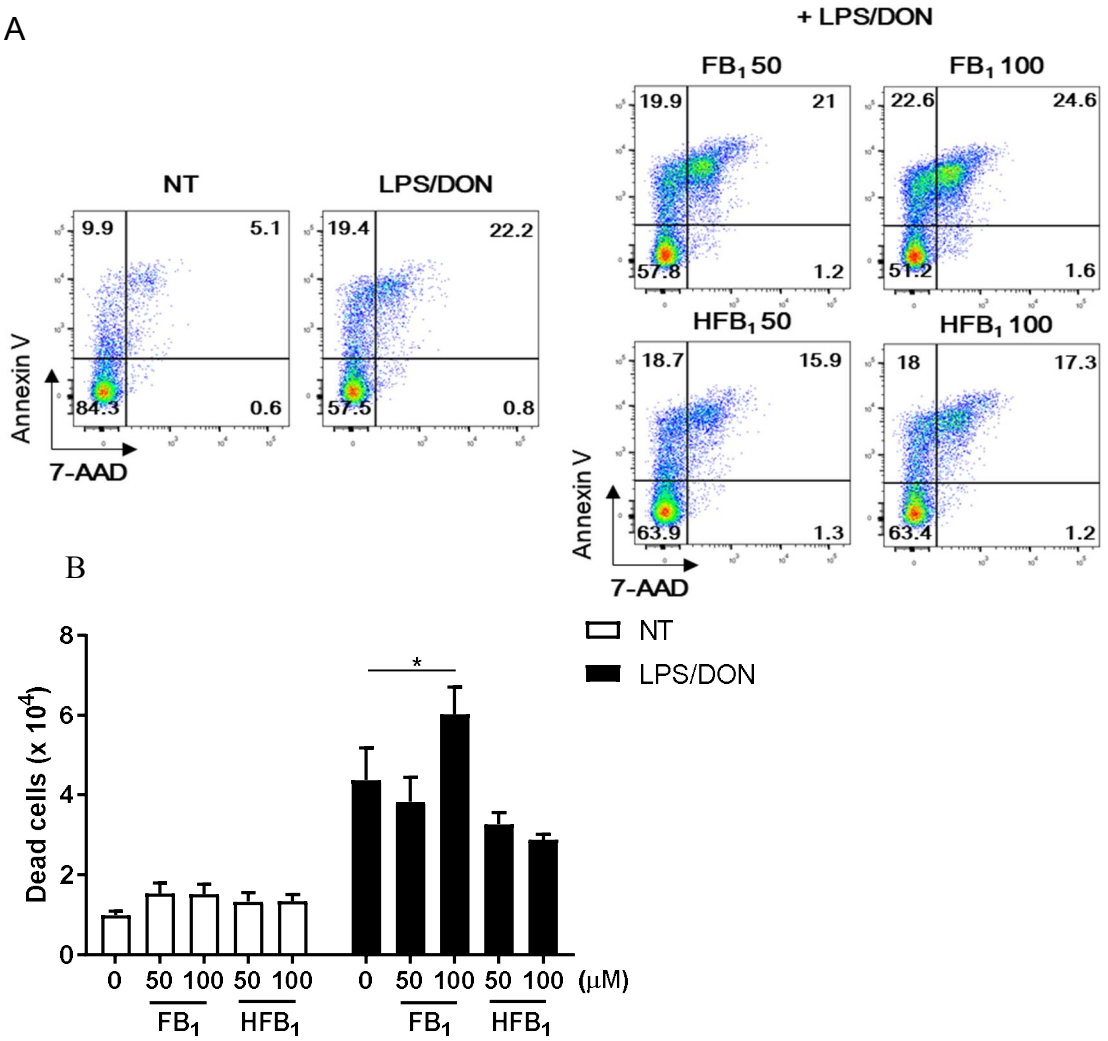
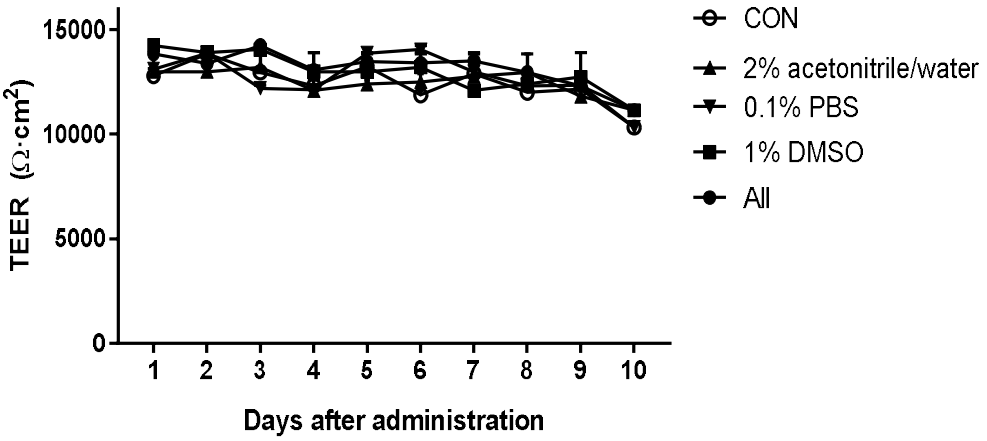
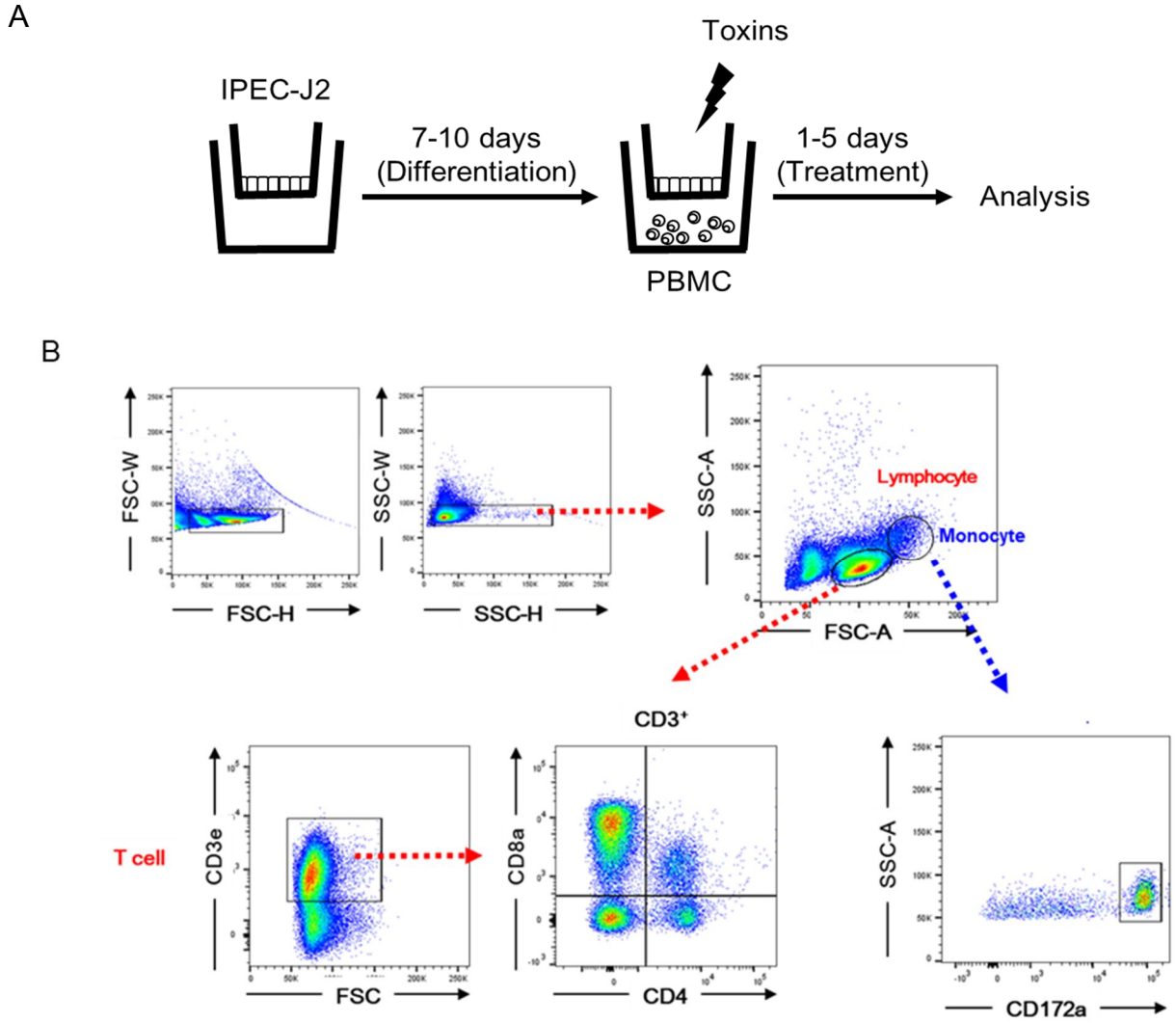


Figure 11. Cell death of PBMCs in the IPEC/J2-PBMC co-culture model after treated with FB_1 or HFB_1. Using the co-culture model, IPEC-J2 in the inserts were treated with FB_1 or HFB_1 (50 and 100 μM) in the presence or absence of LPS (10 μg/mL) and DON (5 μM). Then, PBMCs at the bottom wells were analyzed for cell death using Annexin-V/7AAD staining by flow cytometry. The data represent the (A) percentage and (B) number of dead cells (Annexin-V^+7AAD^+) from total PBMCs at 5 days after the treatment (n=4). *$P<0.05$, compared to 0 μM within LPS/DON group.

V. Supplementary Results

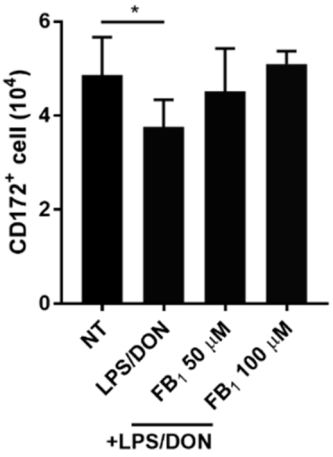


Supplementary figure 1. Permeability of IPEC-J2 treated with different solvents. IPEC-J2 were seeded onto trans-well membrane inserts and treated with 2% acetonitrile/water (to be used for FB_1 and HFB_1) 0.1% PBS (to be used for LPS), 1% DMSO (to be used for DON) and all solvents (2% acetonitrile/water + 0.1% PBS + 1% DMSO), for 10 days. Data represent mean ± SD of TEER. (n=3).

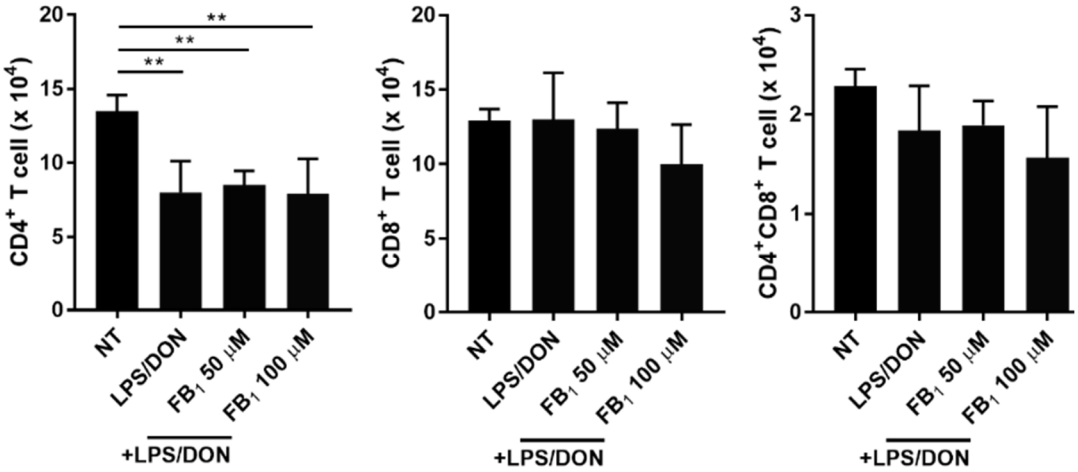


Supplementary figure 2. Experimental scheme for co-culture model and gating strategy for PBMCs. (A) IPEC-J2 cells were seeded onto inserts and incubated for 7-10 days to be differentiated, and then co-cultured with PBMCs at the bottom well. Then, toxins were treated onto differentiated IPEC-J2. The culture supernatant was examined by ELISA, and PBMCs at the bottom well were analyzed using real-time PCR, and flow cytometry. (B) Gating strategy of PBMCs for $CD3^+$ T cells ($CD4^+$, $CD4^+CD8^+$, and $CD8^+$) and $CD172a^+$ myeloid cells.

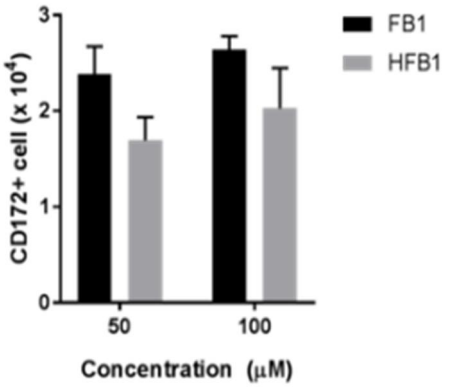
A

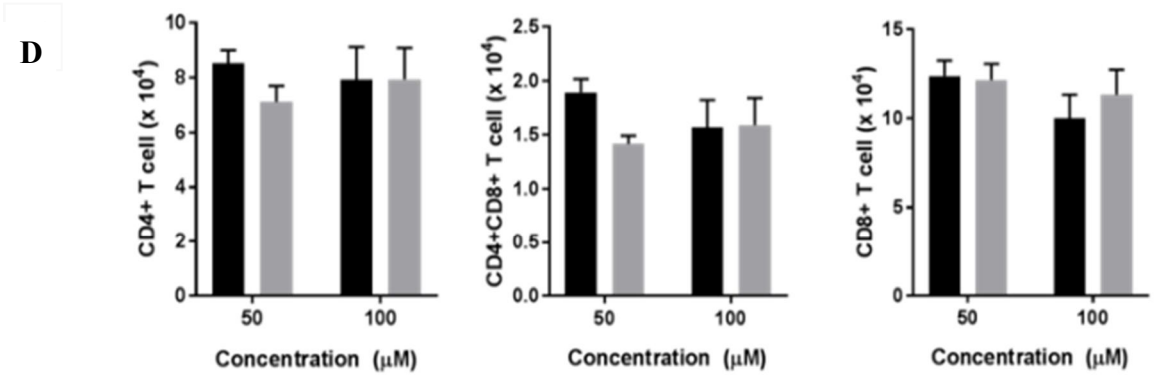

*
CD172+ cell (10^4)
6
4
2
0
NT
LPS/DON
FB1 50 µM
FB1 100 µM
+LPS/DON

B


**
**
**
CD4+ T cell (x 10^4)
CD8+ T cell (x 10^4)
CD4+CD8+ T cell (x 10^4)
20
15
10
5
0
3
2
1
NT
LPS/DON
FB1 50 µM
FB1 100 µM
+LPS/DON

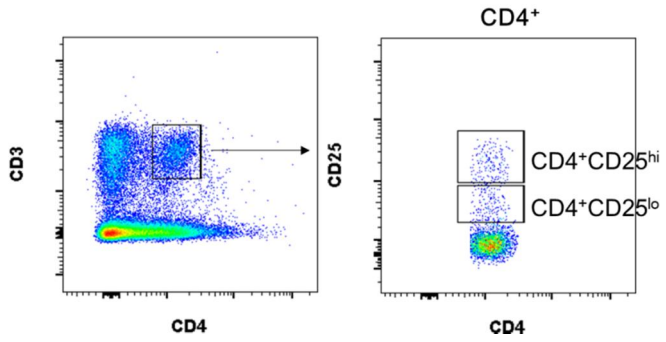
C


FB1
HFB1
CD172+ cell (x 10^4)
3
2
1
0
50
100
Concentration (µM)

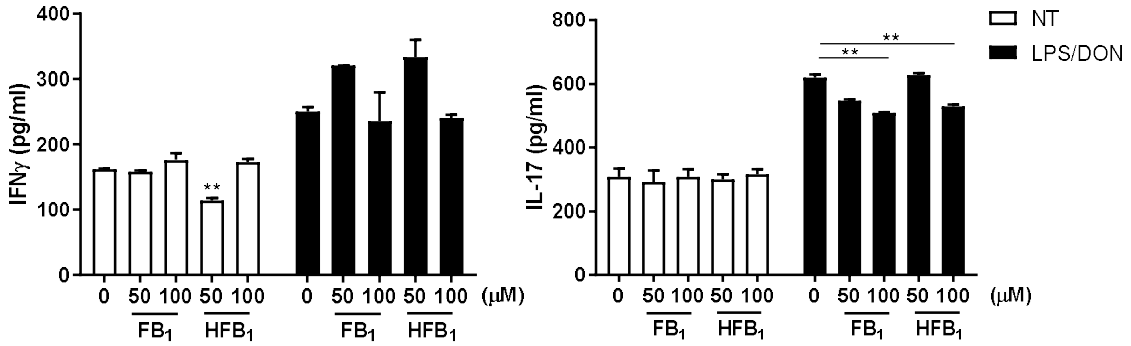


Supplementary Figure 3. Change of PBMC subpopulation in the IPEC-J2/PBMC co-culture model after treated with FB_1 or HFB_1. (A, B) Using the co-culture model, IPEC-J2 were incubated for 7-10 days for differentiation and, then, co-cultured with PBMCs at the bottom well. Then, PBMCs at the bottom well were analyzed for myeloid and T cells after FB_1 treatment in the presence of both LPS (10 μg/mL) and DON (5 μM). The data represent the number of (A) myeloid, and (B) T cells at 3 days after the treatment (n=4). *$P<0.05$, **$P<0.01$. compared to NT (C, D) On the co-culture model, IPEC-J2 in the inserts were treated with FB_1 or HFB_1 (50 and 100 μM) in the presence of LPS/DON for 3 days and PBMCs were analyzed for myeloid and T cells by using flow cytometry. The data represent the number of (C) myeloid and (D) T cells (n=4).

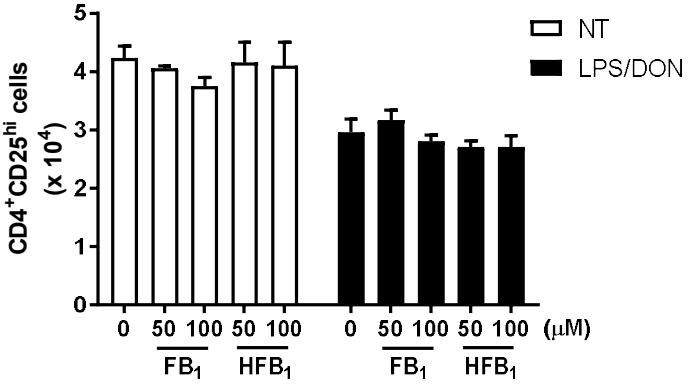
A


CD4+
CD3
CD25
CD4
CD4+CD25hi
CD4+CD25lo
CD4

B


IFNγ (pg/ml)
IL-17 (pg/ml)
NT
LPS/DON
0 50 100 50 100 0 50 100 50 100 (μM)
FB1 HFB1 FB1 HFB1
**

C


CD4+CD25hi cells (x 10^4)
NT
LPS/DON
0 50 100 50 100 0 50 100 50 100 (μM)
FB1 HFB1 FB1 HFB1

Supplementary Figure 4. Regulatory T cells and cytokine expression in the IPEC-J2/PBMC co-culture model after treated with FB_1 or HFB_1. Using the co-culture model, IPEC-J2 in the inserts were treated with FB_1 or HFB_1 (50 and 100 μM) in the presence or absence of LPS (10 μg/mL) and DON (5 μM). Then, PBMCs at the bottom wells were analyzed by flow cytometry after 3 days. (A) Gating strategy of $CD4^+CD25^{hi}$ T cells. (B) The data represent the number of $CD4^+CD25^{hi}$ T cells (n=4). ***P<0.01* compared to 0 μM within LPS/DON group. (C) Supernatant from bottom well at 3 days after the treatment was examined for IFN-γ and IL-17 production by ELISA (n=4).

VI. Discussion

Fusarium mycotoxins such as DON and FB_1 are the major contaminants in animal feed, and their toxic effects are mostly related to subclinical symptoms in animals. We aimed to examine the toxicity of FB_1 in the absence or presence of LPS/DON in porcine IECs and to examine the neutralizing effect of HFB_1, the hydrolyzed metabolite of FB_1, using an IPEC-J2/PBMC co-culture system. Studies on the impacts of mycotoxins on the porcine GIT have been hampered by limitations of available models, intestinal epithelial cell lines (e.g., IPEC-1 and IPEC-J2), or intestinal tissues from pigs fed with mycotoxin-contaminated feed. Recently, a co-culture model using IECs and PBMCs mimicking the intestinal environment was suggested for the investigation of damaged epithelium in the intestinal immune environment (Gu et al., 2016). The purpose of using a co-culture model is to examine the interaction and cross-talk of both cell types under damaged and/or inflammatory conditions. Under induction of excessive inflammation, both the innate and adaptive immune responses are disturbed, resulting in abnormal functioning of the body (Rescigno et al., 2011).

The impact of LPS and DON individually and the combination of LPS/DON have been well documented for their damaging effects on gut integrity in relation to the induction of chemokines and proinflammatory cytokines (Grenier and Applegate, 2013). It is likely that the GIT of an animal fed with average feed will be exposed to LPS and/or DON continuously, since endotoxins are cell wall components of gram-negative bacteria that reside in the gut, and 79% of feed is known to be contaminated with DON (Streit et al., 2012). DON is a representative trichothecene mycotoxin, the largest group of all identified mycotoxins, with more than 200 toxins, and is considered as an indicator of mycotoxin contamination in food and feed (Audenaert et al., 2014). Trichothecenes act as protein synthesis inhibitors targeting

high-turnover cells, such as skin cells, IECs, and immune cells in the lamina propria (Springler et al., 2016). In addition, FUMs are the second most prevalent mycotoxins detected in grains (Streit et al., 2012), resulting in liver, kidney, and GIT toxicity. FUMs are produced by *Fusarium* species, which also produce trichothecenes. Therefore, the feed industry is keen to examine co-occurrence cases of DON and FUMs in both basal feed ingredients and compound feed.

In this study, the effects of HFB_1 and FB_1 were compared with regard to gut integrity and immune modulation. Exposure to FB_1 for longer than 8 days reduced the barrier integrity of IECs, which could be explained by sphingolipid depletion causing a loss of integrity, as sphingolipids are structural components of TJs (Bouhet et al., 2004). As shown in **Figure 12**, accumulation of sphingoid bases associated with the depletion of complex sphingolipids is the major mechanism underlying the toxicity of FB_1 (Soriano et al., 2005; Voss et al., 2007). The aminopentol (AP) backbone of FB_1 is structurally similar to the sphingoid base, and the tricarballylic acid (TCA) side chains of FB_1 interfere with binding of fatty acid acyl-CoA (Desai et al., 2002; Humpf et al., 1998). Unexpectedly, we showed that 10 μM of FB_1 with 10 μg/mL LPS induced less damage in IPEC-J2 cells compared to 10 μM of FB_1 alone. It is probable that the low level of FB_1 may contribute to the induction of more sphinganine-1-phosphate, which reduces toxicity, as it can be mitogenic (Desai et al., 2002). LPS has a mitogenic activity and, perhaps together with a low level of FB_1, could have delayed the damage to IPEC-J2 cells. Because it is a long chain structure similar to sphingoid bases and a relatively large molecular weight, FB_1 is not well absorbed in the GIT (e.g., 3–6% in the porcine gut; Grenier and Applegate, 2013) without the help of bile salts and/or phospholipids. This was also demonstrated in *in vitro* experiments using a trans-well plate culture model by De Angelis et al. (2004). Moreover, this could explain why FB_1 or HFB_1 only induced damage in IPEC-J2 cells after 6 days in the current study.

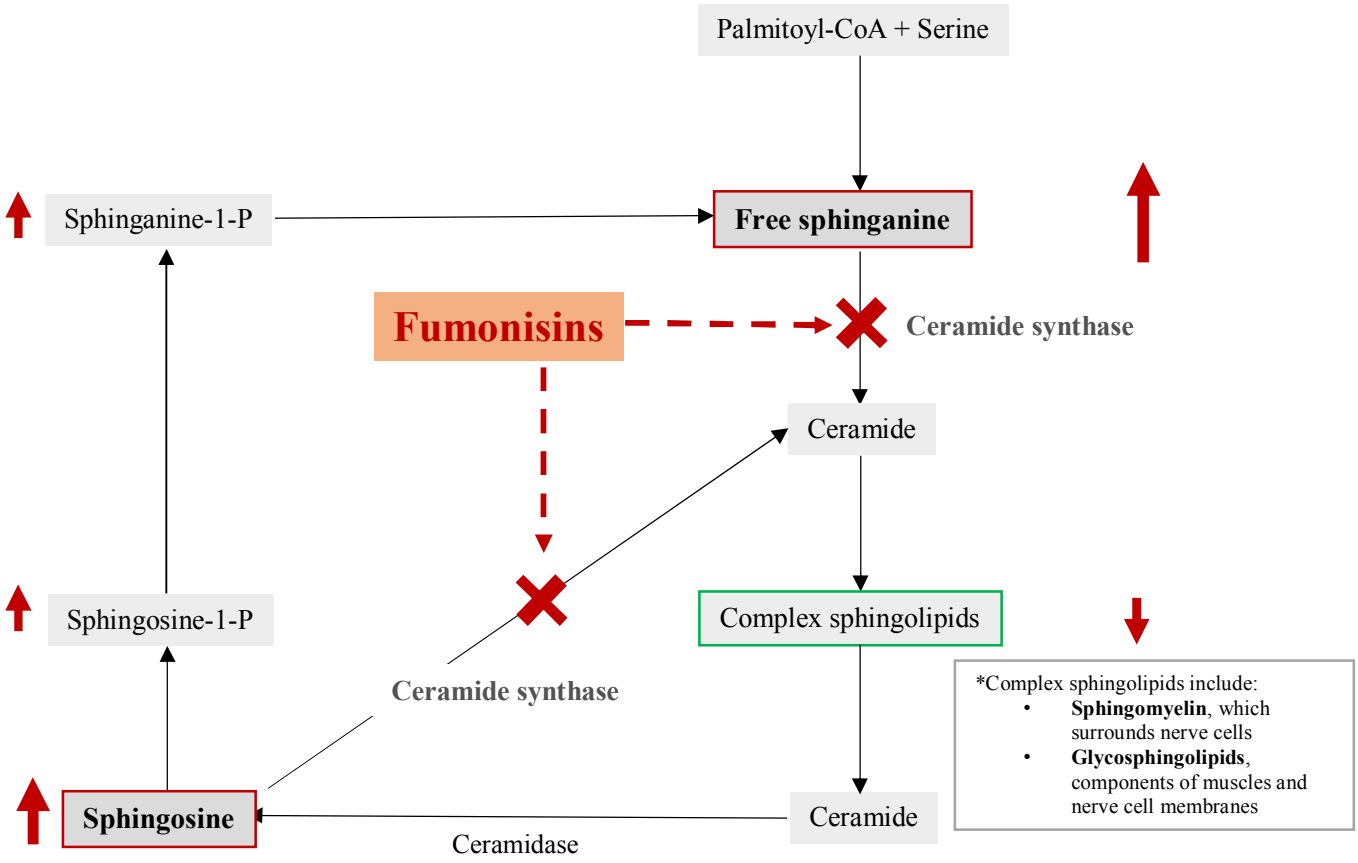


Figure 12. Schematic diagram of sphingolipid metabolism showing the inhibition of ceramide synthase (×) by fumonisins (Soriano et al., 2005; Voss et al., 2007).

Cleavage of TCA side chains from FB_1 leading to the formation of HFB_1 has been suggested as a method to diminish the toxicity of FUMs via enzymatic degradation (Desai et al., 2002; Masching et al., 2016). HFB_1 would likely cause less disruption to gut permeability than FB_1. However, unexpectedly, both FB_1 and HFB_1 caused decreased transepithelial electrical resistance in the differentiated epithelial cells, independent of the presence of LPS and DON. Although the exact mechanism was not defined in the present study, we offer two

hypotheses as to how HFB_1 was still able to damage IPEC-J2: (1) There is a strong possibility that the AP backbone without the TCA side chains attached still competes for the binding of the sphingoid base substrate and inhibits ceramide synthase synthesis, resulting in sphingoid base accumulation in cells (Desai et al., 2002; Espaillat et al., 2015). (2) In the process of hydrolyzation of FB_1 into HFB_1, TCA side chains exist with APs in the product after the enzymatic reaction. If the TCA side chains are still present as separated compounds together with APs, they could also support acylation of ceramide synthase (Espaillat et al., 2015). Nonetheless, how FB_1 and HFB_1 dysregulate the barrier integrity is yet to be fully elucidated. Given that ceramide has been shown to interact with atypical protein kinase C to regulate the epithelial junctions (Wang et al., 2009), further investigation is needed to define the molecular mechanism underlying the toxicity of FB_1 and HFB_1 in relation to their interaction with the ceramide pathway and the formation of epithelial junctions.

As expected, inflammatory mediators (i.e., IL-8 and TNF-α) were significantly elevated when cells were apically treated with FB_1 in the presence of LPS/DON in both IPEC-J2 alone and in the co-culture system. This was supported by the finding that DON+FB_1 increased IL-8 and TNF-α expression in IPEC-1 when compared to FB_1 alone (Wan et al., 2013), and by the *in vivo* challenge trial by *Clostridium perfringens* infection and DON+FB_1-contaminated feed in broilers, which showed a substantially increased inflammatory reaction in the gut, leading to a higher lesion score for necrotic enteritis (Greiner et al., 2011). It is well documented that LPS and DON co-treatment in porcine epithelial cells induces elevation of IL-8 (Cano et al., 2013; Paszti et al., 2014). When PBMCs are exposed to inflammatory stimuli, TNF, IL-1, IL-6, IL-8, and IL-12 cytokines are secreted (Duque and Descoteaux, 2014). Therefore, it is conceivable that the proinflammatory cytokine IL-8 is detected in IPEC-J2/PBMC apically treated with FB_1/LPS/DON, causing epithelial damage. Also, the results of FB_1/LPS/DON treatment of the IPEC-J2–PBMC co-culture in the current study

resulted in increases in both IL-8 and TNF-α. These findings suggest that interactive downstream signals induced by the inflammatory stimuli from the damaged IEC resulted in the elevation of IL-8 and TNF-α in a GIT-mimicking environment.

Co-incubation of FB_1/LPS/DON in our setting induced an increase in the expression of IL-8, CCL20, and MCP-1, whereas HFB_1/LPS/DON did not induce such an increase in IPEC-J2 cells. The best explanation for this phenomenon is that the AP backbone structure with attached TCAs could be responsible for the induction of chemokines after FB_1 treatment. Moreover, we showed that, compared to FB_1, HFB_1 had lower cytotoxicity and caused downregulation of pro-inflammatory cytokines such as IL-8 in immune cells. Supporting these results, there was minimal induction of intestinal toxicity and little alteration of the inflammatory cytokines IL-8 and IL-10 in animals fed FB_1 and FUM*zyme*® (a fumonisin esterase that converts FB_1 into HFB_1) when compared to FB_1-fed animals (Grenier et al., 2012; Grenier et al., 2017). In this *in vivo* feeding trial, purified enzyme together with FB_1 was mixed in the animal diet, which differed from our study, in which HFB_1 was used in an *in vitro* cell culture setting. However, the trends identified by Grenier et al. (2012; 2017) were comparable with the findings of our study, confirming the effect of hydrolyzation of FB_1 to reduce cytotoxicity and lower the production of proinflammatory cytokines in the gut environment. Biotransformation is the most promising practice to remove feed-borne mycotoxins. For example, the DON-degrading eubacteria BBSH 797 has been on the market since the early 2000s. Its use was approved by the European Union committee in 2013 as a feed additive to counteract trichothecenes in pigs (EFSA Scientific Opinion, 2013). Following the success of DON biodegradation in the animal GIT, a more sophisticated method has been developed based on molecular engineering techniques to produce purified enzymes. Fumonisin esterase (FUM*zyme*®) is the first patented and European Union-approved purified mycotoxin-biotransforming enzyme (EFSA Scientific Opinion, 2014).

It has been suggested that DON induces the Th17-mediated response in a porcine intestinal explant by decreasing $CD4^{+}CD25^{hi}$ regulatory T cells (Cano et al., 2013; Kaser et al., 2008). A decrease in $CD4^{+}CD25^{hi}$ T cells among $CD4^{+}$ T cells, and upregulation of IL-17 production by LPS/DON was also observed in the current study. However, FB_1 and HFB_1 did not affect the production of IFN-γ and IL-17 (Supplementary Figure 4). This result confirmed the fact that DON, in the presence of LPS, can be a powerful tool to induce a proinflammatory reaction along the GIT by modulating Th17-mediated immune responses, which is related to homeostasis in the gut. It was anticipated that FB_1 would also alter TH17-mediated responses in the current study based on previous studies of PBMCs and bone marrow-derived dendritic cells (BMDCs) treated with FB_1 (Devriendt et al., 2009; Li et al., 2007; Marin et al., 2007). Dendritic cells are the most important antigen-presenting cell type, and premature dendritic cells exist in the intestine with high endocytic activity. FB_1 decreased the LPS-induced expressions of MHC-II and CD80/86 molecules in BMDCs, as well as the T-cell stimulatory capacity of BMDCs (Li et al., 2007).

The current study is the first *in vitro* experiment testing DON/FB_1 together with LPS treatment in the IPEC-J2 cell line and in an IPEC-J2/PBMC co-culture system. Furthermore, it is the first to test HFB_1, the hydrolyzed form of FB_1, for its ameliorating effect on inflammation compared with FB_1 using the IPEC-J2/PBMC co-culture model. *In vivo* trials are both costly and time-consuming, especially for testing toxic materials such as mycotoxins. In addition, gut health has become a key concept in the agricultural industry since animal production entered the era of post-antibiotic growth promotors. Therefore, any replacements for post-antibiotic growth promotors in feed must effectively protect gut integrity and lessen excessive inflammation reactions in the GIT (Close, 2000; Wang et al., 2010). The co-culture model using IECs and relevant PBMCs offers a platform for testing many different types of materials under identical conditions. More importantly, the

established IPEC-J2/PBMC co-culture model, which mimics the gut immune system, is expected to support the investigation of the molecular and cellular mechanisms of the interactions between IECs and immune cells after exposure to different mycotoxins.

In conclusion, the apical administration of FB_1 together with LPS and DON increased gut permeability in IPEC-J2 cells and led to an aggravated intestinal inflammatory response via the upregulation of inflammatory mediators in IPEC-J2 cells. In the IPEC-J2/PBMC co-culture system, FB_1/LPS/DON treatment induced increased levels of inflammatory cytokines with increased immune-mediated cell death compared to LPS/DON treatment. By contrast, HFB_1/LPS/DON induced fewer inflammation responses than FB_1/LPS/DON in IPEC-J2 monocultured and IPEC-J2/PBMC co-cultured cells, causing lower toxicity in the underlying immune cells. Collectively, these findings suggest that FB_1 aggravates LPS/DON-induced intestinal inflammation and that hydrolyzation of FB_1 may be considered as an effective strategy to reduce intestinal inflammation in pigs.

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VIII. Summary in Korean

곰팡이독소는 곰팡이에서 생산되는 제2차 대사산물로 대부분의 곰팡이들은 여러 가지 곰팡이독소들을 동시에 생산할 수 있으며, 현재까지 완전히 독성이 규명된 곰팡이독소의 수만 600여 개 이상이다. 그 중에서 Deoxynivalenol (DON)은 *Fusarium*곰팡이 종에서 생산되는 trichothecene 곰팡이독소들 중에서 가장 많이 분석되는 trichothecene 곰팡이독소이다. 또한 Fumonisin B_1 (FB_1)은 주로 *Fusarium verticillioides*과 *Fusarium proliferatum*에서 생산되며 옥수수를 비롯한 대다수의 농산물에서 분석된다. 따라서, 인간뿐만 아니라 가축들도 다양한 곰팡이독소 독성에 노출되어 있으며, 그 중에서도 장관 내에서 나타나는 준임상증상들과 깊은 관련이 있는 것으로 알려져 있다.

본 연구는 돼지 장 상피세포 (IPEC-J2)와 돈 혈의 말초 혈액 단핵 세포 (PBMC)를 공동 배양하는 모델을 구축하여 장 상피세포에 DON과 FB_1의 독성 효과를 규명하고자 했는데 특히, 장 온전성 (integrity)과 면역력 조절 작용을 중점적으로 살펴보았다. 첫 번째로, endotoxin (Lipopolysaccharides: LPS)과 DON를 함께 처리한 경우 TR (transepithelial electrical resistance) 치로 알아본 결과, 장 투과성 (permeability)이 높아지고 또한 염증성 사이토카인, IL-8의 생산이 높아짐을 알 수 있었다. FB_1를 추가로 처리 했을 때는 장 장벽 기능 (gut barrier function)이 유의 차 있게 더 약화되고 염증 반응도 더 심화되는 것을 발견했다. 공동 배양 모델에서, FB_1/LPS/DON 처리 구는 LPS/DON 처리구보다 PBMC내에서 더 많은 수의 면역 세포 사멸을 가져왔고, 더 많은 염증성 사이토카인들의

생산을 유도했다. 이와반대로, 효소 작용에 의해 가수 분해된 FB_1 (HFB_1)은 LPS/DON과 함께 처리 했을때 FB_1/LPS/DON 처리 구와 비교해서 더 낮은 염증성 사이토카인들과 체모카인 (chemokine) 생산을 유도했으며, 매우 제한적인 면역세포의 사멸을 나타내었다.

결론적으로, FB_1은 LPS/DON으로 유도된 장 염증 작용을 악화시켰으나, 효소 처리에 의한 FB_1의 가수분해는 FB_1에 의한 돼지 장 내 염증을 완화시키는 데 기여할 수 있음이 증명되었다.