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# Biotransformation Approaches To Alleviate the Effects Induced by *Fusarium* Mycotoxins in Swine

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**ABSTRACT:** Mycotoxin mitigation is of major interest as ingestion of mycotoxins results in poor animal health, decreased productivity, as well as substantial economic losses. A feed additive (FA) consisting of a combination of bacteria (*Eubacterium* BBSH797) and enzyme (fumonisin esterase FumD) was tested in pigs for its ability to neutralize the effects of mono- and co-contaminated diets with deoxynivalenol (DON) and fumonisins (FB) on hematology, biochemistry, tissue morphology, and immune response. Forty-eight animals, allocated into eight groups, received one of eight diets for 35 days: a control diet, a diet contaminated with either DON (3 mg/kg) or FB (6 mg/kg), or both toxins, and the same four diets with FA. Inclusion of FA restored the circulating number of neutrophils of piglets fed the FB and DON + FB diets. Similarly, FA counteracted the minor changes observed on plasma concentrations of albumin and creatinine. In lung, the lesions induced by the ingestion of FB in mono- and co-contaminated diets were no longer observed after addition of FA in these diets. Lesions recorded in the liver of pigs fed either of the contaminated diets with FA were partly reduced, and the increased hepatocyte proliferation was totally neutralized when FA was present in the co-contaminated diet. After 35 days of exposure, the development of the vaccinal response was significantly improved in animals fed diets supplemented with FA, as shown by results of lymphocyte proliferation, cytokine expression in spleen, and the production of specific Ig. Similarly, in jejunum of animals fed diets with FA, occurrence of lesions and upregulation of pro-inflammatory cytokines were much less obvious. The ameliorative effects provided by FA suggest that this approach would be suitable in the control of DON and FB that commonly co-occur in feed.

**KEYWORDS:** *deoxynivalenol, fumonisin, co-contamination, biotransformation, vaccinal and intestinal responses*

## INTRODUCTION

Mycotoxins are structurally diverse low-molecular-weight metabolites produced by fungi. As secondary metabolites, they are not essential to fungi growth but may contaminate animal and human feeds at all stages of the food chain. Their global occurrence is considered to be a major risk factor affecting both human and animal health, and additionally causing substantial economic losses.[1]

Among mycotoxins, deoxynivalenol (DON) and fumonisins (FB) are both produced by *Fusarium* species (mostly *F. graminearum* and *F. verticillioides*, respectively). The major problem associated with animal feed contaminated with mycotoxins is not acute disease episodes, but rather the ingestion of low level of toxins which may cause an array of metabolic, physiologic, and immunologic disturbances.[1,2] For instance, ingestion of realistic doses of DON has been reported to alter functions of the gut epithelium, such as permeability, mucosal immunity, and nutrient utilization.[3−6] At low doses, FB interfere with mechanisms of resistance and immune function, predisposing animals to pulmonary infections and increasing intestinal pathogen colonization.[7−9] Besides, both DON and FB have been reported in separate studies to impair the development of vaccinal response.[10,11]

As evidenced by recent surveys, animals are usually exposed to more than one mycotoxin.[12−14] The effects resulting from mycotoxin co-contamination are poorly documented, but toxicity of combinations of mycotoxins cannot be predicted from the individual toxicities of the toxins.[15] With regard to the effects of DON and FB in association, a few studies demonstrated that these two toxins act in an additive manner on the induction of hepatic lesions, on the modulation of intestinal functions, or on the reduced efficacy of vaccination.[16−19]

Good feed management practices on the farm are major goals in controlling mycotoxicosis in livestock and poultry.[20,21] However, despite substantial efforts, prevention of mycotoxin formation in stored feeds is not always feasible. Methods based on the inclusion of feed additive to inhibit the intestinal absorption of mycotoxins have gained popularity over the past

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decade.[22] Given the frequent occurrence of multitoxin contaminated feed, the feed additive must be able to target different types of mycotoxins at the same time. Due to the diverse structures and properties of these metabolites, this issue remains critical in the development of control strategies. In line with that, adsorbents such as clays (HSCAS, bentonite), yeast, or bacteria were demonstrated to work very effectively with regard to preventing aflatoxicosis.[23−25] By contrast, the adsorption and the subsequent elimination of other mycotoxins, particularly trichothecenes (DON, T-2 toxin) and fumonisins, are much less obvious.[23,25] New strategies for mycotoxins other than aflatoxin must be therefore considered. To this end, a promising approach would be the use of microorganisms or enzymes able to convert mycotoxins into much less toxic metabolites. Recently, some publications showed the ability of some microorganisms to degrade zearalenone, trichothecenes, ochratoxin A, and fumonisins.[26−29] Among them, the bacterium designated BBSH 797 isolated from rumen content was the first to be developed as a feed additive microorganism for detoxification of mycotoxins. This bacterial strain is able to biotransform the epoxide group of trichothecenes into a diene. DON is enzymatically reduced by a de-epoxydase of *Eubacterium* BBSH 797 to the nontoxic metabolite de-epoxy-deoxynivalenol (DOM-1).[27,28] Very recently, we showed that hydrolyzed fumonisin, initially obtained from a treatment of fumonisin with the fumonisin esterase FumD, was nontoxic when orally given to piglets.[30] This enzyme possesses the ability to remove the tricarballylic acid side chains from fumonisins.[31]

The present work was designed to evaluate the efficacy of a feed additive to simultaneously reduce the adverse effects of DON and FB in pigs. The feed additive (FA) consisted of a combination of both *Eubacterium* BBSH 797 and fumonisin esterase FumD, targeting specifically DON and FB, respectively. The effect of FA was especially investigated on blood hematology/biochemistry, histopathological observations (liver, lung, small intestine), and immune response.

## MATERIALS AND METHODS

**Piglets, Housing, Diets, and Experimental Settings.** Forty-eight 4-wk-old weaned castrated male pigs (Pietrain X Duroc X Large-white) were obtained locally, acclimatized for 1 week, individually weighed, and randomly allocated into 8 experimental groups at the animal facility of the INRA ToxAlim Laboratory (France, Toulouse). For 35 days, pigs were kept in batch pens, had *ad libitum* access to water, and were offered eight dietary treatments: (1) control diet, (2) control diet supplemented with FA, (3) DON diet, (4) DON diet supplemented with FA, (5) FB diet, (6) FB diet supplemented with FA, (7) DON + FB diet, (8) DON + FB diet supplemented with FA. Concentrations of mycotoxins in different diets are reported in Table 1. Nutrient concentrations met or exceeded minimum requirements for piglets as previously described.[18]

The formulation of the four diets without feed additive FA has been previously described in two papers.[18,19] The formulation of the four other diets with FA was prepared at the same time using the same sources of FB and DON. In short, two strains of *Fusarium*, *F. graminearum*, and *F. verticillioides* were used to produce DON and FB, respectively. These strains were grown separately on rice. The FB containing culture material was directly used to contaminate the basal feed, whereas DON was purified prior to its addition to feed by extracting the culture material with ethyl acetate and drying on silica gel 60 (Merck, Darmstadt, Germany). Homogenous mixing of the feed with mycotoxins led to the eight different treatments, and to similar inclusion and consistent concentrations of DON and FB between treatments (Table 1). FA in these diets consisted of a combination of bacteria and recombinant bacterial enzyme [FA, *Eubacterium* BBSH 797 and fumonisin esterase FumD (FUM*zyme*, EC 3.1.1.87); BIOMIN Holding GmbH]. Regarding inclusion of FA, freeze-dried *Eubacterium* BBSH 797 strain ($6.25 \times 10^8$ CFU/kg of feed) was simultaneously incorporated with raw materials and/or mycotoxins during feed processing. With respect to the fumonisin esterase FumD, the enzyme was produced by fermentation of recombinant *Pichia pastoris* as described before,[31] except that expression of the *fumD* gene was driven by the GAP promoter rather than the AOX1 promoter, and the cells were cultured in a lab-scale fermenter. Culture supernatant containing FumD was freeze-dried for storage and shipping. The freeze-dried enzyme preparation was dissolved in water (26.4 g/L) and directly sprayed on a monolayer thin of finished feed (10 mL/kg of finished feed equivalent to 0.264 g/kg or 100 U/kg, where one unit is the enzymatic activity that releases 1 μmol tricarballylic acid per minute from 100 μM $FB_1$ in 20 mM Tris-Cl buffer pH 8.0 with 0.1 mg/mL BSA at 30 °C). Enzyme spraying was applied at the animal facility just prior to giving feed to piglets. The measured FumD activity per kg finished feed, determined as described in the unit definition above, was 40 U.

**Table 1. Analyzed Mycotoxin Concentrations of Experimental Diets[a]**

| | concentrations of mycotoxins in the diets (in μg/kg feed) | | |
|---|---|---|---|
| formulated diet | deoxynivalenol | fumonisin $B_1$ | fumonisin $B_2$ |
| Without Feed Additive (FA−) | | | |
| control | 539 | <LOD | <LOD |
| DON | 2847 | <LOD | <LOD |
| FB | 421 | 4113 | 1799 |
| DON + FB | 3091 | 4512 | 2025 |
| With Feed Additive (FA+) | | | |
| control | 500 | <LOD | <LOD |
| DON | 2953 | <LOD | <LOD |
| FB | 476 | 4856 | 2180 |
| DON + FB | 2707 | 4090 | 1843 |

[a]LOD = limit of detection corresponding to 25 μg/kg for both fumonisin $B_1$ and $B_2$. In addition, deoxynivalenol, zearalenone, and enniatin were found to be naturally present in the cereals used, resulting in concentrations of 500, 50, and 100 μg/kg of feed, respectively. All other mycotoxins, including aflatoxins, T-2 toxin, HT-2 toxin, and ochratoxin A, were below the limit of detection.

The experimental design used in this study was randomized with six repetitions (each animal represented one repetition). To evaluate the vaccinal response, an immunization protocol was performed with subcutaneous injections of ovalbumin (OVA) to all piglets. A first injection of 1 mg OVA was done at day 4 of the experiment, followed by a second one of 2 mg OVA at day 16. Both inoculations consisted of OVA dissolved in sterile PBS and mixed with incomplete Freund's adjuvant (Sigma, St-Quentin Fallavier, France). After 35 days of dietary exposure, blood was collected in EDTA or heparin tubes from the left jugular vein of piglets, for blood and plasma analysis. The next day pigs were euthanized by electrical stunning followed by exsanguination. Samples of lungs and liver were collected from all groups and fixed in 10% buffered formalin for histopathological analysis. A portion of spleen was flash-frozen in liquid nitrogen and stored at −80 °C until processed for measurements of cytokine mRNA. In addition, the small intestine from all animals was removed, aligned, and measured on a table. A 15-cm tissue segment was collected in the middle of the small intestine corresponding to mid-jejunum, divided in two subsamples, and stored either in formalin or at −80 °C for the analysis of histopathology or cytokine mRNA levels, respectively.

All animal experimentation procedures were carried out in accordance with the European Guidelines for the Care and Use of Animals for Research Purposes (Directive 86/609/EEC). Two of the authors have an official agreement from the French Veterinary Services for animal experimentation.

**Hematology, Biochemistry, Sa/So Ratio.** The total white blood cell population and the neutrophils subpopulation were counted as already reported.[18] Similarly, plasma concentrations of creatinine and albumin were determined as described in our previous study.[18]

**Table 2. Nucleotide Sequence of Primers for Real-Time PCR**

| gene | primer sequence | Genbank no. | ref |
|---|---|---|---|
| RPL32 | F (300 nM) TGCTCTCAGACCCCTTGTGAAG R (300 nM) TTTCCGCCAGTTCCGCTTA | NM_001001636 | 4 |
| $\beta$2-$\mu$globulin | F (900 nM) TTCTACCTTCTGGTCCACACTGA R (300 nM) TCATCCAACCCAGATGCA | NM_213978 | 9 |
| IL-12p40 | F (300 nM) GGTTTCAGACCCGACGAACTCT R (900 nM) CATATGGCCACAATGGGAGATG | NM_214013 | 9 |
| IL-8 | F (300 nM) GCTCTCTGTGAGGCTGCAGTTC R (900 nM) AAGGTGTGGAATGCGTATTTATGC | NM_213867 | 18 |
| IL-1$\beta$ | F (300 nM) GAGCTGAAGGCTCTCCACCTC R (300 nM) ATCGCTGTCATCTCCTTGCAC | NM_001005149 | 9 |
| IL-6 | F (300 nM) GGCAAAAGGGAAAGAATCCAG R (300 nM) CGTTCTGTGACTGCAGCTTATCC | NM_214399 | 18 |
| IFN-$\gamma$ | F (300 nM) TGGTAGCTCTGGGAAACTGAATG R (300 nM) GGCTTTGCGCTGGATCTG | NM_213948 | 46 |
| TNF-$\alpha$ | F (300 nM) ACTGCACTTCGAGGTTATCGG R (300 nM) GGCGACGGGCTTATCTGA | NM_214022 | 40 |

**Table 3. Effects of Mycotoxin-Contaminated Diets with or without Feed Additive (FA) on Hematology and Biochemistry Parameters at Day 35 Post-Exposure[a]**

| | hematology | biochemistry | | |
|---|---|---|---|---|
| diet | neutrophils proportion of WBC (in %) | creatinine ($\mu$mol/L) | albumin (g/L) | Sa/So ratio |
| control | 34.4 ± 3.9a | 102.5 ± 5.3a | 34.3 ± 0.7a | 0.20 ± 0.01a,b |
| control with FA | 33.8 ± 3.2a | 101.7 ± 3.7a | 34.6 ± 1.1a | 0.23 ± 0.02a,b |
| DON | 35.1 ± 1.8a | 98.0 ± 4.1a | 29.2 ± 1.5b | 0.22 ± 0.02a,b |
| DON with FA | 33.3 ± 3.3a | 96.5 ± 6.9a | 33.4 ± 2.1a,b | 0.20 ± 0.02a,b |
| FB | 21.7 ± 2.3b | 120.5 ± 5.6b | 35.1 ± 2.1a | 1.04 ± 0.09c |
| FB with FA | 36.4 ± 4.9a | 100.0 ± 3.1a | 35.3 ± 2.8a,b | 0.18 ± 0.01a |
| DON + FB | 25.0 ± 1.4b | 101.6 ± 5.5a | 32.8 ± 2.1a,b | 0.92 ± 0.06c |
| DON + FB with FA | 37.8 ± 4.0a | 101.6 ± 5.1a | 30.2 ± 1.0b | 0.24 ± 0.02b |

[a]Results are expressed for 6 animals ± SEM for hematology and Sa/So ratio, and for 5 animals ± SEM for creatinine and albumin. Means within a column with no common letter are significantly different ($P < 0.05$). Sa, sphinganine; So, sphingosine; WBC, white blood cells.

The concentrations of the sphingoid bases sphinganine (Sa) and sphingosine (So) were determined in plasma samples collected at the end of the trial. In addition, the Sa/So ratio was calculated for each animal. Sample preparation and HPLC-FLD analysis were carried out as previously described.[30]

**Histopathology of Hepatic, Pulmonary, and Intestinal Tissues: Lesions and Cell Proliferation.** The different tissues collected at slaughter and stored in formalin were then dehydrated through graded alcohols and embedded in paraffin wax. Classic staining of 3-$\mu$m sections by hematoxylin/eosin (HE) allowed us to record several types of lesions depending on the tissue studied. The recorded lesions were characterized according to their severity and their extent, leading to the establishment of a lesion score per animal. Published methods were used to calculate the lesion score in liver and lung,[18] and in jejunum.[19] For each tissue, the minimal scores were 0, and the maximal scores were 21, 33, and 38 for liver, lung, and jejunum, respectively.

The cellular proliferation was assessed by counting $K_i$-67-positive nuclei on formalin-fixed embedded liver sections as already described.[18] The number of $K_i$-67-positive nuclei among the total of 100 nuclei was counted on the sections under light microscopy at 40× magnification. The proliferative index was calculated by $K_i$-67-positive cells/total cells × 100. In the jejunum, epithelial cell proliferation was estimated by counting the number of mitosis figures in the enterocytes in ten random fields on hematoxylin/eosin-stained slides using 40× magnification.

**Determination of the Immune Responses: Lymphocyte Proliferation, Antibody Synthesis, Cytokine Expression.** Blood samples obtained at day 35 were subdivided and used either for blood culture or for plasma harvest. To evaluate the lymphocyte proliferation upon antigenic stimulation, total blood was initially incubated for 48 h in culture medium supplemented with the OVA antigen, and then for a further 24 h period with medium supplemented by [$^3$H]-thymidine. As previously reported,[10] incorporation of thymidine was measured with a liquid scintillation beta counter (Kontron Instruments, St. Quentin en Yvelines, France), and results were expressed as counts per minute in stimulated culture/cpm in control nonstimulated culture.

In addition to the analysis of the specific cellular response, the humoral response developed following immunization was evaluated through the measurement of anti-OVA immunoglobulins in plasma. As already described,[18] titers of specific anti-OVA antibodies were determined by ELISA, and two subsets of immunoglobulins, IgG and IgA, were investigated.

To complete the immune analysis, the profile of cytokine expression was investigated in spleen which is considered as a peripheral lymphoid organ. The different steps and conditions required to the expression analysis, namely tissue RNA processing, quality, and concentrations of extracted RNA, cDNA production, and real-time PCR, were performed as previously described.[18] The sequence of the primers used is detailed in Table 2. Amplification efficiency and initial fluorescence were determined by DART-PCR method.[32] Then, values obtained were normalized by both house-keeping genes beta2-$\mu$globulin and ribosomal protein L32 (RPL32). Finally, gene expression was expressed relative to the control group without FA. Likewise, the same procedure was applied on the jejunum to examine the levels of these immune mediators after mycotoxin and/or feed additive ingestion.

**Statistical Analysis.** Following the Fisher test on equality of variances, one way ANOVA using Statview software 5.0 (SAS Institute, Cary, NC) was used to analyze the differences between the different treatment groups of animals at each time point. $P$ values of 0.05 were considered significant.

## RESULTS

**Evaluation of FB, DON with and without Feed Additive on the Hematological/Biochemical Parameters.** Analysis of blood at the end of the trial revealed that ingestion of the mono FB- and co-contaminated diets reduced the proportion of neutrophils (relative to the total white blood cells, Table 3, control without FA versus FB and DON + FB without FA, $p$ = 0.018 and 0.046, respectively). In both of these diets, addition of FA significantly restored the neutrophil count, showing similar values to the control groups (Table 3, FB without FA versus FB with FA, $p$ = 0.025, and DON + FB without FA versus DON + FB with FA, $p$ = 0.013).

Analysis of some biochemical analytes at the end of the trial showed minor effects, such as a decreased albumin concentration in DON without FA group and an increased creatinine

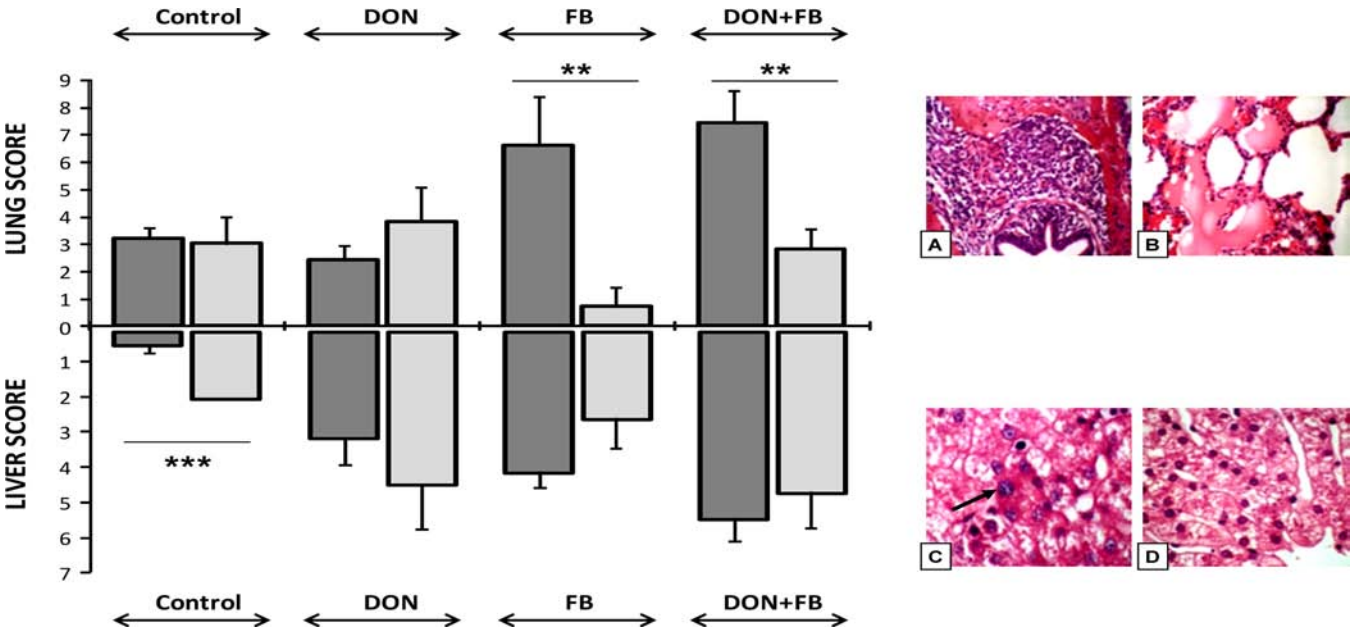



**Figure 1.** Effect of a 35 day exposure to mycotoxin-contaminated diets with and without feed additive (FA) on the lung and liver histology. Pigs received four different diets (control, DON, FB, DON + FB) without FA (dark gray) or the same four diets with FA (light gray). (A) BALT depletion and (B) alveolar edema. HE 40×. (C) Hepatocyte megalocytosis (arrow) and (D) hepatocyte cytoplasmatic vacuolization. HE 40×. Lesion scores were established after histological examination according to the severity and the extent of the lesions. Values are mean ± SEM for five animals: *, $p$ value <0.05; **, $p$ value <0.01; ***, $p$ value <0.001.

concentration in FB without FA group. Addition of FA in these diets prevented the aforementioned changes in piglets (Table 3, DON without FA versus DON with FA, $p = 0.141$ for albumin; FB without FA versus FB with FA, $p = 0.012$ for creatinine).

As expected, at day 35 both mono- and co-contaminated diets containing FB led to a significant increase of the sphinganine (Sa)/sphingosine (So) ratio in plasma (4.5−5.2-fold increase when compared to control group, $p < 0.001$). Conversely, this marked elevation of Sa/So was no longer observed in animals fed these diets supplemented with FA (Table 3, FB without FA versus FB with FA, $p < 0.001$; DON + FB without FA versus DON + FB with FA, $p < 0.001$). Ingestion of DON had no influence on this ratio.

**Evaluation of FB, DON with and without Feed Additive on the Microscopic Lesions in Pulmonary and Hepatic Tissues.** Lesions recorded in the different organs, lungs, liver, and jejunum were considered as mild to moderate for animals fed any of the diets.

Fumonisins are well-known to induce pulmonary edema at high doses (within 4−7 days when fed >90 mg FB/kg of feed) in pigs.[33] In the present experiment, FB present at low doses (6 mg FB/kg of feed) in either mono- and co-contaminated diets induced lesions in lungs, especially depletion of bronchiole-associated lymphoid tissue and vascular disorders. As exemplified in Figure 1, these microscopic alterations resulted in a higher score in comparison to the control group. Occurrence and extent of these lesions were strongly diminished after addition of FA (Figure 1, lesion score observed in FB and DON + FB diets without FA: 6.6 ± 1.7 and 7.4 ± 1.2, respectively, versus 0.7 ± 0.7 and 2.8 ± 0.7 for FB and DON + FB diets with FA, respectively, $p = 0.007$ and $p = 0.01$, respectively).

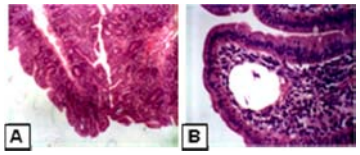
The lesions recorded in the liver were mostly disorganization of hepatic cords, cytoplasmatic and nuclear vacuolization of hepatocytes, and megalocytosis. Combination of DON and FB resulted in higher occurrence and extent of these lesions, as shown by lesion scores in Figure 1. Addition of FA revealed a partial recovery in the mono-FB contaminated diet (38% less). In the other contaminated diets lesion scores were not different from those without FA. Surprisingly, the lesion scores in both control groups were significantly different (Figure 1). Although much less pronounced than for the contaminated diets, a few lesions (megalocytosis and disorganization of hepatic cords) were recorded in hepatocytes of animals from the control group with FA.

Additionally, FA showed a beneficial effect on proliferation of hepatocytes. The marked increase of hepatocyte proliferation induced by the co-contamination of toxins was no longer observed when FA was added in this diet (mean proliferation indexes: 19.0 ± 0.9 versus 39.4 ± 12.8, $p = 0.002$, for DON + FB with and without FA, respectively).

**Evaluation of FB, DON with and without Feed Additive on the Intestinal Disorders.** Like for liver and lung, the same method was applied to score the intestinal morphology. In the jejunum of animals fed the contaminated diets without FA, the histopathological analysis revealed the occurrence of many lesions, mostly villi flattening and fusion, and lymphatic vessels dilation.[19] Inclusion of FA in these diets markedly decreased the occurrence and extent of lesions (Table 4). Although a few animals fed contaminated diets with FA exhibited interstitial edema in the lamina propria, the occurrence of villi flattening and fusion as well as cytoplasmatic vacuolation and necrosis almost disappeared, especially in animals from the DON and DON + FB groups. In these groups, lesion scores were not significantly different from the ones of control groups (Table 4), and account for the overall significant positive effect of FA in comparison to diets without FA ($p = 0.02$).

Similarly in the jejunum, the enterocyte proliferation assessed through the number of mitosis was totally restored when FA was added in the contaminated diets (Table 4). A reduction of

**Table 4. Effects of Mycotoxin-Contaminated Diets with or without Feed Additive (FA) on the Lesion Score and on the Enterocyte Proliferation in the Jejunum at Day 35 Post-Exposure[a]**




| | lesion score[b] | no. of mitosic cells |
|---|---|---|
| control | 0.67 ± 0.33a | 2.37 ± 0.36a,e |
| control with FA | 0.83 ± 0.40a | 1.41 ± 0.18c |
| DON | 6.17 ± 1.72b | 1.73 ± 0.26b |
| DON with FA | 2.50 ± 1.02a,b | 2.18 ± 0.28a,d |
| FB | 4.17 ± 1.14b,c | 1.67 ± 0.21b,c |
| FB with FA | 3.17 ± 0.95b,c | 2.35 ± 0.26a,e |
| DON + FB | 6.17 ± 2.02b,c | 1.91 ± 0.19b,d |
| DON + FB with FA | 2.00 ± 0.68a,c | 2.72 ± 0.19e |

[a]Results are expressed for 6 animals ± SEM. Means within a column with no common letter are significantly different ($P < 0.05$). Enterocyte proliferation is the mean number of mitosic cells per microscopic field. For the graphic: A indicates flattening of villous tips and B indicates lymphatic vessel dilation. HE 10×. [b]Lesion scores were established after histological examination according to the severity and the extent of the lesions.

enterocyte proliferation in the presence of FA was also observed in the control group ($p = 0.04$, Table 4).

At the end of the trial, transcriptomic analysis was also performed in jejunal samples to investigate the expression of four pro-inflammatory cytokines, IL-1$\beta$, IL-6, IFN-$\gamma$, and TNF-$\alpha$. A significant upregulation of cytokines was observed in the small intestine of piglets fed the contaminated diets. This increase was partially or totally abolished in the animals receiving diets supplemented with FA (Figure 2). The statistical analysis between all diets without FA and with FA resulted in a $p$ value <0.05, except for IL-6 mRNA which only showed an effect following DON consumption. The FA added to mono-DON-contaminated diet reduced the expression of IL-1$\beta$ and IL-6 by 34% and 43%, respectively (Figure 2). Similarly, a reduction of 34% and 32% on the mRNA levels encoding for IFN-$\gamma$ and TNF-$\alpha$, respectively, was observed in the mono-FB-contaminated diets with FA. When both DON and FB were co-contaminated in the diet, the inclusion of FA reduced the expression of IFN-$\gamma$ and TNF-$\alpha$ by 31% and 39%, respectively (Figure 2).

**Evaluation of FB, DON with and without Feed Additive on Specific Immune Response.** After 35 days of exposure to mycotoxins, the vaccinal response to OVA was assessed in animals receiving the different diets with or without FA. Lymphocyte proliferation was evaluated following the *in vitro* stimulation of blood lymphocytes with OVA. Vaccination of animals with OVA must have resulted in activation of specific lymphocytes in secondary lymphoid tissue, through priming with OVA-presenting cells. Ingestion of DON and FB, alone or in combination, resulted in low indexes of proliferation (close to 1), suggesting that lymphocytes from these animals were unable to proliferate upon antigenic stimulation (Table 5). The ability of lymphocytes to proliferate was increased by 1.9-, 1.5-, and 2.2-fold when FA was added into the DON, FB, and DON + FB diets, respectively (Table 5). Because of the high individual variability, the increase between diets supplemented or not with FA was only significant in the case of the DON + $FB_1$ contaminated diets.

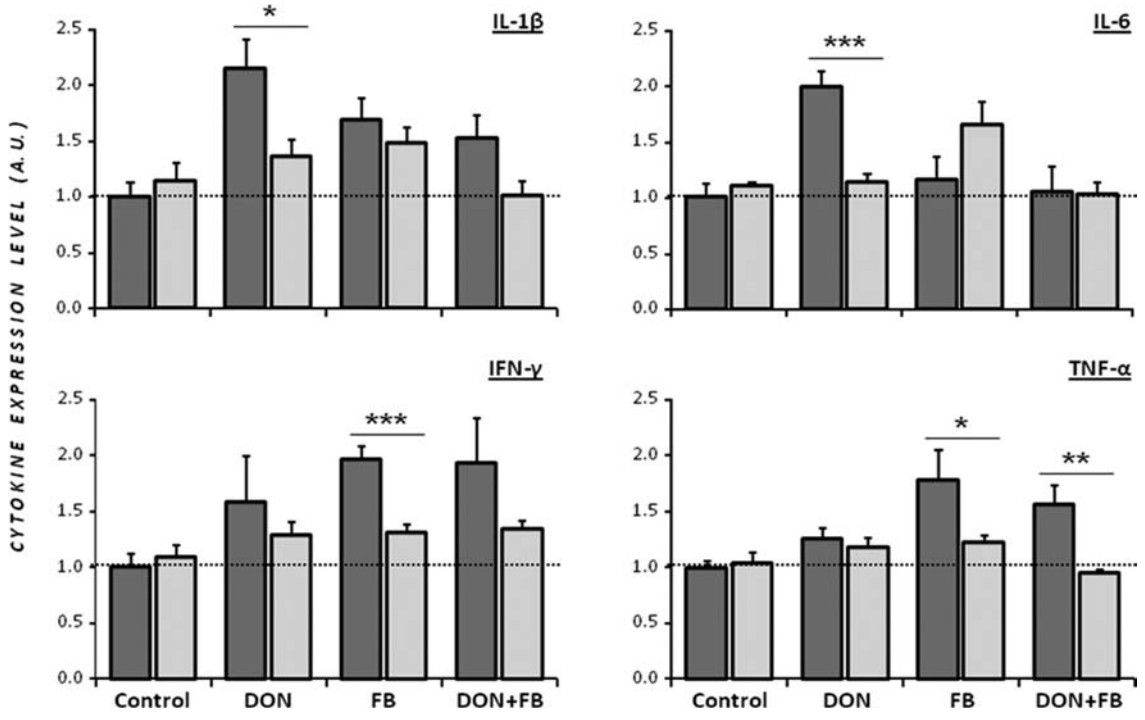



**Figure 2.** Effect of a 35 day exposure to mycotoxin-contaminated diets with and without feed additive (FA) on the cytokine expression in jejunum at day 35 postexposure. Pigs received four different diets (control, DON, FB, DON + FB) without FA (dark gray) or the same four diets with FA (light gray). Quantification of the relative cytokine mRNA level for each sample is expressed in arbitrary units (AU). Values are mean ± SEM for five animals: *, $p$ value <0.05; **, $p$ value <0.01; ***, $p$ value <0.001.


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**Table 5. Effects of Mycotoxin-Contaminated Diets with or without Feed Additive (FA) on Specific Immune Parameters at Day 35 Post-Exposure[a]**

| | | immunoglobulins subsets | |
|---|---|---|---|
| | lymphocyte proliferation index (ova stimulation) | IgG anti-OVA (AU) | IgA anti-OVA (AU) |
| control | 3.79 ± 0.83a | 1545 ± 113a | 20.6 ± 2.5a |
| control with FA | 2.78 ± 0.45a | 1387 ± 86a | 22.1 ± 2.3a |
| DON | 1.53 ± 0.32b,c | 1333 ± 108a,b | 33.1 ± 2.8b |
| DON with FA | 2.94 ± 0.97a,b | 1464 ± 80a | 25.4 ± 3.0a,b |
| FB | 1.25 ± 0.10b | 1101 ± 75b,c | 21.5 ± 0.6a |
| FB with FA | 1.89 ± 0.44a,b | 1166 ± 138a,c | 22.8 ± 3.8a |
| DON + FB | 1.32 ± 0.27b | 789 ± 210c | 22.5 ± 2.2a |
| DON + FB with FA | 2.85 ± 0.64a,c | 1052 ± 84b,c | 22.6 ± 2.3a |

[a]Results are expressed for 5 animals ± SEM. Means within a column with no common letter are significantly different ($P < 0.05$). Ig, immunoglobulin; OVA, ovalbumin.

Two classes of antibodies, IgG and IgA, recognizing specifically OVA were assessed in plasma at the end of the trial. The level of anti-OVA IgG was affected by mycotoxin consumption, the stronger effect being observed when mycotoxins were combined (Table 5). Inclusion of FA in the DON + FB diet allowed 33% recovery of the anti-OVA IgG level. Regarding anti-OVA IgA, only the diet mono-contaminated with DON diet affected the plasma concentration of this isotype. Inclusion of FA in this DON-contaminated diet partially restored the anti-OVA IgA level (Table 5).

Expression of several cytokines was determined in the spleen following the euthanasia of animals. Overall, ingestion of contaminated diets resulted in a reduced expression of IL-6, IL-1$\beta$, IL-12p40, and IL-8. The effect was more pronounced in the diet contaminated with both DON and FB (Figure 3). When the diets were supplemented with FA, the profile of mRNA levels encoding IL-6, IL-1$\beta$, IL-12p40, and IL-8 was restored, and the cytokine expression levels were similar to the ones observed in the control group (Figure 3). The greater recoveries were noticed in the co-contaminated diet ranging from 51% to 107%.

## DISCUSSION

The aim of the present work was to investigate the efficacy of FA to prevent the toxic effects of two fusariotoxins, DON and FB. So far, mycotoxin decontamination/detoxification has mainly been studied in the context of the exposure to a single mycotoxin. However, most fungi are able to produce several mycotoxins simultaneously, and it is a common practice to use multiple grain sources in animal diets.[15] As a consequence, animals are commonly exposed to more than one mycotoxin. In this respect, the efficacy of a decontamination procedure or a feed additive is also based on its ability to target several mycotoxins at the same time, and subsequently to overcome the toxicity caused by multitoxin contaminated feed. This is a real challenge considering the diverse structure of mycotoxins. To this end, the use of a blend of microorganisms or purified enzymes in a feed additive is an interesting approach.

We previously reported the effects of diets mono- and co-contaminated with DON and FB.[18,19] Among mycotoxins, these two toxins from *Fusarium* species are of major concern worldwide in terms of occurrence, as well as impact on animal health and production. We showed that subclinical doses of

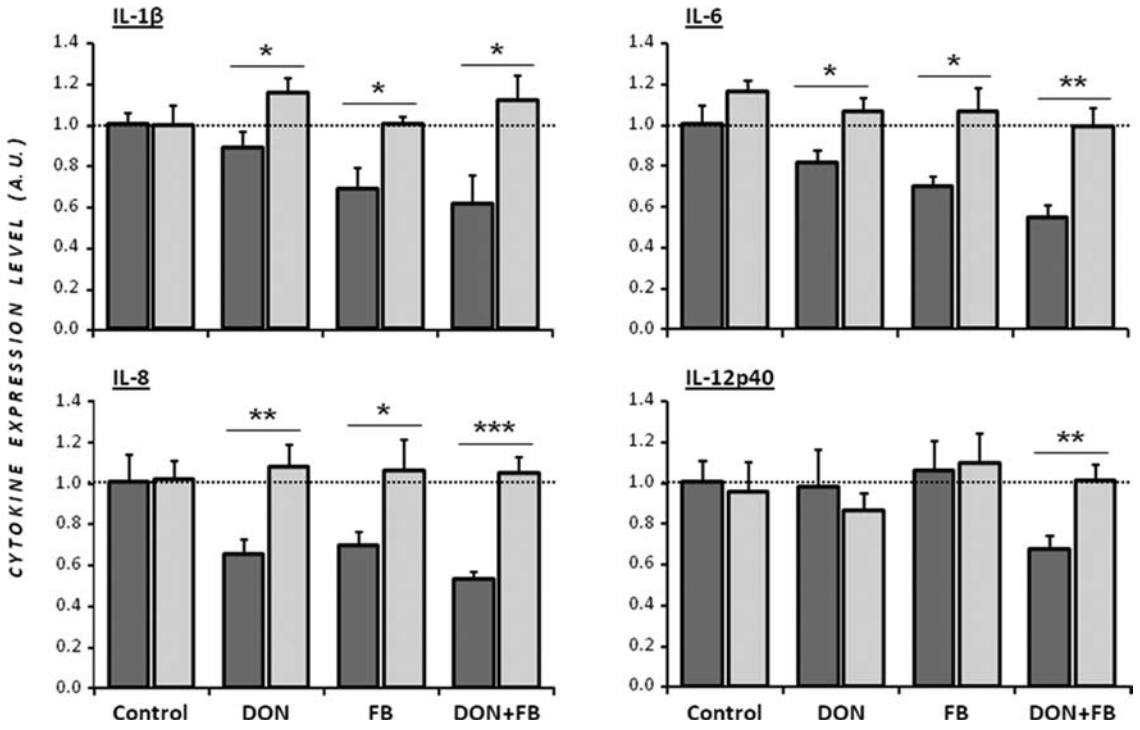


**Figure 3.** Effect of a 35 day exposure to mycotoxin-contaminated diets with and without feed additive (FA) on the cytokine expression in spleen. Pigs received four different diets (control, DON, FB, DON + FB) without FA (dark gray) or the same four diets with FA (light gray). Quantification of the relative cytokine mRNA level for each sample is expressed in arbitrary units (AU). Values are mean ± SEM for five animals: *, $p$ value <0.05; **, $p$ value <0.01; ***, $p$ value <0.001.

DON and FB affect several key functions, ranging from a reduced vaccination efficacy, and an increased occurrence of lesions in many tissues, to a chronic inflammatory disorder. Depending on the end point assessed, the association of DON and FB led to different types of interaction. However, the ingestion of the co-contaminated diet always led to greater toxicity than the mono-contaminated diets. It was thus of interest to conduct the present experiment and to determine the ability of a FA to neutralize the effect of the co-contaminated diet.

The FA incorporated in the feed was a combination of two biological systems, bacteria and recombinant bacterial enzyme. Many reports showed that the live bacterium used, *Eubacterium* BBSH797, is able to convert DON into DOM-1, a metabolite much less toxic.[27,28] In the past, some *in vivo* trials also showed beneficial effects of this bacterium when added in contaminated feed with DON or other trichothecenes.[34−36] With regard to the *Eubacterium* itself, no concerns were noted in tolerance studies (with piglets and chickens) in which the additive was supplied at 10-fold dose.[37] The bacterial enzyme consists of fumonisin esterase FumD, which is able to degrade FB into hydrolyzed FB.[31] Recently, this metabolite ($HFB_1$) resulting from an initial treatment with the enzyme did not cause any toxicity when given orally to piglets.[30] This work was in agreement with conclusions of previous studies using pure $HFB_1$.[38] With regard to inclusion of the FA in the diets, the freeze-dried microorganism BBSH 797 was incorporated during feed milling at 0.25%. Fumonisin esterase FumD was incorporated into feed through the enzyme spraying on a monolayer of grains. This was a practical way of application for experimental purpose, and clearly would not be reflective of a suitable approach for commercial application. Although the results of the experiment suggested that FumD was active in the porcine gastrointestinal tract, in a technological application it might be possible to use a lower FumD dose if the enzyme is provided with protective coating. Accordingly, further work on the dietary supplementation of this enzyme needs to be done. It must be mentioned that samples for mycotoxin analysis were taken before and after enzyme spray application, and the FB concentration of feed prior to feeding the pigs was found not to be reduced.

In the present experiment, the low dose of mycotoxins used did not cause feed refusal and did not interfere with animal growth.[18,19] Similarly, the incorporation of FA in the feed did not affect the growth of animals (data not shown). The minor changes induced by mycotoxins on hematological and biochemical parameters were overcome by the addition of FA. This beneficial effect of FA was already reported on the decreased albumin concentration after DON and ZEA exposure.[39] FA was able to totally counteract the increase of Sa/So ratio induced by FB. Indeed, ingestion of FB disrupts sphingolipid metabolism by inhibiting ceramide synthase, leading to an increased Sa/So ratio in plasma and tissues.[30,38]

The lung is a specific target of FB in swine,[33] and although less studied, ingestion of low doses of toxin induced pulmonary lesions.[7,18] In the present experiment, addition of FA showed a beneficial effect, most likely through the action of the fumonisin esterase FumD as the histopathological alterations induced by FB were no longer noted in piglets fed the FB and DON + FB diets supplemented with FA. As a target organ for many mycotoxins, the liver was also affected in the experiment. The combination of both DON and FB caused hepatic lesions greater than the ones observed with the individual toxins. This effect was slightly reversed after ingestion of this diet with FA. Addition of FA provided a partial protection against the induction of hepatic lesions in the mono-FB contaminated diet. Lesion scores for both control groups (with and without FA) differ significantly; however, the score in the group containing FA was relatively low (score = 2) and in the same range of the one obtained in the control group of another separate experiment.[30] In line with that, the FA had no effect on the index of hepatocyte proliferation, and helped to counteract the effect observed in the co-contaminated diet.

As previously reported, activation of the immune system is needed to observe the effects of low doses of mycotoxins.[10,18,40] Immunization of animals leads to the development of a specific response, involving the recruitment, activation, and proliferation of immune competent cells. Most mycotoxins alter protein synthesis, and therefore make dividing and activated cells, such as immune cells, specific targets. In line with that, the literature is rich with examples of how mycotoxins compromise immune functions, such as depressed T- or B-lymphocyte activity, suppressed antibody production, and impaired macrophage/neutrophil−effector functions.[2] As a consequence, this immunomodulation may eventually decrease resistance to infectious diseases, reactivate chronic infections, and/or decrease vaccine and drug efficacy.[2] In the present experiment we observed that the mono- and co-contaminated diets induced a reduced expression of several cytokines. Addition of FA was able to restore the expression of mRNA levels encoding for IL-8, IL-6, IL-1$\beta$, and IL-12p40 to the level observed in control animals. These data might account for the effects observed in the *in vitro* proliferation of lymphocytes. Indeed, the specific lymphocytes generated in secondary lymphoid organs were unable to proliferate upon OVA stimulation, whereas those from piglets fed the contaminated diets with FA showed a better ability to respond to the same stimulation. It might be suggested that, within the spleen, the interaction and subsequent activation between antigen-presenting cells and T-lymphocytes did not fail as much as in animals fed the contaminated diets without FA. As a consequence, these animals showed reduced concentrations of specific anti-OVA IgG in their plasma, most notably those fed FB and DON + FB diets. However, the FA added in these respective diets was not able to totally restore the concentrations of this specific immunoglobulin isotype. The improvement was mainly observed when both DON and FB were present. Very recently, the ameliorative effect of a similar FA on antibody titers was reported in case of intoxication of broiler chicks with 10 mg DON/kg of feed.[41]

Beyond the toxicity on the immune response, DON and FB are known to affect the gut epithelium of piglets.[6,42,43] The gastrointestinal tract is the first organ coming into contact with mycotoxins of dietary origin and should be expected to be affected by mycotoxins with greater potency as compared to other organs. This aspect is often a neglected area on mycotoxin research, although the nonabsorbed part of mycotoxins remaining in the intestine and their dynamic within the gastrointestinal tract strongly suggest that these metabolites are able to compromise key functions of the intestine.[6] In this respect, the FA must be rapidly released from the stomach and be active for an immediate action in the intestine of animals. A few intestinal parameters were then investigated to evaluate how FA acts in the small intestine and could prevent the intestinal toxicity of DON and FB. Histopathological observations in the jejunum showed that substantially fewer microscopical lesions were recorded in animals fed the contaminated diets with FA, especially the DON and DON + FB diets. Furthermore, the

restoration observed in enterocyte proliferation might explain why animals fed with FA did not exhibit villi flattening.

The chronic ingestion of low doses of mycotoxins might result in persistent inflammatory condition in the intestine.[44] Among alterations reported, the upregulation of pro-inflammatory cytokines all along the small intestine is one of the prominent features. In the present experiment, mRNA levels of IL-1$\beta$, IL-6, TNF-$\alpha$, and IFN-$\gamma$ were found upregulated in jejunum of piglets fed either the mono- or co-contaminated diets. Conversely, addition of FA in these diets allowed the gut epithelium maintaining the cytokine response. Interestingly, IL-6, a cytokine known to drive the synthesis of IgA which is mostly found in the mucosal area, was not upregulated after inclusion of FA in the DON-contaminated diet. This finding may account for the reduced elevation of IgA-induced DON in the plasma of animals fed the DON diet with FA. As widely reported, the DON effect on IgA can lead to the accumulation of this immunoglobulin isotype in the kidney, such as what has been observed in IgA nephropathy.[45] In the present experiment, the expression of tight junctions (TJ) was not analyzed as was previously reported in the intestine of pig exposed to DON or FB.[19,43] However, considering the regulation of TJ by pro-inflammatory cytokines, we can anticipate that FA prevents the alteration of the expression of TJ induced by mycotoxins.

In conclusion, the results presented herein demonstrate that a FA combining two specific biotransformation processes is able to partially to totally neutralize the toxic effects induced by DON and FB, two structurally different mycotoxins from *Fusarium*. Inclusion of FA could allow animals to develop an appropriate immune response following an antigenic challenge. Vaccination and pathogenic challenges are two common situations encountered in animal husbandry, and FA may help the animal to face mycotoxin-induced impairment of the immune response. Inclusion of FA in contaminated feeds may also reduce intestinal disorders (inflammation and malabsorption) due to mycotoxins. More importantly, animals are usually exposed to several mycotoxins at the same time,[13−15] and therefore the data presented here indicate that combination of specific biotransforming enzymes may counteract the toxic effects induced by the coexposure to different mycotoxins.

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### Notes

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## ABBREVIATIONS

DON, deoxynivalenol; FA, feed additive; FB, fumonisins; OVA, ovalbumin; Sa, sphinganine; So, sphingosine

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