ASSESSMENT OF THE POTENTIAL OF A BORON-FRUCTOSE ADDITIVE IN COUNTERACTING THE TOXIC EFFECT OF FUSARIUM MYCOTOXINS

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Running title: Nutritional additives and mycotoxins
ABSTRACT

Trichotecenes are mycotoxins produced by species of genus *Fusarium*, which may contaminate animal and human feeds. A feeding trial was conducted to evaluate the effect of a *fusarotoxins*-contaminated diet and to explore the counteracting potential of a calcium fructoborate additive (CFrB) on performance, typical health biochemistry parameters and immune response in weaned pig. A natural contaminated corn containing low doses of deoxinivalenol, zearalenone, fumonisins and T-2 toxin (1790, 20, 0.6 and 90 ppb) was included in a corn-soybean diet and given *ad libitum* to 8 weaned piglets (2 groups-4 pigs/group) for a period of 24 days. CFrB was administered to one of the contaminated group and to another 4 piglets as a daily supplement following the manufacturer recommendation. At this concentration of feed toxins a decrease in performance was observed in intoxicated animals, which was ameliorated by the dietary CFrB supplementation. *Fusarium* toxins also altered the pig immune response by increasing (P<0.05) the *ex vivo* peripheral mononuclear blood cells proliferation (111.7% in comparison with control), the respiratory burst of porcine granulocytes (15.4% responsive cells vs 5.1% for unstimulated cells and 70.95 vs 22.65% for stimulated cell respectively), the percentage of peripheral T, CD3+, CD3+CD4+ and CD3+CD8+ subsets and the synthesis of IL1-β, TNF-α and IL-8 (123.8, 217.1 and 255.1% respectively). The diet containing the calcium fructoborate additive reduced these exacerbated cellular immune responses induced by *Fusarium* toxins. However, consumption of CFrB did not counteract the effect of mycotoxins on biochemistry parameters and increased plasma IgM, IgG of intoxicated pigs.
INTRODUCTION

Mycotoxins are one of the most frequent natural contaminants of cereals. A list of mycotoxins which are of concern for the safety of animal feed in the European Union (EU32/2003) has been already published. For instance, *Fusarium culmorum* and *Fusarium graminearum* are ubiquitous species of the *Fusarium* genus that can release a large array of mycotoxins: fumonisins, toxin T-2, toxin HT-2, ochratoxin A, deoxinivalenol, zearalenone, etc. (1). Deoxinivalenol (DON) and zearalenone (ZEA) have a particular importance by their high frequency (particularly in the temperate climate) and very high toxic effect at concentrations able to affect the animal health (immunologic effects, susceptibility to infectious diseases) and zootechnical performance (diarrhea, vomiting, anorexia, reduced weight gain, nutrients malabsorption), (2; 3).

Despite the intense efforts of prevention, mycotoxin contamination of feed occurs in the field (4). Therefore various detoxification strategies have been developed during time; these procedures can be grouped into three categories: physical, chemical and biological methods (5). Dietary strategies including biological mitigating agents are one of the most recent approaches in reducing the adverse effects of mycotoxins. The extraordinary development in this area has been lately aided by recent advances in molecular biology, genetic engineering and microbial genomics (6; 7; 8). A lot of studies have investigated the ability of several binding dietary agents (activated carbons, hydrated sodium calcium aluminosilicate, bentonite, zeolites) in reducing the negatives effects of mycotoxins in feed (9; 10; 11). While these researches demonstrated good results against aflatoxins (especially aluminosilicate) their efficacy to bind others toxins or more than one mycotoxins in case of co-contamination must be confirmed. Some of available mycotoxin-binders failed in sequestering the in vitro/in vivo *Fusarium* mycotoxins in pig, for example (12). Recently there has been an increasing interest in the use of bacteria, yeast, and fungi as binders to reduce successfully the toxic effect of mycotoxins (13). It was also shown that substances which do not interact with mycotoxins, i.e. several plant extract and spice oils, antioxidant compounds (selenium, vitamins, provitamins), feed/food components (phenolic compounds, coumarin, chlorophyll and its derivatives, fructose, aspartame) are efficacy in counteracting the toxic effect of contaminants, but again availability data are provided from in vitro studies and especially focus on aflatoxin B1 (14). It is clear that much more work must be conducted to identify especially in vivo the detoxification ability of the above mentioned control agents, their mode of action and their economical and technical feasibility.
Therefore, the objectives of the current study were to evaluate \textit{in vivo} the ability of a nutritional complex compound (a protein concentrate additive enriched in calcium fructoborate-CFrB, a natural ester of boric acid with fructose) in counteracting the toxic effect of feeding a diet naturally contaminated with \textit{Fusarium} toxins (especially DON) in starter pig.

As a great consumer of cereals pig is one of a broad range of farm animals frequently exposed to mycotoxins. It is also considered one of the mammalian species most susceptible to mycotoxins and other contaminants especially during the weaning period, in which changes in nutrition and regrouping are significant and stressors (15). Intake of contaminated feed in this period could leads to decrease of animal disease resistance and increase of infection susceptibility.

The effect of \textit{Fusarium} mycotoxins and of the organic additive on production parameters, organ weights, plasma biochemistry and immune response of peripheral blood cells was investigated. We hypothesize that the use of nutritional complex compounds with polyvalent properties might be promising alternatives in reducing mycotoxin effects.
Experimental animals and diets

Sixteen, crossbred starter piglets, with an average body weight of 11.00 kg ± 0.58 kg were identified by ear tags and housed in floored indoor pens. Piglets were divided into 4 experimental group (4 piglets/group) and randomly assigned to one of the four treatments: M-CFrB- (diet without mycotoxin and without calcium fructoborate additive); M-CFrB+ (diet without mycotoxin, with calcium fructoborate additive); M+CFrB- (mycotoxin contaminated diet without CFrB); M+CFrB+ (mycotoxin contaminated diet with calcium fructoborate additive) for 24 days. The control diet was formulated to meet all nutritional requirements of 8 to 30 kg starter pigs (NRC, 1988) with a total corn content of 53.31 g/kg (Table 1). The mycotoxin contaminated diet was prepared by replacing “uncontaminated” control corn with Fusarium mycotoxins contaminated corn. The contaminated diet contained 1.79 ± 0.56 mg Fusarium toxins/kg compound feed whereas the control diet contained 0.427 ± 0.15 mg Fusarium toxins/kg compound feed.

In order to test the capacity of the mitigating additive in reducing the possible negative effects of mycotoxin contaminated diet, a protein concentrate additive enriched in calcium fructoborate (Vetabor®) was added daily into the diet of intoxicated animals according to the manufacturer instructions (1.65g/pig). Calcium fructoborate (CFrB) has been identified as Ca [(C₆H₁₀O₆)₂B] 2•4H₂O, a natural plant ester of boric acid with fructose. Feed samples were taken at the beginning of the experiment and were analyzed for Fusarium mycotoxins and nutrient content. The respective treatments were administrated for 24 days. At the end of experiment pigs were weighed individually and cumulative feed consumption was measured for each pen. In order to study the effect of various treatments on plasma biochemistry, innate and acquired immune response, blood samples were aseptically collected by jugular venipuncture from all animals (heparinized Vacutainer tubes, Vacutest®, Arzergrande, Italy).

Analysis of mycotoxins

The content of deoxynivalenol (DON) in the feed was analysed by high performance liquid chromatography (HPLC) with UV detection after clean-up with an immune-affinity column and a detection limit of 0.03 mg/kg (Table 1). FB, T2, OTA toxins were analysed by ELISA using ELISA kit Veratox (Neogen, MI, 48912, USA/Canada) with a detection limit of 50, 25, 1, 2 ppb respectively (Table 1).
Plasma biochemical parameters

Plasma concentrations of sodium (Na), potassium (K), chloride (Cl⁻), calcium (Ca), phosphorus (P), manganese (Mg), total protein, urea, glucose, bilirubin, and concentrations of alkaline phosphatase (ALKP), glutamate pyruvate transaminase (TGP), glutamate oxaloacetate transaminase (TGO) were determined on a BS-130 Chemistry analyzer (Bio-Medical Electronics Co., LTD, China).

Plasma total immunoglobulin subsets (IgG, IgA, IgM)

Total concentration of immunoglobulin (Ig) subsets was measured by ELISA (Bethyl, Medist, Montgomery, TX, USA) after plasma dilution: 1/4000 (IgA), 1/60000 (IgG) and 1/6000 (IgM) as previously reported (16) and according to the manufacturer’s instructions. Absorbance was read at 450 nm using a microplate reader (TECAN SUNRISE, Austria).

Isolation of pig PBMC

The effect of *Fusarium* toxins on cellular viability, cell proliferation and T lymphocyte phenotypes has been studied on porcine blood peripheral mononuclear cells (PBMC). Blood collected from all animals was mixed with an equal volume of Dulbecco’s phosphate buffered saline – PBS (Sigma-Aldrich Chemical Co., Steinheim, Germany), than laid over Ficoll-Hypaque (1.077, Sigma) and centrifuged at 2700 tours/min, 20 minutes at room temperature. PBMC were collected, washed twice in PBS and resuspended in RPMI-1640 (Sigma) supplemented with glutamine (2mM), penicillin (100 U. mL⁻¹), streptomycin (50 µg.mL⁻¹) and 10 % foetal calf serum FCS (Sigma). Cells were counted and viability assessed using trypan blue (Sigma).

PBMC proliferation

The ability of isolated and mitogen-activated PBMC to proliferate *ex vivo* was measured by the [methyl⁻³H]-thymidine proliferation assay. PBMC (1x10⁶ cells/mL), stimulated or not with 10 µg/mL concanavalin A (ConA) (Type IV, Sigma, Steinheim, Germany) were cultured for 72 hours at 37⁰ C and 5 % CO₂ in 96-well flat-bottom tissue culture plates (NUNC, Langenselbold, Germany). For the last 18 hrs of cultivation, cells were labeled with 1 µCi /well of [methyl⁻³H]-thymidine and than harvested through glass-fiber filters (Skatron, Sterling, UK). Incorporation of [methyl⁻³H]-thymidine was measured with a
Canberra-Packard Beta Counter (PerkinElmer Life and Analytical Science, Downers Grove, IL, USA) and the results were expressed in counts per minute (cpm).

**Immune phenotyping of lymphocytes**

The percentage of peripheral T lymphocyte subsets CD3+CD4+ and CD3+CD8+ was assessed by flow cytometry. Antibodies were purchased from Becton Dickinson Pharmingen (San Diego, CA, USA). Briefly, 100µL of blood harvested in EDTA-coated vacutainers (Vacutest®, Arzergrande, Italy) were incubated for 20 minutes at room temperature, in the dark with 0.6 µg (1.2 µL) of FITC-conjugated mouse anti-pig CD3ε monoclonal antibody (clone BB23-8E6-8C9) and 0.5µg (2.5 µL) R-PE-conjugated mouse anti-pig CD4a monoclonal antibody (clone 74-12-4) or 0.5µg (2.5 µL) R-PE-conjugated mouse anti-pig CD8a monoclonal antibody (clone 76-2-11). FITC-conjugated mouse IgG2aκ and R-PE-conjugated mouse IgG2bκ were used as isotype controls (clones G155-178 and 27-35). Thereafter, samples were treated 15 minutes with Cell Lyse (BD Biosciences, Heidelberg, Germany) for red blood cells removal. After 2 washes with 2 mL Cell Wash (Becton Dickinson), cells were finally fixed with 400 μL Cell Fix (Becton Dickinson) and analyzed by flow cytometry using a FACSCanto flow-cytometer (Becton Dickinson, New Jersey, USA) and the CellQuest software (Becton Dickinson). At least 10,000 events were analyzed.

**The respiratory burst of granulocytes**

The intracellular production of hydrogen peroxide by peripheral granulocytes was performed by flow-cytometry in whole blood using the fluorogenic substrate dihydrorhodamine (DHRA) 123 (BurstTest kit, ORPEGEN Pharma, Heidelberg, Germany). Briefly, ice-cold heparinised blood (100µL) was activated with unlabeled opsonised *E. coli* for 10 min at 37.0°C. DHRA 123 was then added and incubation continued for another 10 min at 37.0°C. Erythrocytes were lysed and fixed for 20 min at room temperature. Samples were washed twice with washing solution by centrifugation (5 min, 1200 rpm, 4 °C), and supernatant was discarded. Finally, 200 µL of propidium iodide-PI (DNA staining solution) were added whilst samples were kept on ice, in a dark place. Cell analysis was done by flow cytometry using a FACSCanto flow-cytometer (Becton Dickinson, New Jersey, USA) and the CellQuest software (Becton Dickinson). At least 10,000 events were analyzed. By manual gating, we first selected from forward scatter versus side scatter plot the population of granulocytes. Then we selected single cells and we excluded aggregation artifacts, as defined by PI incorporation. Finally, flow-cytometry data were expressed as percentage of responsive.
cells under basal conditions or to a particular *ex vivo* stimulus, meaning the percentage of
cells with fluorescence intensity above a defined threshold (M2). The mean intensity of
cellular response was calculated as geometrical mean of fluorescence channel.

**Measurement of cytokine production**

Samples of liver were weighed and homogenized in phosphate buffer containing
igepal 1%, sodium deoxycholate 0.5%, SDS 0.1% and complete (EDTA-free) protease
inhibitor cocktail tablets. The homogenates were kept 30 min on ice and then centrifuged at
10 000 g at 4°C for 10 min. TNF-α, IL-1β, and IL-8 concentration in the supernatants were
determined by ELISA using the commercially available kits (R&D Systems, Minneapolis,
MN 55413, USA) according to the manufacturer’s instructions. Optical densities were
measured on an ELISA reader (Tecan, Sun Rise, Austria) at a wavelength of 450 nm. Dilution
of recombinant swine TNF-α, IL-1β, and IL-8 were used as standards and data were analyzed
against the linear portion of the generated standard curve. Results were expressed as
picograms of cytokine/mL supernatant.

**Statistic analyses.**

Student’s t-tests were used to analyze the differences in term of weight, plasma
biochemistry parameters, cell proliferation, immunoglobulin synthesis, and so on, between the
*M-CfrB-, M-CfrB+, M+CfrB-*, and *M+CfrB+* groups. P-values of 0.05 were considered
significant.

**RESULTS**

**Animal performance**

We first investigated the effect of dietary treatment on clinical signs and animal
performance. Piglets were fed with control or *Fusarium* mycotoxin contaminated diets (1.79
ppm) and mitigating additive, CFrB for a period of 24 days. Control animals as well as piglets
fed mycotoxin contaminated diet appeared clinically normal during the whole experiment and
no deaths resulted from the intoxication; but, at the end of the feeding period the performance
of the intoxicated piglets were lower than those in the control and CFrB groups (Figure 1).
Indeed, *Fusarium* mycotoxin significantly retarded average daily weight gain (AWG) from
0.458 ± 0.12kg for the control animal to 0.292 ± 0.17kg for the *Fusarium* mycotoxin treated
animals. Gain to feed ration was also reduced in pigs fed contaminated diets (2.312 ± 0.67 for the control vs 3.029 ± 1.43 for the intoxicated pigs). However, this reduction was not significant and the effect was alleviated by the supplementation of feed with the mitigating additive (0.431 ± 0.11kg and 2.912 ± 1.01, respectively).

Organ weights

The weight of internal organs was also measured in animals from the different diet groups. The absolute weights of liver, kidney, spleen and lung were lower in pigs that received the contaminated diet compared with controls (Table 2). The expression of the internal organs as a percentage of body weight was still reduced in the pigs fed contaminated diet (data not shown). The supplementation of the contaminated diets with the fructoborate additive restored the decreased organ weights with a significant difference for lung weight of pigs fed this diet in comparison to that of animals receiving Fusarium mycotoxin diet ($P < 0.05$).

Plasma biochemistry

Biochemistry analysis was also performed in plasma of animals receiving the different diet treatments. Exposure to Fusarium mycotoxin had no effect on the total glucose, urea, phosphorus, alkaline phosphatase. By contrast, the intoxication with mycotoxin has increased significantly plasma concentration of total protein, sodium, calcium and ($P<0.05$) and a decreased tendency in bilirubine and chlorine concentration compared to the control. ASAT and ALAT enzymes increased also from 37.9 ± 4.8U/L and 47.8 ± 9.8 U/L in control groups to 41.8 ± 1.7 U/L and 55.2 ± 1.9 U/L respectively in piglets feeding the contaminated diet (Table 3). The affected biochemistry parameters were not attenuated by the addition of CFrB to the contaminated diet.

Plasma immunoglobulin concentration

Table 4 shows that the concentration of the different plasma immunoglobulin subsets (IgA, IgG, and IgM) ranged within the normal values for this age of animals, but the comparison between the control and Fusarium contaminated diet resulted in 25.9% increase in IgG and 29.8% in IgM level in mycotoxin group 3 at day 24 of the experiment. Ingestion of the diet with CFrB increased with 29.8% and 25.9% the concentration of IgM and IgG suggesting that fructoborate compound per se stimulate the immunoglobulin synthesis; but it was not able to prevent increased plasma immunoglobulin level of pigs that consumed
contaminated diet which remain higher (P<0.05) than the control (3.23±0.6 and 10.17±3.2 vs 2.05±0.4 and 6.36±2.5). A slight decrease in plasma IgA level provoked by DON and reversing by mitigating additive was observed.

Proliferation of peripheral blood mononuclear cell

The [methyl-3H]-thymidine proliferation assay used to determine the ex vivo proliferation of the PBMC derived from pigs fed 24 days with experimental treatments and cultured for 72 hours at 37°C and 5% CO₂ showed a significant effect of Fusarium contaminated diet on cell proliferation which dramatically increased by 111.7% in pig fed this treatment in comparison with control pig (P<0.05). By contrast, the proliferation of PBMC derived from pig receiving contaminated diet and supplemented with mitigating calcium fructoborate compound did not exceed the control level (P<0.05). These porcine PBMC manifested the same tendency of proliferation when they were stimulated in vitro with Con A (Figure 2). In order to verify that the PBMC increased proliferation was associated with an increased cell number, trypan blue exclusion experiment were performed. We observed that the cell number increased from 7.9 ± 1.2 x 10⁶ cells/mL in control PBMC to 15.9 ± 3.9x10⁶ cells/mL in cells derived from mycotoxin treated pigs (P<0.05) and to 14.5 ± 1.0x10⁶ cells/mL in cells of pigs fed contaminated diet and the CFrB supplement (P<0.05). Interestingly, even the number of these cells is higher that of the control, they are less reactive, the [methyl-3H]-thymidine incorporation assay showing a decreased capacity to proliferate.

Immune phenotyping of peripheral T lymphocytes

The effect of DON on T lymphocytes subsets defined by the expression of CD3+, CD3+CD4+ and CD3+CD8+ was measured by flow cytometry with fluorescently labelled anti-CD4 and anti-CD8 antibodies (Figure 3). The results indicated the capacity of the Fusarium mycotoxins diet and especially of that containing the mitigating additive to increase the percentage of peripheral T, CD3+, CD3+CD4+ and CD3+CD8+ subsets of treated pigs in comparison to the control; while the increase provoked by mycotoxin diet was not significant, that produced by fructoborate additive was significant (P<0.05). The addition of the organic additive to the contaminated feed determined a cumulative effect on the CD3+ (P<0.05) and CD3+CD8+ (P<0.05) lymphocyte subsets percentage which increased to 133.25 and 142.70% in comparison to the control.
The respiratory burst of granulocytes

To assess the effect of mycotoxins on innate immune response the intracellular production of hydrogen peroxide induced ex vivo by opsonised *E.coli* was evaluated by flow cytometry in peripheral granulocytes derived from different diet groups (Figure 4). Mycotoxins increased the percentage of responsive cells in both unstimulated (15.4% vs 5.1% in control) and especially under stimulated conditions (70.95 vs 22.65% for the control). The diet containing the calcium fructoborate additive seemed to normalise the exacerbated cellular immune response induced by *Fusarium* toxin (decreased with 42.6%), (*P*< 0.05).

Liver cytokines synthesis

As expected, a synthesis of IL1-β, TNF-α and IL-8 was revealed by ELISA measurement in liver of pigs but this cytokine production was altered in samples from animals which ingested the mycotoxins. As already observed for other immune parameters the exposure for 24 days to *Fusarium* mycotoxins increased the synthesis of IL1-β, TNF-α and IL-8 by 123.8, 217.1 and 255.1% respectively; the increase was significant for IL-8 and TNF-α in comparison to the control (*M-CFrB-*) and mitigating additive group (*M-CFrB+*), (*P*<0.05). In contrast, the ingestion of the toxin associated with the fructoborate supplement significantly decreased (*P*<0.05) the synthesis of investigated cytokines (Figure 5).

DISCUSSION

Feed consumption is the first zootechnical parameters affected by Fusarium mycotoxins which decrease for example for DON from level higher than 1ppm in the feed of pigs (17; 18). A direct consequence of the reduced feed intake is the alteration of pig growth rate (19; 20; 21). Indeed, we demonstrated in the present study that growth rate of pigs fed contaminated diet with *Fusarium* mycotoxin, mostly DON, over 24 days decreased with 36.2% compared to the controls and feed efficiency increased with 30.98%, confirming that the alteration of feed consumption represents the main effect of DON on pig. The lowered performance induced by *Fusarium* toxins was alleviated in the pigs of M+CFrB+ group following the supplementation with fructose and boron compound. Our results are in agreement with those of Armstrong et al., (22) and Armstrong and Spears (23) who also observed an increase in AWG and feed intake in pigs that consumed boron supplemented...
diets following an i.m. injection of LPS. Other studies testing the efficacy of polysaccharides products showed either beneficial or no effect for DON counteracting. Dried apple pomace (a by-product of apple juice production rich in pectin and other non starch polysaccharides) incorporated at the level of 8% in *Fusarium* mycotoxins contaminated feed (3100 ppb DON and 65 ppb ZEA) was able to restore the growth rate of weaner piglets (24) while polymeric glucomannan (0.05, 0.1, 0.2 and 1% in feed) had no beneficial effect on zootechnical performance of growing pigs fed DON contaminated diet (19; 25). However, in these studies the level of mycotoxin was higher than in the present work (5.5, 2-2.5 ppm respectively).

The supplementation of calcium fructoborate compound to the DON diet counteracted organ weight alterations in the current study, but it had no beneficial effect on the biochemistry parameters modified by mycotoxin. The inability of 0.1 and 0.2% of dietary glucomannan polymer to prevent some of the *Fusarium*-induced alterations in serum chemistry and even the lower percentage of liver weight in pig was also reported by Swamy et al., (19). In spite of that, these authors observed that the same concentrations of glucomannan polymer were more effective in reversing the alterations in hematology, serum chemistry and biliary IgA concentrations in broiler chickens (26).

Similar to other mycotoxins, the presence of DON in animal feed could have serious consequences on their immune functions (27). Many *in vivo* and *in vitro* studies have described effects of DON on cellular and humoral response leading to the alteration of immunoglobulin and cytokine synthesis, decreased lymphocyte proliferation, etc (28; 29). Of particular interest is the effect of DON on antibody synthesis which seems to be related to the induction of certain cytokines such as IL-2, IL-5 and IL-6 at Payer’s patch level (3). In pig, the results are contradictory showing either an increase in serum Ig (30; 31; 32; 33) or no effect in Ig levels (34; 35; 36; 37). In our experiment the feeding of contaminated diet to pig increased significantly (P<0.05) plasma IgG and IgM concentration and did not influence plasma IgA level (Table 4). The elevated IgM and IgG concentration provoked by *Fusarium* toxins in the present investigation are in accordance with the experiments conducted by Goyarts et al., (31), Swamy et al., (19) and Pinton et al., (32) with growing pig fed higher concentration of DON (5.7, 5.5 and 2.5 ppm of DON respectively). A stimulative effect on humoral and cellular immune response was also determined by calcium fructoborate alone (increased plasma IgG and IgM concentration and increased percentage of CD4+ and CD8+ lymphocytes subsets), but the additive was not effective in preventing these increased parameters in pigs that consumed *Fusarium* toxins contaminated diet (Table 4, Figure 3).
contrast to our results the supplementation of *Fusarium* contaminated feed with glucomannan was able to counteract the increased IgM and IgG in the experiment of Swamy et al., (19).

It was shown that DON increases the synthesis of interleukin 2 (37; 19), cytokine with a key role in cell proliferation; this fact could explain the effect of DON on lymphocytes proliferation observed in the present and in other similar works. Thus, a series of studies have already showed the non specific capacity of lymphocytes to proliferate is dramatically increased in the growing pigs receiving 1.6 and 3.6 ppm DON (38; 39). Data from the current trial showed that the oral administration of 1790 ppb of DON for 24 days resulted in significantly higher *ex vivo* proliferation response of peripheral blood lymphocytes to a mitogen stimulation or under basal conditions (P<0.05). The dietary fructoborate supplementation was very effective to reverse the alteration in PBMC proliferation as well as in the respiratory burst of circulating granulocytes and in the production of liver pro-inflammatory cytokines, important aspects of the immune response modified under the mycotoxin action in the present trial. *In vitro* studies of Scorei et al., (40; 41) highlighted the anti-inflammatory and antioxidant properties of the fructoborate showing that the addition of calcium fructoborate (0.2–1 mM) to cultured RAW 264.7 macrophages reduced the secretion of IL-6, IL-1 beta and NO by LPS-stimulated cells in a dose-dependent manner and markedly diminished the intracellular reactive oxygen species (ROS) in keratinocytes. These authors support the hypothesis that the soluble carbohydrate compounds of boron formed by the complexion of boric acid with free glucydes, glycolipids, and glycoproteins, buffer the reactive species of oxygen by developing organic peroxyborates. Other authors (42; 43; 44) also suggested that boron decreased the production of hydrogen peroxide generated during the respiratory burst by the up-regulating the activity of some enzymes (superoxide dismutase and glutathione peroxidise) involved in the respiratory burst cascade. In our feeding experiment, the dietary boron compound supplementation significantly decreased the synthesis of liver TNF-α and IL-8 and the exacerbated respiratory burst developed in the peripheral granulocytes.

To our knowledge, boron esters (fructoborate) or other feeds containing fructose have never been tested as possible antidotes to mycotoxins in pigs. We consider that its efficacy to alleviate the performance and some aspects of the immune response of pigs altered by *Fusarium* toxins may derive from the specific and various properties of its components (proteins, boron and fructose) and from the mechanism that such complex compound could start. It was hypothesised that carbohydrates might trap the mycotoxin molecule preventing toxin absorption and might have beneficial effect at the intestine level (increase the amount of
water in the gut lumen, induce higher rates of protein synthesis in small intestinal mucosal cells and stimulate cell division) (24). Boron provides support (cofactor) to important enzymes involved in the antioxidant processes and demonstrated immunomodulatory function (anti-inflammatory and Ig-stimulant) which enhanced the resistance of animals to mycotoxin. Boron is implicated also in calcium metabolism and these two elements affect similar systems including cell membrane characteristics, trans-membrane signalling etc (45). Although we can conclude that the addition of complex compounds to animal feed provides versatile tools to prevent mycotoxicosis, this study has to be completed with further research aiming to establish whether fructoborate additive remains efficient against a higher dose of mycotoxin or under mycotoxin co-contamination conditions.

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FIGURE LEGEND

Figure 1. Influence of dietary mycotoxins and CFrB additive on body weight.
Pigs received control diet (□), CFrB additive diet (■) or diet contaminated with Fusarium mycotoxins (▲) or Fusarium mycotoxins and CFrB additive (■). Animals were weighed at the end of the feeding trial (24d) and results are expressed as mean body weight (n=4) ± SEM. Student's t-tests were performed to compare the different groups. The table presents the performance (AWG and feed:gain ratio) obtained by pigs under the action of the four treatment.

Figure 2. Effect of dietary mycotoxins and CFrB additive on PBMC ex vivo proliferation.
PBMC derived from pigs receiving control diet (□), CFrB additive diet (■) or diet contaminated with Fusarium mycotoxins (▲) or Fusarium mycotoxins and CFrB additive (■) were stimulated or not with 10 µg/mL concanavalin A (ConA) and cultured (1x10^6 cells/mL) for 72 hours at 37°C and 5% CO2. The ex vivo proliferation of PBMC was measured by the [methyl-^3H]-thymidine incorporation assay and the results are expressed in counts per minute (cpm) (n=4) ± SEM. Student's t-tests were performed to compare PBMC proliferation in control and treated animals; a,b,c = statistical difference between control and treated animals under basal condition, P < 0.05; *statistical difference between contaminated diet and contaminated diet plus CFrB additive in ConA stimulated condition, P < 0.05.

Figure 3. Flow cytometric identification of CD3+ (□), CD3+CD4+ (□) and CD3+CD8+ (□) blood T lymphocytes following treatments with Fusarium mycotoxins and CFrB additive.
Blood samples taken after 24 days from pigs fed M-CFrB- diet, M-CFrB+ diet or M+CFrB- diet and M+CFrB+ diet were stained for CD3ε, CD4a and CD8a subsets and analyzed by flow cytometry. Data (mean ± SEM) represents the average of the percentage of CD3+ (□), CD3+CD4+ (□) and CD3+CD8+ (□) blood T lymphocytes at 24 days following treatments with Fusarium mycotoxins and CFrB additive. Student's t-tests were performed to compare T
cell subsets percentage between control and treated animals; a,b,c = statistical difference $P < 0.05$.

**Figure 4. Effect of dietary mycotoxins and CFrB additive on respiratory burst of circulating granulocytes.**

Ice-cold heparinized blood (100 µL) was activated with unlabeled opsonized *E. coli* for 10 min at 37.0°C. The intracellular production of hydrogen peroxide by peripheral granulocytes was performed by flow-cytometry in whole blood using the fluorogenic substrate dihydrorhodamine (DHRA) 123 and propidium iodide-PI as DNA staining solution. Cell analysis was done by flow cytometry using the CellQuest software. At least 10,000 events were analyzed. Data were expressed as percentage of responsive cells under basal conditions or in response to a particular ex vivo stimulus (*E. coli*), meaning the percentage of cells with fluorescence intensity above a defined threshold (M2).

**Figure 5. Effect of dietary mycotoxins and CFrB additive on the synthesis of cytokines, IL-8, TNF-α and IL-1β, in liver of pigs.**

Samples of liver were weighed and homogenized in phosphate buffer containing igepal 1%, sodium deoxycholate 0.5%, SDS 0.1% and complete (EDTA-free) protease inhibitor cocktail tablets. The homogenates were kept 30 min on ice and then centrifuged at 10,000 g at 4°C for 10 min. TNF-α, IL-1β, and IL-8 concentration in the supernatants were determined by ELISA using R&D Systems kits (according to the manufacturer’s instructions. Optical densities were measured on an ELISA reader (Tecan, Sun Rise, Austria) at a wavelength of 450 nm.

a,b statistical difference between treatments, $P < 0.05$. 
Tabel 1. Composition of experimental diet (%)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control</th>
<th>Contaminated diet</th>
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<tbody>
<tr>
<td>Wheat</td>
<td>15.00</td>
<td>15.00</td>
</tr>
<tr>
<td>Corn</td>
<td>53.31</td>
<td>0.00</td>
</tr>
<tr>
<td>Contaminated corn</td>
<td>0.00</td>
<td>53.31</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>3.00</td>
<td>8300</td>
</tr>
<tr>
<td>Sunflower meal</td>
<td>8.00</td>
<td>8.00</td>
</tr>
<tr>
<td>Powder milk</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Gluten</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Full fat Soybean</td>
<td>9.00</td>
<td>9.00</td>
</tr>
<tr>
<td>Salt</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Monocalcium phosphate</td>
<td>1.30</td>
<td>1.30</td>
</tr>
<tr>
<td>Feed grade limestone</td>
<td>1.60</td>
<td>1.60</td>
</tr>
<tr>
<td>Methionine premix</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Lysine premix</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Choline premix</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Vitamin mineral premix 1</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Analyzed composition

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Contaminated diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein (g/kg)</td>
<td>164.0</td>
<td>164.0</td>
</tr>
</tbody>
</table>

Mycotoxins (µg/kg)

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>427</td>
<td>1790</td>
</tr>
<tr>
<td>ZEA</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>FB</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>T-2</td>
<td>50</td>
<td>90</td>
</tr>
<tr>
<td>OTA</td>
<td>0.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>

1Vitamin-mineral premix / kg diet: 0-24 days: 10,000 UI vit.A; 2000 vit. D; 30 UI vit. E; 2 mg vit. K; 1.96 mg vit. B₁; 3.84 mg vit. B₂; 14.85 mg pantothenic ac.; 19.2 mg nicotinic ac; 2.94 mg vit. B₆; 0.98 mg folic ac; 0.03 mg vit.B₁₂; 0.06 biotin; 24.5 mg vit.C; 40.3 mg Mn; 100 mg Fe; 100 mg Cu; 100 mg Zn; 0.38 I; 0.23 mg Se.
Tab. 2. Effect of *Fusarium* mycotoxins and calcium fructoborate additive on organs weight

<table>
<thead>
<tr>
<th></th>
<th>M-CFrB-</th>
<th>M-CFrB+</th>
<th>M+CFrB-</th>
<th>M+CFrB+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>655.0 ± 30.1</td>
<td>506.7 ± 71.3</td>
<td>481.7 ± 76.9</td>
<td>555.0 ± 44.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>103.3 ± 13.3</td>
<td>100.0 ± 15.3</td>
<td>95.0 ± 12.6</td>
<td>81.7 ± 21.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>46.7 ± 4.4</td>
<td>41.7 ± 8.8</td>
<td>35.0 ± 5.8</td>
<td>46.7 ± 10.9</td>
</tr>
<tr>
<td>Lung</td>
<td>251.7 ± 6.7</td>
<td>215.0 ± 43.7</td>
<td>196.7 ± 24.2</td>
<td>308.3 * ± 29.1</td>
</tr>
</tbody>
</table>

*=Mean value was significantly different from that of the control group: *P<0.05
Table 3. Effect of dietary *Fusarium* mycotoxins and Fructoborate additive administration on selected blood biochemical parameters in piglets*

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>M-CFrB-</th>
<th>M-CFrB+</th>
<th>M+CFrB</th>
<th>M+CFrB+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mg/dL)</td>
<td>139.5b ± 2.0</td>
<td>193.0a ± 19.6</td>
<td>209.7a ± 13.4</td>
<td>229.0a ± 13.9</td>
</tr>
<tr>
<td>Chloride (mg/dL)</td>
<td>110.0 ± 1.6</td>
<td>74.5 ± 15.9</td>
<td>87.3 ± 10.4</td>
<td>99.0 ± 8.6</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>10.3b ± 0.4</td>
<td>9.9b ± 0.5</td>
<td>11.1a ± 0.2</td>
<td>10.8ab ± 0.3</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>9.3b ± 0.1</td>
<td>10.2ab ± 0.6</td>
<td>9.1b ± 0.4</td>
<td>11.0c ± 0.3</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>5.1c ± 0.2</td>
<td>5.0c ± 0.2</td>
<td>5.8b ± 0.1</td>
<td>6.6a ± 0.3</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>20.4ab ± 4.3</td>
<td>15.0b ± 1.6</td>
<td>28.3a ± 2.9</td>
<td>15.5b ± 0.4</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>93.5 ± 2.9</td>
<td>89.5 ± 14.9</td>
<td>96.0 ± 6.1</td>
<td>82.5 ± 6.9</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.06 ± 0.0</td>
<td>0.06 ± 0.0</td>
<td>0.04 ± 0.0</td>
<td>0.08 ± 0.0</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>149.0 ± 30.6</td>
<td>144.0 ± 8.9</td>
<td>140.0 ± 9.8</td>
<td>137.5 ± 5.3</td>
</tr>
<tr>
<td>Aspartate aminotransferase (IU/L)</td>
<td>47.8 ± 9.8</td>
<td>57.4 ± 1.6</td>
<td>55.2 ± 1.9</td>
<td>63.0 ± 0.1</td>
</tr>
<tr>
<td>Alanine aminotransferase (IU/L)</td>
<td>37.9 ± 4.8</td>
<td>47.8 ± 1.3</td>
<td>41.8 ± 1.7</td>
<td>38.2 ± 7.7</td>
</tr>
</tbody>
</table>

* At the end of the experiment plasma from the piglets (4/group) was used to measure the blood biochemical parameters. Data are means ± SEM. Comparison between control and treated animals, a,b,c = Mean values within a row with unlike superscript letters were significantly different (P<0.05)
Tabel 4. Immunological values in pigs fed diets contaminated with *Fusarium* mycotoxins and fructoborate additive

<table>
<thead>
<tr>
<th></th>
<th>M-CFrB⁻</th>
<th>M-CFrB⁺</th>
<th>M+CFrB⁻</th>
<th>M+CFrB⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig A (mg/mL)</td>
<td>0.77 ± 0.1</td>
<td>0.63 ± 0.0</td>
<td>0.74 ± 0.0</td>
<td>0.71 ± 0.1</td>
</tr>
<tr>
<td>Ig M (mg/mL)</td>
<td>2.05ᵇ ± 0.3</td>
<td>2.66ᵃᵇ ± 0.1</td>
<td>2.43ᵇᶜ ± 0.1</td>
<td>3.23ᵃ ± 0.3</td>
</tr>
<tr>
<td>Ig G (mg/mL)</td>
<td>6.36ᵇ ± 1.5</td>
<td>8.01ᵃᵇ ± 1.0</td>
<td>7.81ᵃᵇ ± 1.0</td>
<td>10.17ᵃ ± 1.9</td>
</tr>
</tbody>
</table>

*Pigs were fed for 24 days with a control diet or a diet contaminated with *Fusarium* mycotoxins (179 ppb of DON). Results are expressed as Ig A, G or M content in the plasma of piglets, mean ± SEM (n= 4). Student's t-tests were realized to analyze the effect of mycotoxin treatment on immunoglobulin levels.ᵃᵇᶜ = Mean values within a row with unlike superscript letters were significantly different (P<0.05).
Groups | AWG | Gain:Feed
--- | --- | ---
M-CFrB- | 0.458 ± 0.12 | 2.312 ± 0.67
M-CFrB+ | 0.511 ± 0.05 | 1.803 ± 0.12
M+CFrB- | 0.292 ± 0.17 | 3.029 ± 1.43
M+CFrB- | 0.431 ± 0.11 | 2.912 ± 1.01
Taranu et al. Fig. 3

The figure shows a bar graph depicting the percentage of cells for different conditions labeled as M-CFrB-, M-CFrB+, M+CFrB-, and M+CFrB+. The x-axis represents these conditions, and the y-axis represents the percentage of cells. The graph includes error bars, indicating variability. The figure legend indicates groups labeled as CD3+, CD4+, and CD8+.
Taranu et al. Fig. 4

- %PMN responsiveness
- No stimulus
- E.coli

Groups:
- M-CFrB-
- M-CFrB+
- M+CFrB-
- M+CFrB+

Significance:
- a
- b
- c
Taranu et al. Fig. 5

The graph shows the concentration of IL-1β, IL-8, and TNF-alfa in different conditions. The concentrations are measured in pg/mL. The bars labeled with 'M-CFrB-' and 'M-CFrB+' indicate different groups, with 'M+CFrB-' and 'M+CFrB+' showing the effect of another factor. The bars are labeled with letters 'a' and 'b' to indicate statistical significance. The graph suggests that there are significant differences in the concentration of these cytokines under the different conditions.