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Assessment of the Multi-mycotoxin-Binding Efficacy of a Carbon/Aluminosilicate-Based Product in an in Vitro Gastrointestinal Model

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A laboratory model, set to simulate the in vivo conditions of the porcine gastrointestinal tract, was used to study the small intestinal absorption of several mycotoxins and the effectiveness of Standard Q/FIS (a carbon/aluminosilicate-based product) in reducing mycotoxin absorption when added to multitoxin-contaminated diets. Mycotoxins were quickly absorbed in the proximal part of the small intestine at levels of 105 and 89% for fumonisins B_1 and B_2, respectively, 87% for ochratoxin A, 74% for deoxynivalenol, 44% for aflatoxin B_1, and 25% for zearalenone. Addition of Standard Q/FIS to the diet (up to 2%, w/w) significantly reduced mycotoxin absorption, in a dose-dependent manner, up to 88% for aflatoxin B_1, 44% for zearalenone, and 29% for the fumonisins and ochratoxin. Standard Q/FIS was ineffective in reducing deoxynivalenol uptake. These findings suggest that Standard Q/FIS can be used as a multitoxin adsorbent material to prevent the individual and combined adverse effects of mycotoxins in animals.

KEYWORDS: Mycotoxins; aflatoxins; ochratoxins; fumonisins, deoxynivalenol; zearalenone; mycotoxin binders; mycotoxin detoxification

INTRODUCTION

In animal feed, mycotoxins rarely occur as single contaminants. Many mycotoxigenic fungi can grow and produce their toxic metabolites under similar conditions. Furthermore, animal feed is made from several grain sources, which may be contaminated by different mycotoxins. Several combinations of mycotoxins frequently occur, as reported in monitoring programs (*1*, *2*), such as the co-occurrence of aflatoxin B_1 with ochratoxin A or that of deoxynivalenol with zearalenone, nivalenol, or other *Fusarium* toxins. Intake of combinations of mycotoxins may lead to interactive toxic effects (*1*, *2*). In swine the combination of aflatoxin B_1 and ochratoxin A may show additive or less than additive effects (*3*), whereas the combination of deoxynivalenol and fumonisin B_1 or deoxynivalenol and fusaric acid shows synergistic interactions (*4*, *5*). Additive toxic effects have been observed in vitro for most combinations of trichothecenes (*2*).

Many attempts have been made to detoxify/decontaminate mycotoxin-contaminated feedstuffs, with little success. A strategy for mycotoxin decontamination involves the addition to the diet of non-nutritive adsorbent materials that tightly bind mycotoxins in the gastrointestinal tract of animals. The extensive use of adsorbent materials in the livestock industry has led to the introduction of a wide range of new products onto the market, which still have no legal status in the European Union (EU) market. Before any feed additive is applied to prevent mycotoxin intoxication, it is essential to establish its reliability and safety while considering the economic feasibility. Mineral clay products such as bentonites, zeolites, and aluminosilicates are the most common feed additives, which are effective in binding/adsorbing aflatoxins (*6*). When saturated with water, the surfaces of these additives attract the polar functional groups of the mycotoxin and trap it on their surface. This isolates the mycotoxin from the digestive process and thereby inhibits its absorption (*7*). Hydrated sodium calcium aluminosilicates at 1.0% of the feed can significantly diminish the adverse effects of aflatoxins in young animals (*7*). Aluminosilicates are allowed by the U.S. Food and Drug Administration when used at a level up to 2% in complete diets as "anti-caking" agents. Aluminosilicates, however, show a number of disadvantages, not least being the impairment of mineral utilization at levels above 2% (*8*) and a narrow range of binding efficacy. In animals, aluminosilicates are selective in their "chemisorption" of aflatoxins and show little or no beneficial effect against zearalenone, fumonisin B_1, ochratoxin A, and trichothecenes, including deoxynivalenol, T-2 toxin, or diacetoxyscirpenol (*9*). In contrast, activated carbons have shown their efficacy in in vivo studies for aflatoxins, ochratoxin A, diacetoxyscirpenol, and T-2 toxin (*9*) and in experiments in vitro using a gastrointestinal model for deoxynivalenol, nivalenol, and zearalenone (*10*−*12*). The latter experiments have recently been performed in our laboratory with a dynamic, computer-

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controlled model (TIM system) simulating the upper gastrointestinal tract of healthy pigs. This gastrointestinal tract model was developed at TNO (Zeist, The Netherlands) (*13*). Validation studies on mycotoxins and sequestrants show a high predictive quality (*10*, *11*, *14*). In our previous studies, the model was used to evaluate the intestinal absorption of zearalenone, deoxynivalenol, and nivalenol, and the effectiveness of activated carbon and cholestyramine in binding mycotoxins (*10*, *11*). The results on the inhibition of intestinal absorption of zearalenone, deoxynivalenol, and nivalenol from contaminated pig feed, with and without these mycotoxin binders, are consistent with pharmacokinetic data from pigs. This means that the TIM system is an effective tool for determining the bioaccessibility of mycotoxins from contaminated feeds and for testing the efficacy of mycotoxin binders in inhibiting this bioaccessibility.

In view of these innovative results and the current importance of investigating the efficacy of adsorbent materials in preventing the biological effects of co-occurring mycotoxins, we performed a study to assess the multitoxin binding reliability of a carbon/aluminosilicate-based product, named "Standard Q/FIS". The TIM system was used to estimate the simultaneous intestinal absorption of aflatoxin B_1 and ochratoxin A from artificially contaminated corn and of deoxynivalenol, zearalenone, fumonisin B_1, and fumonisin B_2 from a blend of naturally contaminated grains. Three levels of Standard Q/FIS were included in the multitoxin-contaminated feeds and tested in the TIM system for their ability to bind mycotoxins, thus reducing their intestinal absorption. Preliminary in vitro tests were also performed to screen the potential mycotoxin-binding capacities of Standard Q/FIS.

MATERIALS AND METHODS

Reagents and Chemicals. All chemicals used were of analytical grade unless otherwise stated. All solvents (HPLC grade) and chemicals were purchased from J. T. Baker (Deventer, The Netherlands). Water was of Milli-Q quality (Millipore, Bedford, MA).

Six mycotoxin standards (aflatoxin B_1, ochratoxin A, fumonisin B_1, fumonisin B_2, zearalenone, and deoxynivalenol) were supplied by Sigma-Aldrich (Milan, Italy). Stock solutions of mycotoxins were prepared by dissolving 0.5 mg/mL of toxin in toluene/acetone (90:10, v/v) for ochratoxin A, toluene/acetonitrile (98:2, v/v) for aflatoxin B_1, acetonitrile/water (1:1, v/v) for fumonisins, and methanol for zearalenone and deoxynivalenol. Standard solutions were prepared at different concentrations in distilled water for the in vitro tests, in methanol for spiking purposes, and in the mobile phase for HPLC calibration.

Standard Q/FIS, a mixture of six components mainly based on carbon and aluminosilicate, was obtained from Feed Industry Service (F.I.S. S.r.l., Lodi, Italy).

Adsorption in Vitro Screening Tests. Eighteen adsorbent materials, including commercial feed additives (Standard Q/FIS, Myco AD A-Z, Mycosorb, CAB 70, Zeovet, Micoprev, Flo Bond, Microton) and silicate minerals (such as phyllosilicates and tectosilicates) were tested in vitro in aqueous solution for their ability to adsorb ochratoxin A and aflatoxin B_1 (**Table 1**). The adsorbent materials were obtained from sources reported in **Table 1**. The binding capacity of 1 mg of adsorbent materials per milliliter of buffer at pH 7 was tested in triplicate with 1, 2, and 10 μg of mycotoxin/mL. After shaking for 1 h at room temperature, the adsorbent materials were separated by centrifugation at 10000 rpm for 10 min. The supernatants were transferred to clean tubes and analyzed for ochratoxin A and aflatoxin B_1 by HPLC as reported below. The amount of bound mycotoxin was calculated for each material as the difference between the initial and the final concentration of mycotoxin in the testing solution and expressed as a percentage of the initial concentration.

To obtain adsorption isotherms for Standard Q/FIS, 1 mL of an aqueous solution containing ochratoxin A or aflatoxin B_1 at a wider range of concentrations was incubated with 1 mg of adsorbent and analyzed by HPLC as reported below. To avoid the problem of solubilizing high concentrations of mycotoxins in water, which may result in precipitation of the toxins, solutions above 10 μg/mL were prepared in ethanol 10% (v/v). Adsorption isotherms were obtained by plotting the amount of adsorbed mycotoxin (mg of toxin/g of adsorbent) against the equilibrium non-adsorbed toxin concentration (mg/L). The binding capacity (B_{max}) was determined by plotting the double-reciprocal transformation of the following data: amount of adsorbed mycotoxin (mg of toxin/g of adsorbent) versus initial mycotoxin concentration (mg/L). B_{max} was derived from the calculated *y*-intercept of the linear regression line through the plot.

Table 1. In Vitro Ability of Adsorbent Materials (0.1% w/v) To Adsorb Ochratoxin A (2 μg/mL) and Aflatoxin B_1 (1 or 10 μg/mL) at pH 7

	adsorbed mycotoxin[a] (%) (mean ± SD)			
	ochratoxin A	aflatoxin B_1		
feed additive	2 μg/mL	1 μg/mL	10 μg/mL	source[b]
Standard Q/FIS	97 ± 0.5	97 ± 0.1	96 ± 1.0	A
Myco AD A–Z	89 ± 2.0	99 ± 0.4	99 ± 0.1	B
cholestyramine	94 ± 0.4	–[c]	–	C
activated carbon	94 ± 0.6	–	–	C
Mycosorb	42 ± 2.5	–	–	D
Attapulgite[d]	26 ± 1.8	80 ± 2.0	74 ± 3.4	A
sodium bentonite[d]	17 ± 1.2	92 ± 3.5	96 ± 0.1	A
Greek bentonite[d]	14 ± 4.8	97 ± 0.4	100 ± 0.3	A
activated bentonite[d]	9 ± 1.7	98 ± 1.5	98 ± 0.8	A
Indian bentonite[d]	6 ± 1.4	91 ± 0.5	98 ± 1.1	A
Clinoptilolite[e]	20 ± 1.5	61 ± 1.3	40 ± 0.6	A
CAB 70[e]	19 ± 1.4	26 ± 5.2	8 ± 0.8	A
Zeovet[e]	30 ± 2.1	–	–	A
Celite	25 ± 2.4	–	–	C
Micoprev[e]	21 ± 1.5	–	–	A
Flo Bond[e]	15 ± 1.3	–	–	E
Microton[e]	14 ± 4.0	–	–	F
glucomannan	7 ± 3.7	–	–	G

[a] Values are means of three replicates. [b] Adsorbent material suppliers: A, Feed Industry Service, Lodi, Italy; B, Special Nutrients Inc., Miami, FL; C, Sigma-Aldrich, Milan, Italy; D, Alltech Ltd., Stamford, U.K.; E, Agri-Tec, Amarillo, TX; F, Estelar, Sao Paulo, Brazil; G, Dan Shen s.a.s., Milan, Italy. [c] Not tested. [d] Phyllosilicate. [e] Tectosilicate.

Adsorption Experiments with the Dynamic Gastrointestinal Model. *Dynamic in Vitro Gastrointestinal Model.* The TIM system, a computer-controlled laboratory model simulating the gastrointestinal conditions of pigs, was used to evaluate the intestinal absorption of ochratoxin A, aflatoxin B_1, zearalenone, deoxynivalenol, and fumonisins B_1 and B_2 from multi-mycotoxin-contaminated feeds and the efficacy of Standard Q/FIS in reducing their bioaccessibility from the small intestine. The setup of the TIM experiments for mycotoxin studies has been described by Avantaggiato et al. (*10*, *11*). In the TIM system, the gastrointestinal temperature, secretion, and pH values, as well as peristalsis and transit of the chyme, simulated the conditions of pigs (**Figure 1**). Products of digestion and water were absorbed from the jejunal and ileal compartments by pumping dialysis liquid through hollow fiber membranes with a molecular weight cutoff of approximately 5000. Mycotoxins released from the feed matrix during digestion could be either absorbed via the semipermeable membrane systems or bound to the adsorbent and passed through the gastrointestinal model without being absorbed or adsorbed.

Preparation of Multi-mycotoxin-Contaminated Feeds. For feeding the TIM system, two multi-mycotoxin-contaminated feeds were prepared. Due to the difficulty in finding high levels of aflatoxin B_1 and ochratoxin A in naturally contaminated grains, an artificially contaminated feed was made by spiking blank corn with standard solutions of toxins in methanol. Three hundred and twenty microliters of aflatoxin B_1 (1.25 mg/mL) and 200 μL of ochratoxin A (2 mg/mL) spiking solutions were spread on 200 g of finely ground blank corn (control, containing <0.1 μg/kg aflatoxin B_1 and <1 μg/kg ochratoxin A). The spiked material was left overnight to allow solvent evaporation and then blended nine times with an additional 200 g of blank material. To

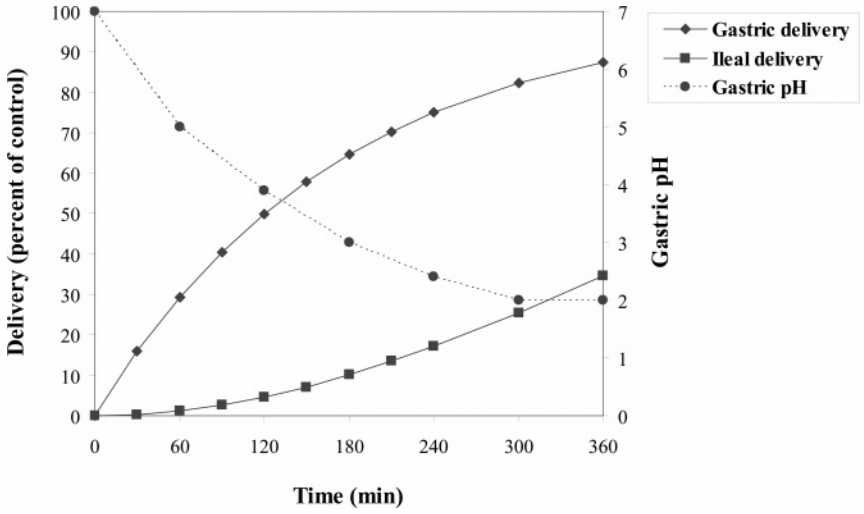

Figure 1. Simulation in the TIM system of the gastric emptying and gastric pH curve and the ileal effluent of the pig feed.

check the homogeneity of the 2 kg corn batch, five samples were taken randomly and analyzed for aflatoxin B_1 and ochratoxin A as described by Stroka et al. (*15*) and Entwisle et al. (*16*), respectively. The performance characteristics of these analytical methods were checked in our laboratory by spiking blank corn with 0.2 mg/kg of aflatoxin B_1 or ochratoxin A. Mean recoveries ($n = 3$) and coefficients of variation were, respectively, 96 and 3% for aflatoxin B_1 and 95 and 4% for ochratoxin A.

The *Fusarium* mycotoxin contaminated feed, containing zearalenone, deoxynivalenol, and fumonisins B_1 and B_2, was prepared by blending fungal cultures and naturally contaminated grains as reported below. For zearalenone contamination, one fungal isolate of *F. graminearum* from the Spanish Collection of Type Cultures (CRCT reference 20486) was used to inoculate wheat samples. Before use, the fungal strain was grown on Petri dishes containing potato dextrose agar medium (PDA) (Oxoid, Basing-stoke, Hampshire, U.K.) for 7 days. Wheat grain was purchased in a retail market and then analyzed to assess the absence of mycotoxins. Three samples of 100 g of cereal kernels were placed in 250 mL Erlenmeyer flasks and moistened overnight at 4 °C after the addition of about 30% water, followed by autoclaving for 20 min at 120 °C. After that, wheat samples were inoculated with pieces of PDA single-spore cultures and maintained at 20 °C for 4 weeks in closed chambers. After the incubation period, cultures were dried at 50 °C for 48 h and finely ground with a laboratory mill. The ground wheat cultures were homogenized and analyzed for zearalenone and trichothecenes (type B) as described by Visconti and Pascale (*17*) and Avantaggiato et al. (*11*), respectively. To prepare the multi-mycotoxin-contaminated feed (2 kg) the following grains were used: 25 g of *Fusarium graminearum* (CRCT 20486 strain) wheat culture prepared as above and containing 88.0 ± 0.6 mg/kg zearalenone ($n = 3$) and <10 μg/kg of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, or Fusarenon-X; 1100 g of naturally contaminated durum wheat containing 10.0 ± 0.2 mg/kg deoxynivalenol ($n = 3$) and 215 g of naturally contaminated corn containing 146.4 ± 0.5 mg/kg fumonisin B_1 plus 45.3 ± 0.3 mg/kg fumonisin B_2 ($n = 3$), obtained from our laboratory (ISPA) following experimental field trials of inoculum with *Fusarium* strains; 660 g of blank wheat. All of these grains were finely ground with a laboratory mill and homogenized. To check the homogeneity of the blended grain batch, five samples were taken randomly and analyzed as described by Visconti and Pascale (*17*) for zearalenone, by Avantaggiato et al. (*11*) for deoxynivalenol, and by Pascale et al. (*18*) for fumonisins. The performance characteristics of the analytical methods were checked by spiking blank cereal grains with standard solutions of toxins at levels of 1 mg/kg zearalenone, 5 mg/kg deoxynivalenol, or 20 mg/kg fumonisin B_1 plus 10 mg/kg fumonisin B_2. Mean recoveries ($n = 3$) and coefficients of variation were, respectively, 95 and 5% for zearalenone, 86 and 3% for deoxynivalenol, and 85 and 1% for fumonisins.

Experimental Design. The TIM experiments with mycotoxin-contaminated feeds included two duplicate control experiments without added adsorbent materials and five duplicate test experiments with the adsorbent material at three inclusion levels. Corn artificially contaminated with 187 μg/kg ochratoxin A plus 193 μg/kg aflatoxin B_1 or naturally contaminated grain containing 19.9 mg/kg fumonisin B_1 plus 5.9 mg/kg fumonisin B_2, 5.6 mg/kg deoxynivalenol, and 1.3 mg/kg zearalenone was used in the gastrointestinal model to estimate the intestinal absorption of mycotoxins. Standard Q/FIS was tested in duplicate in the TIM system for its ability to bind mycotoxins at an inclusion level of 1 or 2% (w/w) toward ochratoxin A and aflatoxin B_1 and of 0.3, 1, or 2% (w/w) toward zearalenone, deoxynivalenol, and fumonisins B_1 and B_2. An additional experiment was performed with a mycotoxin-free feed to check the analytical method of ochratoxin A, aflatoxin B_1, zearalenone, deoxynivalenol, and fumonisins, by collecting mycotoxin-free dialysis fluids that were then spiked to determine the analytical recovery. During the experiments the TIM system was protected from UV light by UV-adsorbing foil. At the start of the experiments 300 g of mycotoxin-contaminated meal was prepared by mixing 60 g of finely ground grain containing ochratoxin A plus aflatoxin B_1 or zearalenone plus deoxynivalenol, fumonisin B_1. and fumonisin B_2, with 90 g of artificial saliva (*10*) and 150 g of distilled water. The meal was pumped into the gastric compartment of the model (containing 10 g of gastric residue at pH 2), and the digestion process was monitored for 6 h. Dialysates from the jejunal and ileal compartments were collected in 2 h aliquots (0−2, 2−4, and 4−6 h). After the collected volume had been recorded, 250 mL of sample material was stored in duplicate at −20 °C for mycotoxin analysis. In addition, pooled samples representative of the entire experiment (0−6 h) were obtained from jejunal and ileal dialysates.

Mycotoxin Analysis in Dialysate Fluids. All below-described analyses were performed in triplicate.

The dialysate samples were filtered through Whatman no. 1 filter paper (Whatman International Ltd., Maidstone, U.K.). For aflatoxin B_1 and ochratoxin A analyses, 20 mL of filtered dialysate was diluted with 30 mL of phosphate-buffered saline (PBS; 0.8% NaCl, 0.16% Na_2HPO_4, 0.02% KH_2PO_4, and 0.02% KCl in distilled water, pH 7) and applied onto AflaTest or OchraTest wide-bore immunoaffinity columns (Vicam L.P., Watertown, MA). The columns were washed with 2 × 10 mL of PBS, and the toxins were eluted with 1.5 mL of methanol. The solvent was then evaporated to dryness under a nitrogen stream at ca. 50 °C, and the residues were reconstituted with 250 μL of the HPLC mobile phases. To check the analytical recoveries, mycotoxin-free jejunal and ileal dialysate were spiked with 1.5 ng/mL for jejunal dialysates and 0.45 ng/mL for ileal dialysates. HPLC analysis of ochratoxin A and aflatoxin B_1 was performed using a reversed phase Symmetry C_{18} column (15 cm × 4.6 mm, 5 μm particles) (Waters, Milford, MA) preceded by a Rheodyne guard 0.5 μm filter. The

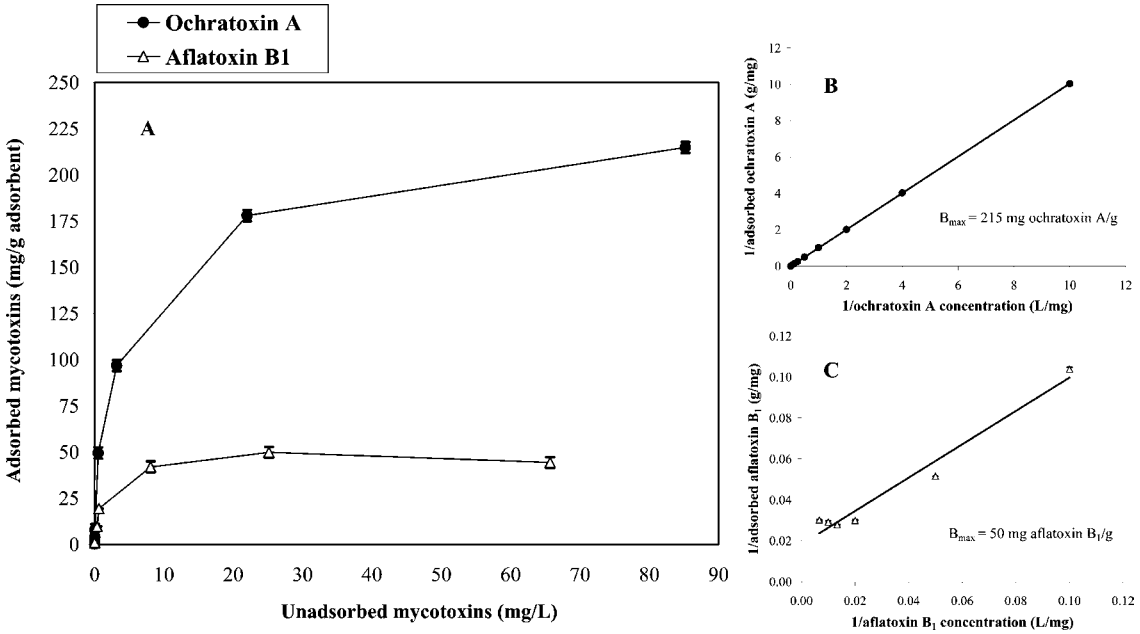

Figure 2. Adsorption isotherms (**A**) of Standard Q/FIS versus ochratoxin A and aflatoxin B_1 and double-reciprocal plots (**B**, **C**) of the adsorbed toxin (mg of toxin/g of adsorbent) versus initial toxin concentration (mg/L). *y*-intercepts are at 4.6×10^{-3} g/mg for ochratoxin A and 19.8×10^{-3} g/mg for aflatoxin B_1. Binding capacities (B_{max}) were derived from the reciprocal of the *y*-intercept of the regression line for each toxin. Data represent the mean value ± SD ($n = 3$); where not shown, error bars are within the size of the symbols.

fluorescence detector was set at 333 nm excitation/460 nm emission for ochratoxin A and at 365 nm excitation/435 nm emission for aflatoxin B_1. The mobile phases were mixtures of acetonitrile, water, and acetic acid (99:99:2, v/v) for ochratoxin A and water, methanol, and acetonitrile (60:20:20, v/v) for aflatoxin B_1, eluted at a flow rate of 1.0 mL/min. Potassium bromide (119 mg) and nitric acid (100 μL) were added to 1 L of the mobile phase of aflatoxin B_1 for the postcolumn derivatization of the toxin (bromination) involving the Kobra Cell (R-Biopharm Rhône Ltd., Glasgow, Scotland), an electrochemical cell fitted on-line between the HPLC column and the detector. Quantification of mycotoxins was performed by measuring peak areas at mycotoxin retention times and comparing them with the relevant calibration curves.

To analyze zearalenone in dialysate fluids, 60 mL of dialysate fluids was passed through ZearalaTest wide-bore immunoaffinity columns (Vicam), followed by 3×5 mL distilled water. Zearalenone was eluted from the column with 1.5 mL of methanol, the solvent was then evaporated, and the residue was redissolved in 250 μL of HPLC mobile phase. To check the analytical recoveries mycotoxin-free dialysate was spiked with 5.0 ng/mL and 2.5 ng/mL zearalenone for jejunal and ileal dialysates, respectively. The HPLC apparatus was the same as used for aflatoxin B_1 and ochratoxin A analyses. The fluorescence detector was set at 274 nm excitation and 440 nm emission. HPLC analysis of zearalenone was performed as described by Visconti and Pascale (*17*).

For fumonisin analysis, 20 mL of dialysate fluids was diluted with 20 mL of PBS and applied onto FumoniTest wide-bore immunoaffinity columns (Vicam). The column was washed with 2×10 mL of PBS, and fumonisins were eluted with 1.5 mL of methanol. After evaporation of the solvent, the residue was reconstituted with 400 μL of acetonitrile/water (1:1, v/v). The analytical recovery was checked by spiking fumonisin-free dialysate with 40.0 ng/mL fumonisin B_1 and 8.0 ng/mL fumonisin B_2 for jejunal dialysates and with 20.0 ng/mL fumonisin B_1 and 4.0 ng/mL fumonisin B_2 for ileal dialysates. HPLC analysis of fumonisins was performed as described by Pascale et al. (*18*).

For deoxynivalenol analysis, the dialysate samples were analyzed as described by Avantaggiato et al. (*11*) with some modifications for the cleanup phase. Three aliquots of 1.6 mL of dialysate were diluted with 8.4 mL of acetonitrile and cleaned up through MycoSep columns (Romer Labs, Union, MO). The three purified extracts were collected from the top of the columns and mixed in a 50 mL tube. Then, 18 mL was transferred to a 4 mL reaction vial and evaporated to dryness under a nitrogen stream at ca. 50 °C. The residue was derivatized with Tri-Sil TBT and analyzed by GC-ECD. The analytical recovery was checked by spiking mycotoxin-free dialysate with 30.0 and 5.0 ng/mL deoxynivalenol for jejunal and ileal dialysates, respectively.

Calculations and Statistics. For the jejunal and ileal dialysate fluids the absolute amount of mycotoxins (μg) was calculated by multiplying the measured sample volume by the analyzed concentration. The relative amount was calculated as a percentage of mycotoxin intake. For each compartment the percentage of mycotoxins in the dialysate was also calculated in comparison to the control experiments without adsorbent to determine the sequestering efficacy of Standard Q/FIS. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the GraphPad Instat statistical software package (Instat, San Diego, CA). The Tukey−Kramer multiple-comparisons post test was used with the differences considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

In Vitro Adsorption Screening Tests. The results of the in vitro screening tests showed that Standard Q/FIS bound almost 100% of both ochratoxin A and aflatoxin B_1 at the concentrations of 1, 2, and 10 μg/mL. Myco AD A-Z, cholestyramine, and activated carbon bound 90% ochratoxin A (**Table 1**). Ochratoxin A adsorption was rather low for all of the other tested products (<40%), most of which were silicate minerals, such as phyllosilicates and tectosilicates (**Table 1**). Myco AD A-Z and phyllosilicates showed a good efficiency in binding aflatoxin B_1 (>80%), whereas the two tested tectosilicates (clinoptilolite and CAB 70) bound <60% aflatoxin B_1 (**Table 1**).

The isotherm plots for ochratoxin A and aflatoxin B_1 adsorption to Standard Q/FIS are shown in **Figure 2A**. The adsorption isotherms showed an exponential relationship, indicating that the binding of the mycotoxins to Standard Q/FIS was a saturable process. The binding capacities (B_{max}) of Standard Q/FIS versus ochratoxin A and aflatoxin B_1 were 215 mg of ochratoxin A and 50 mg of aflatoxin B_1 per gram of adsorbent, as determined by the double-reciprocal plots reported in **Figure 2**, panels **B** and **C**, respectively. The regression plots fitted quite well with the experimental data, having r^2 values of >0.995.

The use of adsorbent materials to detoxify mycotoxin-contaminated diets will become an effective strategy to counteract mycotoxin problems in animal production if these materials have the ability to bind combinations of mycotoxins. Standard Q/FIS has the potential to act as a multitoxin-binding agent, adsorbing in vitro aflatoxin B_1, ochratoxin A, zearalenone, fumonisin B_1, and, to a lesser extent, deoxynivalenol and nivalenol. In previous experiments, Standard Q/FIS was found to be the most promising of the commercial products, showing a quite good binding capacity toward *Fusarium* mycotoxins and high stability over a wide pH range (*12*). In in vitro tests, it bound 390 mg of fumonisin B_1, 66 mg of zearalenone, 1.6 mg of deoxynivalenol, and 0.6 mg of nivalenol per gram of adsorbent (*12*). The ability of Standard Q/FIS to bind mycotoxins can be compared to that of the highly purified activated carbon obtained from Sigma-Aldrich as tested in our previous experiments (*12*). This activated carbon bound 124 mg of fumonisin B_1, 236 mg of ochratoxin A, 112 mg of zearalenone, 10.4 mg of deoxynivalenol, and 2.6 mg of nivalenol per gram of adsorbent. Considering literature data on ochratoxin A adsorption, the binding capacity of Standard Q/FIS (215 mg of ochratoxin A/g of adsorbent) is much higher than that of activated carbons (100−124 mg/g) (*19*, *20*), bentonite (1.5−9.0 mg/g) (*19*), and cholestyramine (9.6 mg/g) (*19*). However, the binding capacity of Standard Q/FIS for aflatoxin B_1 (50 mg/g of adsorbent) is less than that found for activated carbon (120 mg/g) (*21*), but it is in the same range (62−72 mg/g) as that found by Phillips et al. (*22*) for aluminosilicates.

Adsorption Experiments with the Dynamic Gastrointestinal Model. For the purpose of this study, two multimycotoxin-contaminated feeds were prepared and checked for toxin distribution. The homogeneity of the mycotoxins was considered to be satisfactory for the contaminated feeds, and no outlier was detected. Mean contents (±SD, $n = 5$) of mycotoxins in a whole blend of grains naturally contaminated with fumonisins B_1 and B_2, deoxynivalenol, and zearalenone and in a corn feed artificially contaminated with ochratoxin A and aflatoxin B_1 were 19.9 ± 0.5 mg/kg for fumonisin B_1, 5.9 ± 0.2 mg/kg for fumonisin B_2, 5.6 ± 0.3 mg/kg for deoxynivalenol, 1.3 ± 0.1 mg/kg for zearalenone, 0.187 ± 0.010 mg/kg for ochratoxin A, and 0.193 ± 0.049 mg/kg for aflatoxin B_1. On the basis of these contents the mean values of mycotoxin intakes ($n = 10$) in the TIM system were 1187 ± 9 μg for fumonisin B_1, 352 ± 2 μg for fumonisin B_2, 334 ± 2 μg for deoxynivalenol, 75 ± 1 μg for zearalenone, and 11.0 ± 0.1 μg for both ochratoxin A and aflatoxin B_1. Mycotoxin levels in the feeds were in the range of or higher than those known to cause adverse clinical effects in pigs (*23*). Levels of mycotoxins reported to cause toxic effects in pigs are as follows: ≥5 mg/kg fumonisins, ≥0.35 mg/kg deoxynivalenol, ≥0.5 mg/kg zearalenone, ≥0.2 mg/kg aflatoxins, and ≥0.2 mg/kg ochratoxin A.

The performance characteristics of the analytical methods for the mycotoxins in jejunal and ileal dialysates at different spiking levels are reported in **Table 2**. Recoveries ranged from 85 to 119% for jejunal dialysate and from 83 to 109% for ileal dialysate. The coefficients of variation were ≤12%. The limits of quantification (LOQ) of mycotoxin methods, defined here as the smallest amount reproducibly and accurately detected with at least a 6:1 signal-to-noise ratio, were 0.025 ng/mL for aflatoxin B_1 and ochratoxin A, 0.2 ng/mL for zearalenone, 1.0 ng/mL for fumonisins, and 1.5 ng/mL for deoxynivalenol. All samples of jejunal and ileal dialysate gathered from the TIM system contained mycotoxin amounts above the LOQ of analytical methods and were accurately analyzed.

Table 2. Performances of the Analytical Methods of Mycotoxins in Jejunal and Ileal Dialysate Fluids[a]

	jejunal dialysate fluids		ileal dialysate fluids	
	spiking level (ng/mL)	recovery (%), mean ± SD (CV%) ($n = 3$)	spiking level (ng/mL)	recovery (%), mean ± SD (CV%) ($n = 3$)
fumonisin B_1	40	96 ± 1.4 (1)	20	91 ± 3.1 (3)
fumonisin B_2	8	96 ± 1.0 (1)	4	91 ± 1.8 (2)
deoxynivalenol	30	119 ± 7.4 (6)	5	109 ± 12.8 (12)
zearalenone	5	94 ± 0.6 (1)	2.5	86 ± 0.1 (0.1)
ochratoxin A	1.5	100 ± 0.7 (1)	0.45	98 ± 4.5 (5)
aflatoxin B_1	1.5	85 ± 4.3 (5)	0.45	83 ± 1.1 (1)

[a] Mycotoxin-free fluids spiked with toxin standards.

Assessment of the Intestinal Absorption of Mycotoxins from Multitoxin-Contaminated Feeds. During the transit of the multitoxin-contaminated feeds through the model, the mycotoxins were absorbed from the intestinal compartments. Intestinal absorptions of mycotoxins measured in dialysate collected for 6 h of gastrointestinal digestion and expressed as a percentage of intakes are shown in **Figure 3**. When no sequestering material was added to the feed (control), the total intestinal absorptions of mycotoxins (corresponding to the mycotoxin amounts measured in jejunal plus ileal dialysate fluids) were 105% for fumonisin B_1, 89% for fumonisin B_2, 87% for ochratoxin A, 74% for deoxynivalenol, 44% for aflatoxin B_1, and 25% for zearalenone (**Figure 3**). For the controls, the absolute amounts of mycotoxins absorbed from the jejunum and ileum were 1240 ± 14 μg for fumonisin B_1, 312 ± 5 μg for fumonisin B_2, 247 ± 23 μg for deoxynivalenol, 18.5 ± 0.9 μg for zearalenone, 9.7 ± 0.4 μg for ochratoxin A, and 5.0 ± 0.5 μg for aflatoxin B_1 (**Table 3**). The absorption of mycotoxins occurred mainly from the upper part of the small intestine (jejunum) and less from the ileum (**Figure 3**; **Table 3**). Dialysate samples collected at different time intervals (0−2, 2−4, and 4−6 h) showed that, with the exception of zearalenone, maximum absorption of mycotoxins occurred in the first 2 h of digestion (0−2 h), was persistent for the following 2 h (2−4 h), and decreased during the later 2 h (4−6 h) of the experiment (**Figure 4**). Zearalenone was less and more slowly absorbed in comparison to the other mycotoxins. Consequently, its digestion and absorption took place in the distal part of the small intestine as shown by the higher absorption from the ileum compartment (**Figure 3**).

The results of our TIM experiments show that fumonisins, ochratoxin A, and deoxynivalenol were quickly and nearly completely absorbed in the proximal part of the small intestine. In the 6 h of digestion of the contaminated feeds, almost all fumonisins, ochratoxin A, and deoxynivalenol were released from the food matrix and became available for absorption, whereas <50% of the administered dose was absorbed for aflatoxin B_1 or zearalenone. These findings are in agreement with previous studies using the TIM system simulating the gastrointestinal digestion of pigs to estimate the bioaccessibility of zearalenone, deoxynivalenol, and aflatoxin B_1 (*10*, *11*, *14*). The bioaccessibility of zearalenone from three feeds containing 4.1 mg/kg zearalenone (artificial contamination), 0.5 mg/kg zearalenone (natural contamination), and 0.1 mg/kg zearalenone (natural contamination), expressed as a percentage of intake, were 32, 21, and 32%, respectively. In the present study with a naturally contaminated feed containing 1.3 mg/kg zearalenone, the bioaccessibility was 25%. It may therefore be concluded that neither the level of zearalenone in the feed nor the origin

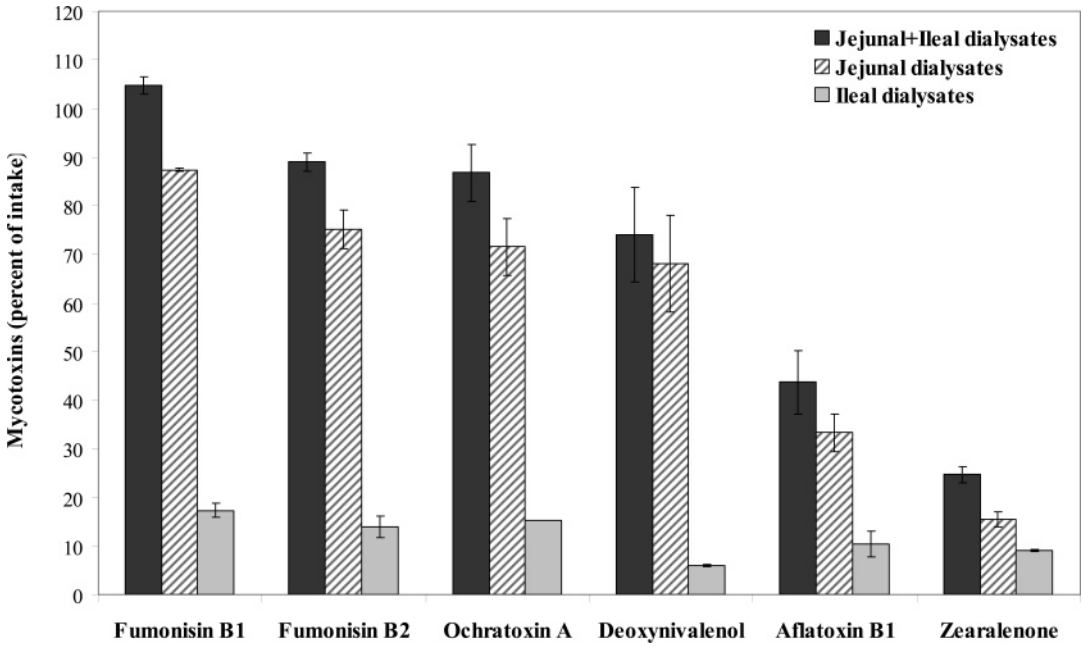

Figure 3. Intestinal absorption of fumonisins B_1 and B_2, ochratoxin A, deoxynivalenol, aflatoxin B_1, and zearalenone. The absorptions are the amounts of mycotoxins (expressed as percentage of the intake) in dialysate samples collected from the TIM system during 6 h of digestion of multi-mycotoxin-contaminated feeds. Bars are the means of duplicate experiments. Error bars represent the difference between the two individual values.

Table 3. Absolute Amounts of Mycotoxins Absorbed from the Jejunal and Ileal Compartments during 6 h of Gastrointestinal Digestion of Multicontaminated Feeds with Standard Q/FIS at Different Inclusion Levels

	mycotoxin amounts[a] (μg)					
inclusion level of Standard Q/FIS	fumonisin B_1	fumonisin B_2	deoxynivalenol	zearalenone	ochratoxin A	aflatoxin B_1
jejunum						
0% (control)	1035 ± 3	264 ± 10	227 ± 24	11.6 ± 0.8	8.0 ± 0.4	3.8 ± 0.3
0.3%	976 ± 48	246 ± 4	210 ± 11	11.9 ± 0.3		
1%	828 ± 15	209 ± 7	205 ± 1	9.3 ± 0.9	6.0 ± 0.1	0.3 ± 0.1
2%	735 ± 5	189 ± 2	187 ± 52	6.8 ± 0.1	5.9 ± 0.3	0.5 ± 0.4
ileum						
0% (control)	205 ± 11	49 ± 6	20 ± 1	6.8 ± 0.1	1.7 ± 0.1	1.2 ± 0.2
0.3%	144 ± 1	41 ± 1	21 ± 1	6.3 ± 0.1		
1%	153 ± 1	42 ± 2	13 ± 1	4.8 ± 0.2	1.3 ± 0.1	0.3 ± 0.1
2%	142 ± 7	39 ± 1	12 ± 1	3.4 ± 0.1	1.2 ± 0.1	0.2 ± 0.1

[a] Values are means ($n = 2$) ± the difference between the individual values of the two replicate experiments.

of contamination (natural or artificial) has an effect on the intestinal absorption of zearalenone (mean value at 28 ± 5% of intake). These results are consistent with in vivo studies (*24*, *25*). Ramos et al. used an in situ technique with the whole small intestine of rats perfused with an isotonic solution of zearalenone at 4 μg/mL. Zearalenone crossed the intestinal mucosa at a high rate by a passive process (*24*). Döll et al. (*25*) showed that increasing concentrations of zearalenone in the diet were correlated with increasing concentrations of zearalenone and its metabolites in biological samples of piglets. Regarding the bioaccessibility of aflatoxin B_1 in the TIM system, Zeijdner et al. (*14*) estimated an aflatoxin B_1 absorption at 47% of intake after ingestion of a 0.02 mg/kg naturally contaminated feed. This matches with our study (44% absorption) using an artificially contaminated feed containing a 10-fold higher level of toxin. Concerning deoxynivalenol, the absorption recorded by Avantaggiato et al. (*11*) using the same model with 2.8 mg/kg artificially contaminated meal, and corresponding to 51% of deoxynivalenol intake, was lower than the 74% absorption found herein with a 5.6 mg/kg naturally contaminated feed. However, both studies showed a high bioaccessibility for deoxynivalenol, also in the case of natural contamination.

Efficacy of Standard Q/FIS in Reducing Intestinal Absorption of Mycotoxins. Absolute amounts of mycotoxins absorbed from the jejunal and ileal compartments of the TIM system during 6 h of digestion of multicontaminated feeds with Standard Q/FIS at three inclusion levels are listed in **Table 3**. The intestinal absorption of mycotoxins was significantly decreased by the addition of Standard Q/FIS in a dose-dependent manner. The reduction was recorded at all inclusion levels from 0.3 to 2%. In particular, the addition of Standard Q/FIS to two multitoxin-contaminated feeds resulted in simultaneous reductions in toxin absorption of 1−44% for zearalenone, 10−29% for fumonisin B_1, 8−27% for fumonisin B_2, 7−20% for deoxynivalenol, up to 29% for ochratoxin A, and 88% for aflatoxin B_1 (**Figure 5**). A relevant and statistically significant reduction in intestinal absorption of mycotoxins was recorded by supplementing the multicontaminated feeds with 1% Standard Q/FIS. The use of an inclusion level of Standard Q/FIS of >1% was economically reasonable only in the case of zearalenone: 2% Standard Q/FIS

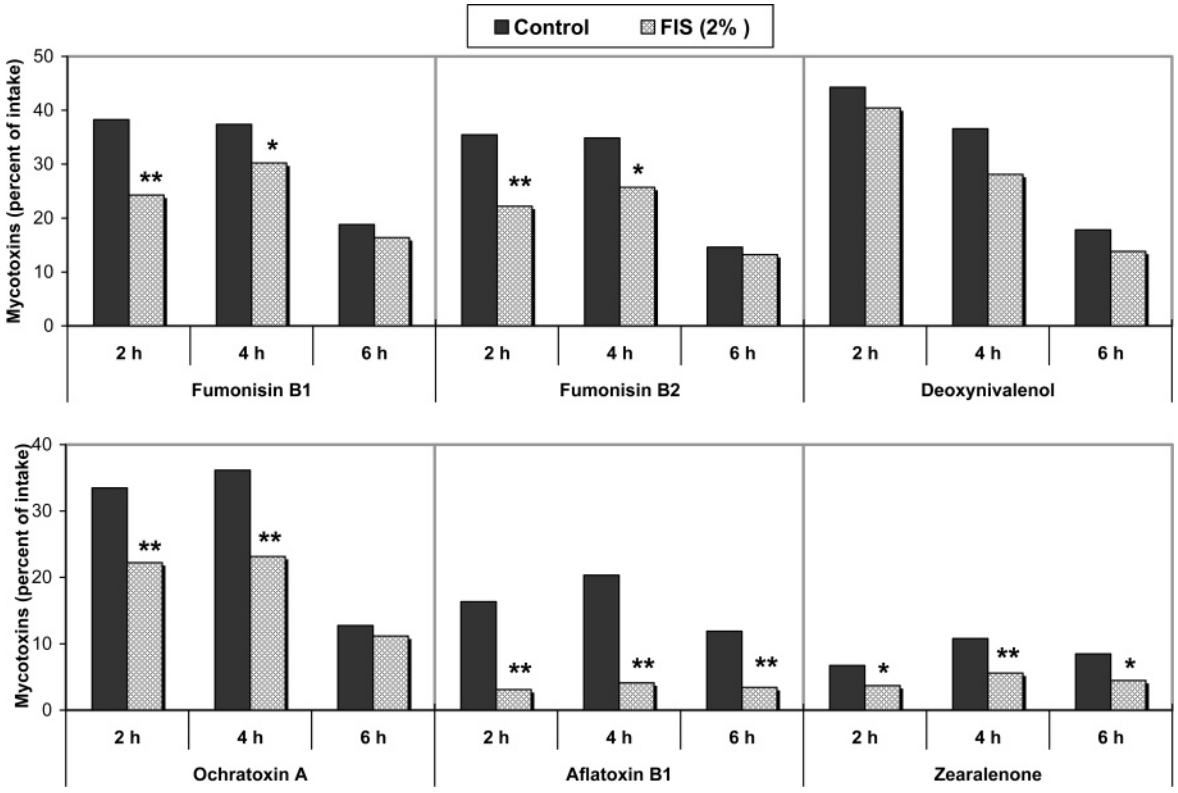

Figure 4. Mean ($n = 2$) content of fumonisins B_1 and B_2, deoxynivalenol, and zearalenone and (in separate experiments) ochratoxin A and aflatoxin B_1 in jejunum plus ileum dialysate in 2 h periods during gastrointestinal passage of the multi-mycotoxin-contaminated feeds with Standard Q/FIS at 0% (control) or 2% inclusion level (*, $P < 0.05$; **, $P < 0.001$, in comparison to the respective control).

significantly lowered zearalenone absorption in comparison to the addition of 1% (44% reduction with respect to the control) (**Table 3**; **Figure 5**). Standard Q/FIS showed the highest binding effectiveness toward aflatoxin B_1. The inclusion of 1% Standard Q/FIS led to a reduction in aflatoxin absorption by 88%.

The progression of intestinal absorption of mycotoxins showed that Standard Q/FIS sequesters mycotoxins at different rates in the gastrointestinal tract. The effect of Standard Q/FIS in reducing mycotoxin absorptions was at its maximum at the time of maximum level mycotoxin absorption (**Figure 4**). For fumonisins and ochratoxin A, the reduction of mycotoxin absorptions reached the highest value in the first 2 h of digestion, persisted for the following 2 h period, and decreased during the final 2 h of the experiments. In particular, in the first 4 h of digestion, when most mycotoxins are absorbed, the addition of 2% Standard Q/FIS to contaminated diets significantly decreased the bioaccessibility of fumonisins and ochratoxin A (35%; $P < 0.001$). A <13% reduction occurred during the later 2 h period (not statistically significant) in comparison to the control. At this time, both fumonisin and ochratoxin A absorptions were negligible and accounted for just 15% of the total mycotoxin uptake. For the less and slowly bioaccessible mycotoxins zearalenone and aflatoxin B_1, a constant and statistically significant reduction of intestinal absorption occurred over the whole experimental period. With the addition of 2% Standard Q/FIS to the feed, the reduction occurring during the 2 hourly intervals of digestion accounted for 80% of aflatoxin B_1 and 47% of zearalenone, with respect to the control (**Figure 4**).

Standard Q/FIS as a Multitoxin-Binding Agent. Because mycotoxins are able to cross the intestinal lumen easily, an adsorbent material effective at sequestering mycotoxins in the digestive tract needs to have a high affinity for mycotoxins under the physiological conditions of the gastrointestinal tract. It should be able to form stable mycotoxin-adsorbent complexes within a short period of time, preferably with different classes of mycotoxins. The results of the present study show that when mycotoxins are released from the feed matrix into the soluble phase of the chyme, they are rapidly and efficiently sequestered by Standard Q/FIS. In particular, Standard Q/FIS was effective in blocking the intestinal absorption of aflatoxin B_1 and significantly reduced the absorption of fumonisins, ochratoxin A, and zearalenone. As expected, it was not effective in reducing deoxynivalenol uptake. The adsorption of mycotoxins by Standard Q/FIS in the lumen of the gastrointestinal tract was not affected by the digestive degradation, solubilization, and interaction with other components of the chyme or by the simultaneous presence of different mycotoxins.

Mycotoxin binding capacities of Standard Q/FIS measured by in vitro screening tests decreased in the order fumonisin B_1 > ochratoxin A > zearalenone > aflatoxin B_1 > deoxynivalenol, whereas in the TIM system they decreased in the order aflatoxin B_1 > zearalenone > ochratoxin A and fumonisins > deoxynivalenol. In spite of the higher in vitro binding capacities of Standard Q/FIS toward fumonisin B_1 and ochratoxin A, the best effectiveness in preventing intestinal absorption of mycotoxins was found for aflatoxin B_1 and zearalenone, whereas for ochratoxin A and fumonisins a slight efficacy in binding and reduction of absorptions was recorded. In agreement with the in vitro screening tests, deoxynivalenol was not adsorbed by Standard Q/FIS in the gastrointestinal compartments of the TIM system. Hydrophobicity of mycotoxins may play a role in both intestinal absorption and binding to Standard Q/FIS. Hydrophilic mycotoxins, such as deoxynivalenol, fumonisins, and ochratoxin A, showed the highest absorption from the intestine, but were less sequestered by the product. Probably due to their high affinity for water, these toxins were rapidly extracted from the

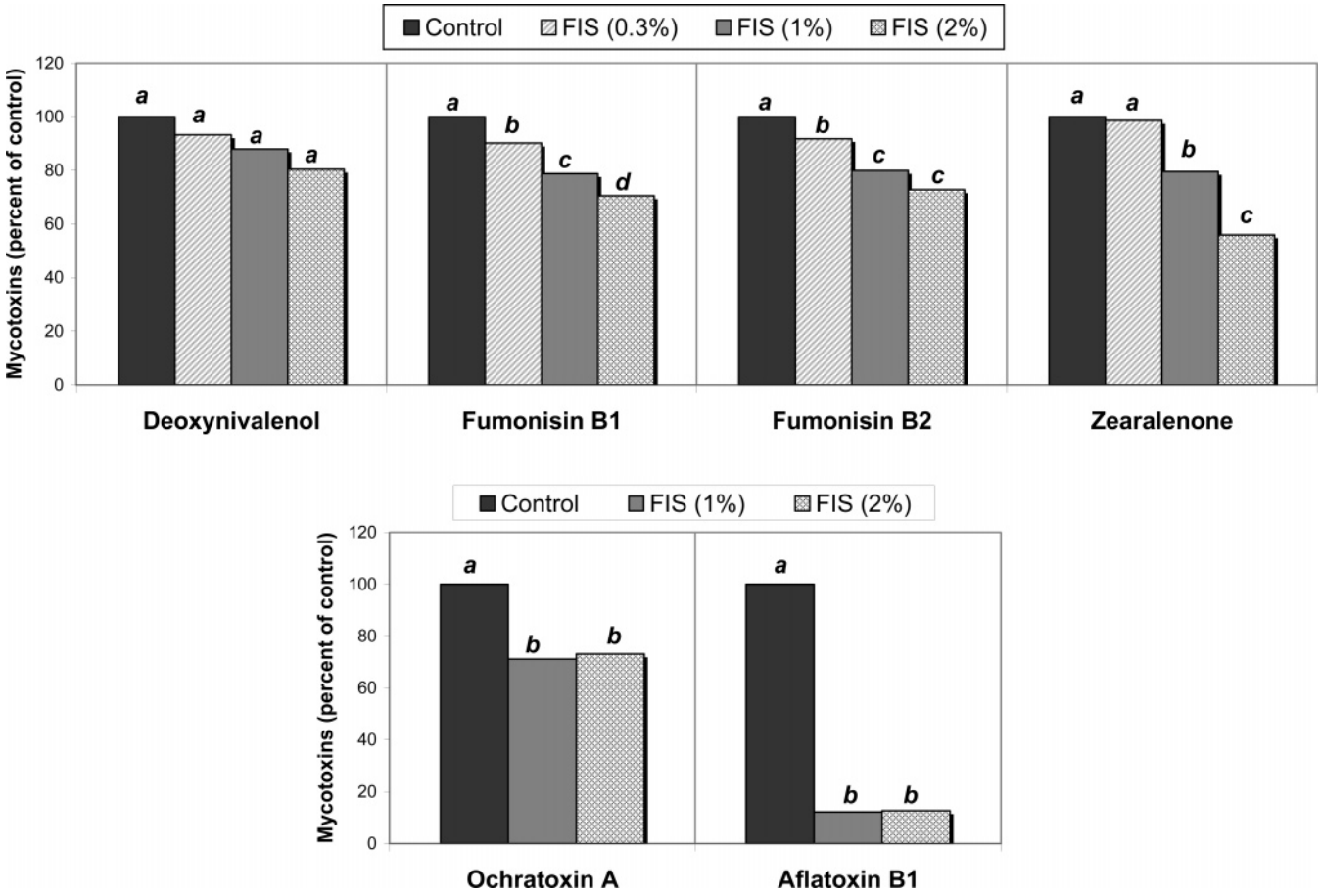

Figure 5. Mean ($n = 2$) percentage of mycotoxins in jejunum plus ileum dialysate collected from the TIM system during 6 h of digestion of multi-mycotoxin-contaminated feeds with Standard Q/FIS at different inclusion levels. Mean amount of mycotoxin in dialysate of control experiments without Standard Q/FIS is set at 100%. Different letters in each group of bars indicate significant differences ($P \leq 0.05$).

matrix when mixed with water during meal preparation or in the stomach and then rapidly delivered with the liquid phase of the gastric content into the small intestine. As a result, their transit rate through the gastrointestinal tract was faster than the solid phase of the chyme (including the adsorbent material), leading to a short contact time between mycotoxin and the binder. On the other hand, hydrophobic mycotoxins, such as aflatoxin B_1 and zearalenone, are delivered with the solid phase of the gastric content, including the transit of Standard Q/FIS. This could explain the efficient sequestering effect of Standard Q/FIS toward aflatoxin B_1 and zearalenone during the first 2 h, which persists for the rest of the intestinal transit.

The effectiveness of Standard Q/FIS in reducing mycotoxin absorption can be compared to that of cholestyramine and activated carbon as tested in our previous TIM experiments (*12*) or in in vivo studies. Our previous study with cholestyramine showed an in vitro binding capacity of 34 mg of zearalenone/g of adsorbent and 52% reduction in the intestinal absorption of toxin at a 2% inclusion level (*14*). Standard Q/FIS bound in vitro 66 mg of zearalenone/g of adsorbent and reduced the intestinal absorption with 44% in the model at a 2% inclusion level. Compared to activated carbon, Standard Q/FIS showed a lower in vitro binding capacity toward zearalenone and deoxynivalenol. This corresponds to a lower efficacy of Standard Q/FIS in preventing intestinal absorption of zearalenone and deoxynivalenol in comparison to that reported for activated carbon (*11*).

Several in vivo studies confirm the effectiveness of activated carbon and cholestyramine in preventing the detrimental effects of mycotoxins on animal health. In an in vivo study, an activated carbon significantly protected mice against the estrogenic effects induced by 35 mg of zearalenone/kg of feed (*26*). Underhill et al. (*27*) showed that the addition of cholestyramine to 6 mg/kg zearalenone contaminated feed decreased by 19% the estrogenic effect of the toxin in prepuberal mice. In in vivo experiments with rats, using the SA/SO ratio as a biomarker for bioavailable fumonisins, the addition of 2% cholestyramine to a diet containing 20 mg/kg fumonisins significantly decreased the mean SA/SO ratios by about 50%, both in kidneys and in urine (*28*). Moreover, cholestyramine increased ochratoxin A excretion in rat (*29*) and inhibited the enterohepatic circulation of this mycotoxin in mice (*30*). Kerkadi et al. (*31*) found that cholestyramine at 0.1, 1, and 5% significantly decreased by 30, 36, and 70%, respectively, the ochratoxin A concentration in the plasma of rats fed a diet containing 1 mg/kg of the mycotoxin. In our study, Standard Q/FIS showed the same binding capacity for ochratoxin A (215 mg/g of adsorbent) and a higher binding capacity for fumonisin B_1 (390 mg/g of adsorbent) in comparison to both activated carbon (236 mg of ochratoxin A and 124 mg of fumonisin B_1 per gram of activated carbon) and cholestyramine (200 mg of ochratoxin A and 190 mg of fumonisin B_1 per gram of cholestyramine). At a 1% inclusion level it significantly reduced by about 30% the intestinal absorption of both toxins in the TIM system.

In conclusion, the reduction of zearalenone, fumonisin, and ochratoxin A bioaccessibility in the TIM system due to Standard Q/FIS is similar to that found in vivo for cholestyramine. In the light of these results, it can be assumed that Standard

Q/FIS may significantly decrease in vivo the bioavailable amounts of zearalenone, fumonisins, and ochratoxin A, and almost completely prevent intestinal absorption of aflatoxins in contaminated feed. Probably due to its composition and the presence of different adsorbent materials, Standard Q/FIS can bind a number of chemically different mycotoxins and can be beneficial in reducing both individual and combined adverse effects of mycotoxins in animals. However, further studies are necessary to prove the effectiveness of this adsorbent material in reducing the toxic effects of mycotoxins without affecting the regular utilization of essential nutrients, such as vitamins and minerals.

This is the first time that the bioaccessibility of different classes of mycotoxins, commonly co-occurring in animal feedstuffs has been simultaneously assessed using a multicompartmental model simulating the kinetic conditions in the gastrointestinal tract of pigs. Our findings can help to interpret the in vivo studies on toxicology and carcinogenicity of mycotoxins and contribute to understanding why pigs are so sensitive to mycotoxin exposure. The analysis of intestinal absorption clearly shows that most mycotoxins are rapidly and efficiently absorbed from the gastrointestinal tract of pigs but that efficient sequestrants can be developed to reduce their availability for absorption under practical conditions.

ABBREVIATIONS USED

TIM, TNO gastrointestinal model; B_{max}, binding capacity; PBS, phosphate-buffered saline; GC-ECD, gas chromatography−electrochemical detection.

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