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The efficiency of mycotoxin binding by sorbents in the *in vitro* model using a naturally contaminated animal feed

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Abstract

Introduction: The productivity of domestic animals and the safety of food products derived from them are jeopardised by mycotoxins in animal feed. To control them, feed additives are used, which limit the absorption of mycotoxins in the gastrointestinal tract of animals by binding to them. The study aimed to evaluate the effectiveness of a new *in vitro* model in experiments on the binding of mycotoxins from buffers and contaminated feed and to confirm the effect of a single sorbent or mixture in binding them. **Material and Methods:** Nine mineral sorbents were tested for their efficiency binding eight mycotoxins. Two *in vitro* experiments were conducted to indicate the mycotoxin-binding capacity of sorbents, each specifying a buffer with one of two different pH levels reflecting gastrointestinal conditions (pH 3.5 and 7.0). The first investigated the sorbent with only the buffer and mycotoxin standards, while the second did so with the sorbent, buffer and feed naturally contaminated with mycotoxins (deoxynivalenol, zearalenone, and ochratoxin A). **Results:** The sorption was significantly lower in the trial with feed. In the first experiment at gastric pH (pH 3.5), activated charcoal bound deoxynivalenol and sepiolite bound zearalenone at 70% and 96%, respectively, whereas in the second experiment with feed, the binding was only 3% and 6%. **Conclusion:** The study underlines the challenge of finding a feed additive that would work comprehensively, binding all mycotoxins regulated by law.

Keywords: mycotoxin, mineral sorbents, mycotoxin binders, feed additives.

Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi of *Aspergillus*, *Fusarium* and *Penicillium* species (31). These toxic metabolites are often found in grains and a substantial percentage of animal feed (18). The most common mycotoxins harmful to animal health are aflatoxin B1 (AFL B1), zearalenone (ZEN), deoxynivalenol (DON), fumonisins B1 and B2 (FB1 and FB2), ochratoxin (OTA) and T-2 toxin (T-2) (6). Different mycotoxins induce different effects on animal health (23). Aflatoxin B1 causes damage to the liver, and long-term consumption is carcinogenic and lethal to animals (12). Zearalenone has an oestrogenic effect in animals, causing reproductive disorders (32). Exposure to DON causes vomiting and weight loss (21). Animal consumption of FB1 and FB2 impairs the body's immune functions and reduces weight gain (30). Ochratoxin is nephrotoxic, hindering the proper functioning of the kidneys (5). As concerns T-2 toxin, it blocks DNA, RNA and protein synthesis, weakening the immune system and consequently making animals more susceptible to diseases (24). It and its metabolite HT-2 cause vomiting, diarrhoea, weight loss, skin problems, as well as necrosis and intestinal mucosal haemorrhaging (20). In general, mycotoxins cause losses in breeding by reducing reproduction and triggering animal diseases, resulting in a decrease in the number of animals on the farm (13, 31).

In order to reduce the harm mycotoxins can do, feed additives are used, which can lessen the negative health effects of feed contamination with mycotoxins by inhibiting or limiting their absorption (28). These additives can be sorbents, which effectively adsorb mycotoxins on their surface, or other agents such as bacteria, fungi or enzymes, which degrade mycotoxins into less harmful metabolites (29). Additives capable of adsorbing mycotoxins are usually substances of high molecular weight. This trait lets them bind these toxins within contaminated feed, limiting their bioavailability and becoming sorbent-mycotoxin complexes which pass through the body's digestive tract (1).

Aluminosilicates, which include bentonite, zeolite, sepiolite and montmorillonite, are the most commonly used sorbents for the control of mycotoxins in animal feed. Activated carbon is a sorbent formed from charcoal and used equally often for this purpose (10, 19, 25). A disadvantage of these sorbents is that they act selectively against one or two mycotoxins (9). The significant inadequacy of feed additive and sorbent selection which has been investigated in the scientific literature is that (before verification *via in vivo* trials) it is mainly based on simple simulations of the animal gastrointestinal tract, which is the buffer–buffer system in the appropriate pH (12, 22).

Using oversimplified *in vitro* models to verify the ability of sorbents to bind mycotoxins can have significant consequences. The insufficiency of the reproduction of the real conditions invites the risk that the investigated sorbents' effectiveness will not be confirmed in animal experiments. Few publications describe a more advanced model or methods using buffers and enzymes or bacteria with feed (3, 27). The results of several researchers only confirm the action of sorbents in buffers (8, 17). As a corollary of the buffer model's inadequacy, scientists are looking for newer, more advanced and more effective *in vitro* methods simulating the binding of mycotoxins. Despite the many limitations of laboratory tests, better future *in vitro* investigative techniques could reproduce the conditions of *in vivo* tests faithfully enough to accelerate mycotoxin-binding sorbent research. Feed additives developed through such work will raise the quality of farming by keeping the animals healthy.

This research aimed to find a single material and mixture of sorbents that would effectively bind eight mycotoxins simultaneously, and also aimed to verify their effects. The research's further aims were to compare the action of feed additives in the gastric and intestinal phases in developed *in vitro* experiments that reflected the pH in the gastrointestinal tract (pH 3.5 in the gastric phase and pH 7.0 in the intestinal phase), as well as to verify the action of the developed model. The final aim was to evaluate the effectiveness of sorbent binding to mycotoxins in an archival sample of naturally contaminated feed.

Material and Methods

Standards, reagents and buffers. Standards of 99.6% pure AFL B1, 99.0% pure DON, 98.6% pure T-2, 98.6% pure HT-2, 99.0% pure OTA, 98.0% pure FB1, 99.8% pure FB2 and 99.7% pure ZEN were obtained from Sigma-Aldrich (Schnelldorf, Germany). Standard solutions of DON, ZEN, T-2 and HT-2 were prepared in acetonitrile, and those of AFL B1 and OTA were prepared in methanol. Solutions of FB1 and FB2 were prepared in 50% acetonitrile. A standard solution containing eight mycotoxin analytes was used in the analysis in concentrations corresponding to the maximum levels of mycotoxins in pig feeds set by the European Parliament and Council and by the Commission (Table 1) (7, 11). In addition, a mixture of seven internal standards was used in a chromatographic analysis. The reason why seven standards were visualised in chromatography instead of eight is that the internal standard FB1 was used for the analysis not only of FB1 but also of FB2. This is justified by the separation of both molecules, *i.e.* FB1 and FB2, in liquid chromatography–tandem mass spectrometry (LC-MS/MS) being similar to that of an internal standard FB1 molecule.

Table 1. Mycotoxin levels in tested feed samples spiked with a mixture of standards

Mycotoxin	Spiking level (µg/kg)
Aflatoxin B1	5
Deoxynivalenol	900
T-2 toxin	50
HT-2 toxin	50
Ochratoxin A	50
Fumonisin B1	250
Fumonisin B2	250
Zearalenone	100

Acetonitrile (LC/MS grade), methanol (LC/MS grade) and acetic acid were purchased from J.T. Baker (Deventer, the Netherlands). Sodium chloride (p.a. grade), potassium chloride (p.a. grade), phosphoric acid (p.a. grade) and ammonium acetate (p.a. grade) came from Sigma-Aldrich (Darmstadt, Germany). The final reagent, disodium hydrogen phosphate (p.a. grade), was obtained from Chempur (Piekary Śląskie, Poland).

The phosphate-buffered saline (PBS) for the analysis of mycotoxin binding by sorbents consisted of 4 g of sodium chloride, 1.8 g of disodium hydrogen phosphate and 0.1 g of potassium chloride, in a 500 mL volume of distilled water and had its pH adjusted to 3.5 or 7 with phosphoric acid. The ammonium acetate solution for the LC-MS/MS phases consisted of 400 mg ammonium acetate and 500 µL of acetic acid in a 500 mL volume of distilled water. The phases for LC-MS/MS analysis were prepared according to the scheme of phase A (ammonium acetate : methanol 95 : 5) 380 mL of ammonium acetate solution and 20 mL of methanol and phase B (ammonium acetate : methanol 5 : 95) 20 mL of ammonium acetate solution and 380 mL of methanol.

The investigated sorbents were bentonite, calcium bentonite, zeolite, calcium lignosulphonate, activated charcoal, sepiolite, attapulgite and ground attapulgite, and silica. Samples of these were supplied by Certech (Niedomice, Poland).

The feed used for the tests was prepared from naturally contaminated grain. The grain became contaminated because the plants were cultivated in inappropriate conditions. The feed samples were prepared and investigated by Tkaczyk *et al.* (26) and comprised contaminated feed with DON at the level

of 1,126 ± 91.43 µg/kg, ZEN at 34.7 ± 4.34 µg/kg and OTA at 226 ± 21.8 µg/kg.

Mycotoxin standard binding by sorbents and sorbent mixtures in the gastric (pH 3.5) and intestinal (pH 7.0) phases. It was assumed that the sorbent would bind mycotoxins at a pH corresponding to the gastric phase. The stability of toxin binding and possible desorption was tested at intestinal pH. Each experimental sample contained sorbent, buffer at the appropriate pH and mycotoxin-standard solution (Fig. 1).

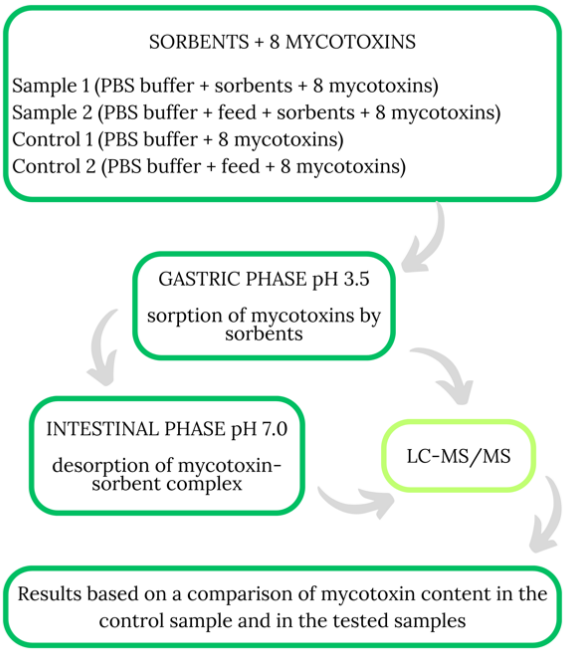

Fig. 1. Scheme of the experiment to evaluate selected sorbents' binding effectiveness to mycotoxin animal feed contaminants
PBS – phosphate-buffered saline; LC-MS/MS – liquid chromatography – tandem mass spectrometry

In the gastric phase (sorption at pH 3.5), duplicate samples with 100 mg of a sorbent were incubated and shaken with 5 mL of pH 3.5 PBS for 2 h at 39°C. The temperature of 39°C reflected the swine body temperature. A control sample was prepared by mixing 10 µL of the pH-adjusted PBS solution with eight mycotoxin standards. After centrifugation at 3,500 rpm for 15 min, 4.5 mL of extract was taken from each sample into new test tubes, and 10 mL of distilled water was added. The extract prepared was applied to solid-phase extraction (SPE) columns with Oasis hydrophilic-lipophilic balance columns (60 mg; Waters, Milford, MA, USA). The columns had been conditioned with 2 mL of MeOH and 2 mL of H_2O. Then, after the extract had passed through, it was washed with 2 mL of H_2O and dried for 1 min. Elution was carried out with 3 mL of MeOH. The extract was evaporated under N_2 at 45°C, and the dry residue was dissolved in 500 µL of phase A and 500 µL of phase B and transferred to vials for LC-MS/MS analysis.

In the intestinal phase (desorption at pH 7.0), the residual sorption precipitate was washed three times with 10 mL of distilled water, the water was poured off, and the pellet was incubated with 5 mL of PBS at pH 7.0 for 2 h at 39°C. The remaining steps of the procedure were of the same as for sorption.

Based on the results presented in Table 2 regarding the mycotoxin-binding ability of sorbents in a pH 3.5 buffer, sepiolite and calcium lignosulphonate were selected for further study as the two sorbents binding greater than 70% of the tested mycotoxins. Mixtures of the two sorbents were made in different proportions (Table 4) to assess which proportion would bind mycotoxins with the best effectiveness. The procedure for testing the sorbent mixtures in mycotoxin binding was the same as described above.

Feedborne mycotoxin binding by the mixture of sepiolite and calcium lignosulphonate in the gastric (pH 3.5) and intestinal phases (pH 7.0). These two sorbents were assessed in combinations for their binding of DON, ZEN and OTA in contaminated feed. Each sample contained naturally contaminated feed, a sorbent mixture and the buffer at the appropriate pH (Fig. 1).

Gastric phase (sorption at pH 3.5). Each experimental sample contained 10 mg of sorbent mixture and 1 g of feed. The 1% proportion of sorbent in the feed mass simulated the percentage of sorbent potentially administered in future practice. The control sample contained only 1 g of feed and PBS. Duplicate samples with 10 mg of a sorbent and feed were incubated and shaken with 30 mL of PBS at pH 3.5 for 2 h at 39°C. After centrifugation at 3,500 rpm for 15 min, 15 mL of extract was taken from each sample into new test tubes, and 10 mL of distilled water was added. The extract prepared this way was applied to the SPE Oasis HLB columns. Conditioning and sample preparation for LC-MS/MS analysis were performed similarly to how they were performed when mycotoxin standards devoid of feed mycotoxin were bound by sorbents.

Intestinal phase (desorption at pH 7.0). The residual sorption sediment was washed three times with 10 mL of distilled water, the water was poured off, and the sediment was incubated with 30 mL of PBS at pH 7.0 for 2 h at 39°C. The remaining steps of the procedure were performed as in the case of sorption.

LC-MS/MS analysis. Detailed conditions for detection and LC-MS/MS analysis are described in the publication by Jedziniak *et al*. (14). Analysis was performed on an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) operating in positive and negative ionisation modes. Chromatographic separation was undertaken at 40°C with a constant flow of 0.3 mL/min in a Kinetex Biphenyl column (100 × 2.1 mm, 2.6 µm) (Phenomenex, Torrance, CA, USA). Separation was performed using a gradient elution of mobile phase A and phase B as described earlier in this section. The volume of the test sample injected was 5 µL, and the chromatographic analysis of one test sample had a 16 min runtime.

Lab Solutions software (version 5.60 SP2) (Shimadzu) was used for data processing. The percentage of mycotoxin binding by sorbents was calculated by comparing the results for the tested samples with those for the control samples.

Results

Mycotoxin standard binding by sorbents in the gastric (pH 3.5) and intestinal (pH 7.0) phases. The results were calculated by comparing test sample results with those for a control sample containing a pH 3.5 buffer and a mycotoxin standard solution. The higher the percentage of mycotoxin binding was, the more effectively the sorbent bound a toxin. In gastric conditions (pH 3.5), activated charcoal bound all tested mycotoxins with high efficiency; the other sorbent with similar efficiency was sepiolite, although it bound DON much less well than activated charcoal (Table 2). No sorbent besides activated charcoal could bind all eight mycotoxins simultaneously, and with the exception of sepiolite, they bound effectively to three or fewer mycotoxins. All sorbents except calcium lignosulphonate showed binding of AFL B1 at 100%. The most difficult mycotoxin to bind was DON; only calcium lignosulphonate (87%) and activated charcoal (70%) could bind this mycotoxin. Bentonite bound fumonisins at a low level of 21–33%; however, calcium bentonite bound these toxins much more effectively at 91–93% (Table 2).

The binding results in the intestinal phase (mycotoxin desorption at pH 7.0) indicated to what extent the mycotoxin-sorbent binding was stable in the later phase of the digestive process. The lower the percentage was, the less the sorbent released mycotoxin to the external environment and the more stable the binding was (Table 3). The results were calculated by comparing test sample results with those for a control sample containing PBS at pH 7.0 and the mycotoxin standard solution. The sorbents that showed the greatest binding stability in the intestinal phase (pH 7.0) were activated charcoal and sepiolite. In the cases of seven of the tested mycotoxins, very good durability of the mycotoxin–activated charcoal bond was observed, DON proving an exception with lower stability at 59% of mycotoxin released. Stable binding to most of the tested mycotoxins at a satisfactory level was also maintained by sepiolite, once again except for binding to DON and HT-2. Calcium lignosulphonate, which bound DON well at pH 3.5, did not show good stability of binding to this mycotoxin in the intestinal phase, and this also related to the other mycotoxins tested. Aflotoxin B1 was a toxin that passed through the conditions in the gastrointestinal tract without its bond with most sorbents being broken. Fairly unstable bonds to ZEN by attapulgite and ground attapulgite were noted at 74% and 72% toxin release, respectively. Fumonisins were mycotoxins to which most of the sorbents bound stably, calcium lignosulphonate being the worst exception. Where the sorbent had not bound to mycotoxin in the gastric phase, the stability of the mycotoxin-sorbent complex in the intestinal phase could not be evaluated.

Feedborne mycotoxin binding by the mixture of sepiolite and calcium lignosulphonate in the gastric phase (pH 3.5). In the experiment using a mixture of sorbents in various proportions, the highest effectiveness was shown by a mixture of sepiolite and calcium lignosulphonate in the proportions of 80:20; this mixture bound to proportions of DON, OTA and ZEN in a 62–82% range (Table 4). The mixture in the proportions of 40:60 sepiolite:calcium lignosulphonate was the most effective in binding DON. The mixture in the proportions of 20:80 was the least able to bind the three tested mycotoxins, sequestering only an average of 46%. The raising of the calcium lignosulphonate proportion mixed with sepiolite improved the ability of the mixture to bind DON. The higher the content of this sorbent, the greater the percentage of DON binding. A similar dependence could be observed in the case of OTA and ZEN: as the proportion of sepiolite in the mixture of the two sorbents increased, the binding capacity also increased.

Table 2. Mycotoxin sorption results for the selected sorbents in the gastric phase (pH 3.5)

	DON	FB1	FB2	HT-2	OTA	AFL B1	T-2	ZEN
Bentonite	10%	21%	33%	9%	0%	100%	4%	5%
Calcium bentonite	4%	93%	91%	10%	14%	100%	12%	12%
Zeolite	1%	71%	85%	3%	3%	100%	1%	1%
Calcium lignosulphonate	87%	-*	-*	54%	52%	0%	31%	17%
Activated charcoal	70%	94%	80%	100%	99%	100%	99%	98%
Sepiolite	3%	91%	83%	72%	94%	100%	93%	96%
Silica	3%	12%	27%	8%	1%	84%	1%	1%
Attapulgite	7%	9%	79%	13%	21%	100%	32%	27%
Ground attapulgite	2%	38%	34%	10%	17%	100%	32%	52%

Legend: 0–20% | 21–40% | 41–60% | 61–80% | 81–100%

* – unexpected contamination of calcium lignosulphonate with fumonisins

DON – deoxynivalenol; FB1 – fumonisin 1; FB2 – fumonisin 2; HT-2 – HT-2 toxin (T-2 toxin metabolite); OTA – ochratoxin; AFL B1 – aflatoxin B1; T-2 – T-2 toxin; ZEN – zearalenone

Table 3. Mycotoxin desorption results for the selected sorbents in the intestinal phase (pH 7.0)

	DON	FB1	FB2	HT-2	OTA	AFL B1	T-2	ZEN
Bentonite	-*	56%	40%	-*	-*	0%	-*	-*
Calcium bentonite	-*	73%	45%	-*	-*	0%	-*	-*
Zeolite	-*	80%	56%	-*	-*	0%	-*	-*
Calcium lignosulphonate	100%	100%	100%	100%	100%	-*	100%	-*
Activated charcoal	59%	2%	2%	0%	0%	0%	1%	1%
Sepiolite	-*	22%	13%	58%	17%	0%	20%	5%
Silica	-*	-*	100%	-*	-*	40%	-*	-*
Attapulgite	-*	-*	24%	-*	77%	0%	95%	74%
Ground attapulgite	-*	46%	23%	-*	83%	0%	87%	72%

Legend: 0–20% | 21–40% | 41–60% | 61–80% | 81–100%

* – where mycotoxin sorption was low in the gastric phase (0–20%, see Table 2), desorption testing was not possible
DON – deoxynivalenol; FB1 – fumonisin 1; FB2 – fumonisin 2; HT-2 – HT-2 toxin (T-2 toxin metabolite); OTA – ochratoxin; AFL B1 – aflatoxin B1; T-2 – T-2 toxin; ZEN – zearalenone

Table 4. Mycotoxin binding results for the mixture of sepiolite and calcium lignosulphonate

	DON	OTA	ZEN
Sepiolite:calcium lignosulfonate 20:80	72%	29%	36%
Sepiolite:calcium lignosulfonate 40:60	75%	63%	41%
Sepiolite:calcium lignosulfonate 60:40	71%	68%	78%
Sepiolite:calcium lignosulfonate 80:20	62%	82%	82%

Legend: 0–20% | 21–40% | 41–60% | 61–80% | 81–100%

DON – deoxynivalenol; OTA – ochratoxin; ZEN – zearalenone

Table 5. Results of mycotoxins binding by sorbents in trial with naturally contaminated feed in the gastric (pH 3.5) and intestinal (pH 7.0) phases

	DON pH 3.5	DON pH 7.0	OTA pH 3.5	OTA pH 7.0	ZEN pH 3.5	ZEN pH 7.0
Bentonite	5%	-*	30%	92%	9%	-*
Calcium bentonite	1%	-*	35%	70%	9%	-*
Zeolite	4%	-*	54%	65%	10%	-*
Calcium lignosulphonate	11%	-*	34%	50%	7%	-*
Activated charcoal	3%	-*	39%	70%	4%	-*
Sepiolite	1%	-*	53%	89%	6%	-*
Silica	4%	-*	49%	81%	11%	-*
Attapulgite	2%	-*	54%	76%	8%	-*
Ground attapulgite	3%	-*	55%	87%	5%	-*

Legend (pH 3.5): 0–20% | 21–40% | 41–60% | 61–80% | 81–100%

Legend (pH 7.0): 0–20% | 21–40% | 41–60% | 61–80% | 81–100%

* – where mycotoxin sorption was low in the gastric phase (0–20%, see Table 2), desorption testing was not possible
DON – deoxynivalenol; OTA – ochratoxin; ZEN – zearalenone

Mycotoxin binding by sorbents in a trial with naturally contaminated feed in the gastric (pH 3.5) and intestinal (pH 7.0) phases. Examining the binding by sorbents of three mycotoxins in naturally contaminated feed samples demonstrated low mycotoxin sorption in the case of two of them, namely DON and ZEN (Table 5). Therefore, no desorption investigation could be performed. All tested sorbents bound OTA in the gastric phase at a level in the range of 30–55%. The mycotoxin-sorbent complex was not stable enough to confirm the good sorption capacity of the sorbents for mycotoxins or the stability of the mycotoxin bond.

In the experiment using contaminated feed in the pH 3.5 phase, there was a significant decrease in the mycotoxin-binding capacity of the tested sorbents compared to the experiment using mycotoxin standards. Calcium lignosulphonate in the buffer with mycotoxin standards bound DON at a satisfactory level of 87%, while when feed was added to the sample, its binding capacity decreased to 11%. In the first step of the experiment modelling sorption, activated charcoal and sepiolite bound OTA and ZEN at very high levels ranging 94–99%. Adding feed resulted in a dramatic decrease in binding. In the case of activated charcoal, the

effectiveness in binding OTA decreased to 39% and that of binding ZEN to merely 4%. A similar decline was observed for sepiolite; for OTA the percentage in binding decreased to 53% and for ZEN to as low as 6%.

Discussion

Our research confirms that sorbents can eliminate AFL B1 with good efficiency and that the mycotoxin most difficult to bind by investigated sorbents is DON. The obtained results from the *in vitro* model confirmed the effectiveness of activated charcoal in binding to mycotoxins as well as the high stability of the mycotoxin-sorbent bond. Sepiolite had comparable properties to active charcoal. The results of the studies using buffers coincided with those obtained by other researchers using a similar *in vitro* research model. When reviewing the available literature regarding the *in vitro* model using buffers for mycotoxin binding by sorbents, it was noted that researchers had mainly focused on AFL B1 and rarely on DON or ZEN. Di Gregorio *et al.* (10) also reviewed the literature and observed that different mineral sorbents could bind and eliminate different mycotoxins from the feed. The present study is one of the first to investigate several sorbents for their binding of all legally regulated mycotoxins.

In the experiment, after elimination of seven sorbents which individually bound too few mycotoxins, the two sorbents (sepiolite and calcium lignosulphonate) which were found to bind many mycotoxins simultaneously were investigated combined in different proportions to test whether proportion changes could optimise binding. The mixture was confirmed to bind mycotoxins well, on average adsorbing 75%. Such a mixture could be a solution for binding multiple mycotoxins simultaneously.

By analysing the results obtained in the experiment using buffers and mycotoxin standards with variable pH, the effectiveness of the sorbents in binding mycotoxins was confirmed. The results were comparable to those obtained and published by other researchers. Most scientists tested a single sorbent for a single mycotoxin, but studies have rarely been conducted on simultaneous binding by sorbents of several mycotoxins. To the best of the authors' knowledge, this is the first study describing the binding of eight mycotoxins by sorbents in an *in vitro* model.

Bocarov-Stancic *et al.* (4) used a pH 3.0 buffer and confirmed the effectiveness of zeolite with AFL B1, 95% of which it bound, and with DON, 50% of this mycotoxin being taken up; we obtained similar results, with binding of 100% of AFL B1 by zeolite. Their studies also showed promising effectiveness in the action of bentonite against AFL B1, DON, ZEN and T-2.

The model with the use of buffers but no feed was also used by Albu and Uzunu (2). The researchers investigated the binding of AFL B1 and ZEN by bentonite, zeolite, activated charcoal and Mycosorb preparation (comprising yeast, sodium calcium aluminosilicate and algae). The activated charcoal could bind 88.5% of AFL B1 and 98.2% of ZEA. Zeolite bound 45% and 53%, respectively, and bentonite exhibited mycotoxin binding activity at the level of approximately 60% (2). Regarding activated charcoal, we obtained similar binding of AFL B1 and ZEN. Also, zeolite bound AFL B1 at a level of 100%; however, bentonite and zeolite bound only 1% of ZEN.

The dependence of mycotoxin adsorption by sorbents on pH was demonstrated by Thieu and Pettersson (26). In a variable-pH buffer model, they confirmed adsorption of AFL B1 by zeolite at over 70% when the pH was 3.0; it was 20% when the pH was 7.0. The opposite was the case with bentonite, where the adsorption at pH 3.0 of 30% was much lower than that at pH 7.0 of over 80%.

In the third part of the study, the sorbents' effectiveness at binding mycotoxins in contaminated feed was examined. The binding capacity of the three mycotoxins DON, OTA and ZEN was analysed in feed naturally contaminated with them. Adding feed to the research model significantly reduced the effectiveness of mycotoxin binding by the tested sorbents. In the available literature, no *in vitro* methodology describes the evaluation of a sorbent in a buffer with pH corresponding to that of the gastrointestinal tract when mycotoxins and feed are in the sample simultaneously. However, Kolawole *et al.* (16) used a more advanced model using digestive enzymes. They tested the mycotoxin binding capacity of commercial feed additives and obtained results for all tested preparations where DON was bound at 22–61%, ZEN at 8–53% and AFL B1 at 29–62%. However, not all tested feed additives bound FB1, OTA and T-2. A similarly advanced model was used by Avantaggiano *et al.* (3) to study the effectiveness of AFL B1, ZEN, FB1, FB2 and OTA binding by carbon and aluminosilicate products (attapulgite, various types of bentonite and clinoptilolite). The products reduced the simulated mycotoxin absorption by the organism in conditions reproducing those in the small intestine, Avantagiano *et al.* (3) noting AFL B1 binding at a 44% level, ZEN at 25%, OTA at 97% and fumonisins at 89–105%. A more complex model was also used by Kihal *et al.* (15). They demonstrated the ability of activated carbon to bind seven mycotoxins (AFL B1, DON, FB1, FB2, OTA, T-2 and ZEN) at the level of 81%, they found evidence of this ability of bentonite at the level of 45%, and observed that of zeolite to be 32%. Clinoptilolite was narrower in its effectiveness, binding 75% of AFL B1, 29% of T-2 and 14% of ZEN. The effectiveness of sepiolite against AFL B1, DON and ZEN was also tested and in percentage terms was 95, 13 and 39, respectively.

Many researchers have proposed various *in vitro* models to test the ability of sorbents to bind mycotoxins. The results of these models vary with the factors in action, the variety of feed additives used, and the complexity of the model. When comparing results of

experiments with a common goal, in this case assessing the effectiveness of sorbents, it is necessary to consider many factors that may cause results to diverge.

Conclusion

In this study, an innovative approach to verifying feed additives' mycotoxin binding abilities was using a model that contained feed naturally contaminated with mycotoxins, a sorbent, and two buffers separately, each with a pH appropriate for a section of the digestive tract. Moreover, a new model for the evaluation of feed additives was proposed, which attempted the assessment of the stability of the mycotoxin-sorbent complex at the pH level of the gastrointestinal tract. A group of sorbents was also tested for simultaneous binding of many legally regulated mycotoxins. Consideration of the results reveals a need to further search for a sorbent or a mixture of sorbents that would simultaneously and effectively bind many mycotoxins. It has been confirmed that in a simple model, sorbents have a high mycotoxin binding capacity; however, when added to actual contaminated feed samples, their activity decreased markedly.

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