JOURNAL OF ADVANCED VETERINARY AND ANIMAL RESEARCH
ISSN 2311-7710 (Electronic)
http://doi.org/10.5455/javar.2019.f322
A periodical of the Network for the Veterinarians of Bangladesh (BDvetNET)

March 2019
VOL 6, NO. 1, PAGES 125–132

ORIGINAL ARTICLE

In vitro studies on gastrointestinal monogastric and avian models to evaluate the binding efficacy of mycotoxin adsorbents by liquid chromatography-tandem mass spectrometry

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ABSTRACT

Objective: The objective of this study is evaluating the efficacies of 11 mycotoxin adsorbent products, marketed in South East Asia. Three prominently occurring mycotoxins; aflatoxin B1 (AFB1), deoxynivalenol (DON), and zearalenone (ZEN) were simultaneously spiked into the samples.
Materials and Methods: Samples were simultaneously tested in vitro in phosphate buffer and simulated at different pH conditions in the gastrointestinal tracts of the porcine and avian model, analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).
Results: All mycotoxin adsorbent products had high efficacy at over 90% for AFB1 adsorption in both GI porcine and avian models. AFB1 could be adsorbed more in acidic condition than the basic condition. ZEN adsorption was determined to be more stable at pH 3 than pH 6.5 or 8.4, in which pH condition might influence on ZEN desorption rate. DON was poorly adsorbed by all tested agents.
Conclusions: The finding showed that the adsorption rate varied depending on the type of adsorbent. Our results might provide useful information regarding the efficacy of mycotoxin adsorbents commercially marketed in the region.

ARTICLE HISTORY

Received November 23, 2018
Revised January 03, 2019
Accepted January 12, 2019
Published February 25, 2019

KEYWORDS

Aflatoxin B1; deoxynivalenol; LC-MS/MS; mycotoxin adsorbent; mycotoxin binder; zearalenone

Introduction

Contamination of food and feedstuffs with mycotoxins as harmful secondary metabolites produced by certain filamentous fungi is a global concerning issue with one-quarter of the estimated agricultural commodities [1]. The negative effects of mycotoxins on animals depend on species, age, dose, duration, and the nutritional and health status in which they are consumed. Contaminated feed can also affect the health functions and promote illness in animals that cause an economic loss. Therefore, a various physical, chemical, and biological methods to offset the adverse effects of mycotoxins have been implemented [2–5].

Using anti-mycotoxin feed additives is an alternative and attractive way of reducing the risk of mycotoxicosis and diminishing the transfer of mycotoxins from feed into animal products [6]. Generally, anti-mycotoxin feed additives are used to decrease mycotoxin absorption and to promote the excretion. The anti-mycotoxin or mycotoxin detoxifying agents can be categorized into two major groups as bio-transforming and adsorbing agents. First, bio-transforming or mycotoxin modifier agents, e.g., bacteria, fungi, yeast, and enzymes act to degrade mycotoxins into non- or less-toxic metabolites. Second, adsorbing agents as mycotoxin binders or adsorbents cause to decrease the absorption of mycotoxins from the gastrointestinal tract into the blood circulation and target organs by adsorbing on their surface. The use of adsorbing agents as technological feed additives has recently been officially

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How to cite: Prapapanpong J, Udomkusonsri P, Mahavorasirikul W, Choochuay S, Tansakul N. *In vitro* studies on gastrointestinal monogastric and avian models to evaluate the binding efficacy of mycotoxin adsorbents by liquid chromatography-tandem mass spectrometry. J Adv Vet Anim Res 2019; 6(1):125–32.

permitted in Europe under the Commission Regulation (EC) No. 386/2009 [7,8].

In farms, the use of mycotoxin adsorbents as feed additives is one of the most famous and popular ways of minimizing the effect of mycotoxin contamination in feed. Among mycotoxin adsorbents, aluminosilicate clays are the largest class and have been well reported in numerous research trials in order to diminish mycotoxicosis. Several aluminosilicates clays such as hydrated sodium calcium aluminosilicate (HSCAS), bentonite, montmorillonite, smectite, and zeolite showed a good binding affinity to mycotoxins [9,10]. These clays have a physicochemical ability to bind smaller mycotoxins, such as aflatoxin and ochratoxin A, but have less effect on larger molecules in certain Fusarium toxins. Numerous studies have reported the ability of various adsorbent materials to form and firmly bind mycotoxin-adsorbent complexes between their layers to reduce toxin bio-availability [11,12].

In tropical areas, aflatoxin B_1 (AFB_1), deoxynivalenol (DON), and zearalenone (ZEN) are commonly seen and active in livestock's production [12–14]. Several commercial mycotoxin binders have been said to be capable of counteracting major mycotoxins, including AFB_1, DON, and ZEN. However, there are limited scientific data to prove these claims, even though products are widely distributed.

Therefore, the objective of this study is to assess the effectiveness and stability of 11 locally marketed mycotoxin adsorbent products with simultaneous multi-mycotoxins in an *in vitro* model. The pH and transit of gastrointestinal (GI)-tract empty time for monogastric and avian species were simulated and analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Chemicals and reagents

AFB_1 and DON standards were purchased from TRC Inc. (Canada). ZEN standard, hydrochloric acid (HCl), dibasic sodium phosphate (Na_2HPO_4), monobasic sodium phosphate ($NaH_2PO_4.2H_2O$), sodium hydroxide (NaOH), and sodium bicarbonate ($NaHCO_3$) were supplied by Sigma. Methanol (MeOH) and formic acid (CH_2O_2) have been purchased from Fisher Scientific (Hampton, NH). Water from a Milli-Q system (Millipore, Bedford, MA) has been obtained. All chemicals used were of analytical grade unless stated.

Adsorbent

Eleven mycotoxin adsorbent products that marketed in South East Asia have been tested for their ability to bind AFB_1, DON, and ZEN at different *in vitro* gastrointestinal tract conditions. To blind beneficial referral by companies, the adsorbents were categorized into three groups depending on the type of clay and its main functional composition (Table 1) as unmodified clay adsorbent (group A; products 1–4), mixture of unmodified clay and yeast cell wall extract (group B; products 5–8), and mixture of modified clay and yeast cell wall extract (group C; products 9–11). Our study attempted to limit unexpected factors during the experiment by dissolving mycotoxin solutions using methanol and also by mixing similarly adsorbent mycotoxins at a ratio equivalent to 1% inclusion rate for a binding assessment of capacity evaluation by Faucet-Marquis et al. [15].

Preparation of phosphate buffer saline

For the monogastric model, three solutions of 0.01 mol/l phosphate buffer saline were prepared at pH 3.0, 7.4, and 8.4 using Na_2HPO_4 and $NaH_2PO_4.2H_2O$. The Henderson–Hasselbalch equation was used to calculate and adjust the pH as necessary with HCl or NaOH. For the poultry model,

Table 1. Composition of the 11 commercial products.

Group	Product	Composition
A (unmodified clay)	1	Bentonite, diatomaceous earth, herb
	2	Aluminosilicate
	3	Aluminosilicate
	4	Smectite clay
B (unmodified clay and yeast cell wall extract)	5	Montmorillonite, yeast cell wall extract
	6	Aluminosilicate, yeast cell wall extract, herb
	7	HSCAS, yeast cell wall extract
	8	HSCAS, yeast cell wall extract
C (modified clay and yeast cell wall extract)	9	Modified aluminosilicate and HSCAS, yeast cell wall extract, enzyme
	10	Modified aluminosilicate and HSCAS, yeast cell wall extract, enzyme
	11	Modified HSCAS, yeast cell wall extract

0.01 mol/l phosphate buffer saline was prepared at pH 3.0, 6.0, and 6.5 and adjusted as necessary using HCl or $NaHCO_3$.

High-performance liquid chromatography (HPLC) operating condition

Separation of analytes was carried out by reverse phase chromatographic technique on a Kinetex® 2.6 µm Biphenyl 100A column. The gradient mobile phase was composed of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in MeOH) at a flow rate of 0.4 ml/min. The gradient elution was programmed as follows: 0–3.0 min, 97% A; 3.0–6.0 min, 3%A; and 6.0–12.0 min, 97%A for a total run time of 12 min.

LC-MS/MS condition

Detection of target analytes was carried out on an AB SCIEX QTRAP® 5500 mass spectrometer with electrospray ionization (ESI) in Scheduled MRM detection mode. A full scan mass spectrometer (MS) was operated under positive and negative ion modes. AFB_1 and DON were obtained under positive ionization mode ($[M + H]^+$), whereas ZEN was obtained under negative ionization mode ($[M - H]^-$). ESI-source parameters were optimized and preset for all measurement were as follows: Source temperature: 450°C, curtain gas 25 psi, gas 1:50 psi, gas 2:50 psi. Ion spray voltage was set to 5,500 V in positive ionization mode and −4,500 V in negative ionization mode.

In vitro experiment design

In vitro binding ability of adsorbents

Binding abilities of 11 commercial adsorbents at different pH conditions were tested *in vitro* using the following procedure. Ten milligrams of each adsorbent were suspended in 0.01 mol/l PBS (1 ml) in different pH at 7.4, 3.0, and 8.4 simulating pH conditions in the oral cavity, stomach, and intestine of monogastric animals, respectively.

A phosphate buffer was also prepared to simulate pH conditions in the crop, stomach, and intestine of poultry at different pH equivalents of 6.0, 3.0, and 6.5, respectively. Experiments were tested in triplicate for each pH to evaluate the ability of mycotoxin absorption by adding the same amount (1 ml) at 0.1 µg/ml of each mycotoxin. After 1-min vortexing, all the test tubes were incubated differently following the gastrointestinal transit time of each species. Suspensions of the monogastric animal model were incubated (at 250 rpm.) at 37°C for 30 min, 1 h, and 3 h, whereas suspensions of the poultry gastrointestinal model were incubated at 40°C for 30 min, 45 min, and 1 h. After centrifugation at 2,040 × g for 10 min, the 500 ml aqueous supernatants were diluted with MeOH:H_2O (ratio 50:50) and then analyzed using LC-MS/MS. Two types of negative controls were performed to ensure the validity of the test results, first by fortification into the PBS solution with 0.1 µg/ml of each mycotoxin, and second by containing only each adsorbent in the solution in the control tube.

Stability of adsorption/desorption of adsorbent-mycotoxin complexes

In order to evaluate the stability of adsorbent-mycotoxin complexes in both acid and basic conditions, we chose an *in vitro* mono-gastrointestinal tract model as a pilot study since this could cover a wide variety of pH conditions from 3 to 8.4. In Brief, 10 mg aliquots of the adsorbents were suspended in 1 ml of 0.01 mol/l PBS and fortified with 0.1 µg/ml of each mycotoxin [diluted in MeOH:H_2O (50:50)]. All the samples were then mixed for 1 min and incubated at 37°C on an automated horizontal shaker for a determined time at 250 rpm. Incubation times of acid conditions were 30 min and 1 h, while basic conditions were 30 min and 3 h. Finally, the samples were centrifuged at 2040 × g for 10 min, and 500 µl of the supernatants were diluted in MeOH:H_2O (50:50) to make up the 1 ml final volume. Then, they were stored at 4°C pending analysis by LC-MS/MS within the same day.

Calculation of mycotoxin adsorption rate (%)

The peak areas at mycotoxin retention times were compared to the corresponding calibration curves. Calculation of mycotoxin adsorption rates (%) was performed according to the following equation:

$$Y = (1 - C_{eq}/C_o) \times 100\%$$

where

Y is the adsorption rate,

C_{eq} is the concentration of free mycotoxin after the incubation period, and

C_o is the initial fortified concentration of the mycotoxin.

Statistical analysis

All data were presented as mean ± SD. The data were subjected to two-way analysis of variance (ANOVA) by SPSS. Statistical significance at $p < 0.05$ was considered to exist.

Results

Mycotoxin adsorbent efficacy in the monogastric model

In order to study the ability of mycotoxin-binding adsorbents, an *in vitro* model was designed to simulate the pH and transit of GI-tract empty time of the oral cavity or crop, stomach, and intestine of monogastric animals and avian species. The effect of pH on the effectiveness of AFB1,

DON, and ZEN adsorption in the GI tract of the monogastric model by different detoxifying agents is shown in Figure 1. All tested agents had high binding affinity to AFB_1, in which average adsorption rates were greater than 85%. Product groups B and C had equal ability to adsorb AFB_1 in the range of 98%–99%.

In contrast to AFB_1, all adsorbents showed significantly ($p < 0.05$) low ability to adsorb DON with adsorption rates less than 18%. Interestingly, AFB_1 and DON were significantly adsorbed in acidic conditions (pH 3.0).

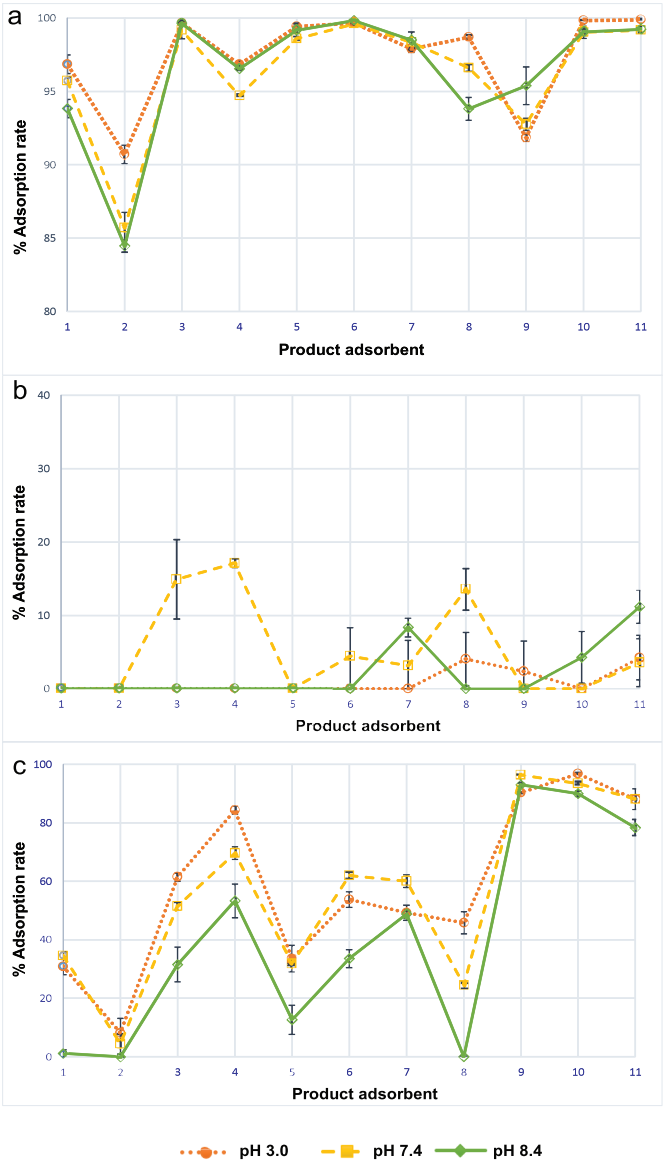

Figure 1. Percentage adsorption rate of mycotoxins in the monogastric model, Aflatoxin B_1 (a), Deoxynivalenol (b), and Zearalenone (c).

ZEN presented a wide variation range of adsorption from 4% to 93%. Group C showed high efficacy, with relatively stable binding capacity at 84%–93% on ZEN detoxification in all pH conditions, whereas groups A and B gave binding capacity rates at 0%–80% and 23%–55%, respectively. Interestingly, ZEN was significantly less adsorbed by the same agent in alkaline than in acid and neutral conditions. Overall, the commercial product, consisting of modified aluminosilicate with HSCAS, yeast cell wall extract, and enzyme showed the satisfactory binding capability to AFB_1 and ZEN in the monogastric model.

Mycotoxin adsorbent efficacy in the poultry gastric model

Results of the 11 commercial products on mycotoxin detoxification in the avian GI tract model are shown in Figure 2. Findings were consistent with the monogastric model and presented adsorbent capacity decreasing in the order AFB_1 > ZEN > DON. Binding capacity of all tested agents against AFB_1 in any pH condition gave high adsorption rates between 83% and 99%.

ZEN adsorption showed high variation efficiency of poor to moderate adsorption, with average adsorption rates at 4%–69% and 23%–52% in groups A and B, respectively, whereas good adsorption was presented at 84%–93% in group C.

Most of the 11 mycotoxin adsorbents were not able to bind DON. However, limited DON adsorption rate at 2%–10% by five commercial products was evident in acidic conditions in contrast to the monogastric model. Similar to the monogastric model results, highest efficacy to detoxify AFB_1 and ZEN in the *in vitro* avian GI tract model was shown by product No 10. AFB_1 and DON were significantly adsorbed in acidic conditions (pH 3.0), while ZEN was significantly adsorbed in weak acid conditions (pH 6.5).

Stability of adsorbent-mycotoxin complexes

To elucidate the stability of adsorbent-mycotoxin complexes, we evaluated the percentage adsorption–desorption rate of mycotoxins in both acidic (pH 3.0) and basic conditions (pH 8.4) for different periods of GI transit time. To evaluate their stability, we simulated passing time as feed presence in the stomach by performing incubation period under acidic conditions for 30 min and 1 h. For basic conditions, the samples were incubated for 30 minutes and 3 h as the duration of the feed presence in the animal's intestinal tract. Thereafter, the mycotoxin levels following incubation periods of each condition were compared. Average adsorption rates of all agents for any time of AFB_1, DON, and ZEN were 96.8%, 0.53%, and 56.6% in acidic conditions and 95.9%, 6.03%, and 46.2% in basic conditions, respectively. Figure 3 showed alteration percentage of adsorption and desorption rate after an incubation time of each product. Our findings indicated that transit time

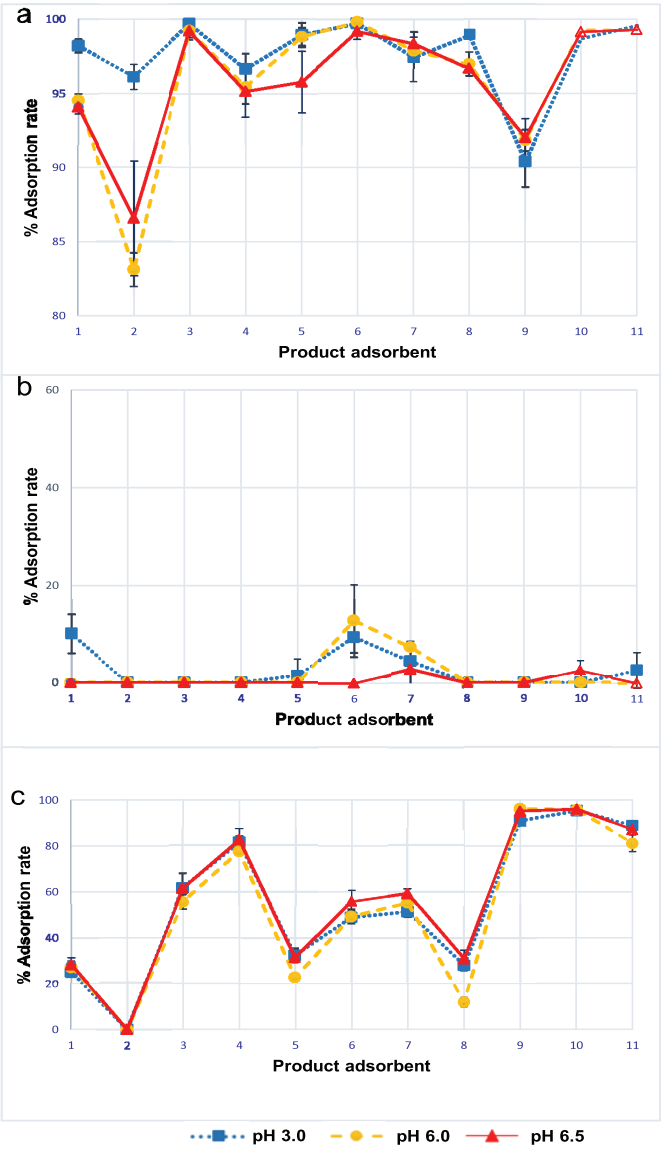

Figure 2. Percentage adsorption rate of mycotoxins in the poultry gastrointestinal model, Aflatoxin B_1 (a), Deoxynivalenol (b), and Zearalenone (c).

period did not significantly affect adsorbent-mycotoxin complexes ($p > 0.05$). Considering acidic conditions, most binding agent-mycotoxin complexes were stable except for one product (No. 2) that showed high desorption rate to ZEN. Results showed that alkaline conditions had an effect on the interaction between ZEN and binding agents with the instability of the adsorption rate. In addition, similar to the adsorption rate under acidic conditions, our results confirmed that alkaline conditions appeared to impede the ZEN-binding complex.

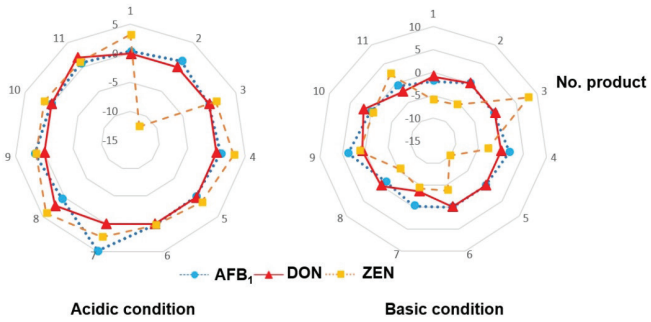

Figure 3. Adsorption–desorption rates of 11 mycotoxins binder products (Number on outer circle line) in acidic (pH 3.0) and basic (pH 8.4) conditions. Number in inner circle means changed percentage of adsorption rate (positive value) and desorption rate (negative value) of each products after incubation period.

Discussion

Effectiveness of 11 commercial mycotoxin adsorbents on three fungal toxins was compared using a gastrointestinal tract model. Results indicated that most products had very high ability to adsorb AFB_1 toxins in both monogastric and avian GI tract models. Only one product, consisting of unspecified aluminosilicate clay, appeared to show inadequate binding affinity to AFB_1 possibly because the source of clay in the product was of poor quality. Therefore, careful product sourcing and batch-to-batch adsorbent material assays are necessary and important to maintain quality. Mycotoxin adsorbent products used here were mainly composed of clay minerals which regulate the adsorption process. Typically, clays are most effective in binding small-sized polar molecules. Adsorbent characteristics such as total charge, charge distribution, size of the pores, and accessible surface area influenced adsorption [5,16]. In addition, the physicochemical properties of mycotoxins including polarity, solubility, size, shape, and charge play important roles for complex binding processes [5,16–19].

Considering the chemical structure of the fungal toxins, AFB_1 is a polar mycotoxin and contains β-carbonyl, which is involved in the adsorption process [20]. According to, the adsorption process involves the exchange of electrons of the metallic cation on the surface of the adsorbent, especially the positive charge of calcium ions on each layer of clay. A hypothesis proposed by Jaynes et al. [21] discussed the possibility that aflatoxins can be captured at multiple locations on HSCAS surfaces, as well as between HSCAS inter-layers. Several studies have shown that the addition of HSCAS to animal feed at 0.5%–2% has a significant effect by preventing harmful aflatoxicosis in animals such

as chicken, turkey, duck, and lamb [22–24] and reducing toxins in cow and goat milk [25–27]. Those findings have confirmed the high capacity to interact with AFB1 in all mineral clay products, including bentonite, montmorillons, and aluminosilicate (HSCAS).

Attempts to classify the huge variety in the clay group have been extensively discussed and examined [16,28]. Smectite, hydrated sodium calcium aluminosilicates (HSCAS), bentonite, and montmorillonite are commonly used in the livestock production and originate in the Phyllosilicate group [29,30]. The inter-layer characteristics known as 2:1 Phyllosilicate clay are made up of an octahedral sheet sandwiched as a planar structure between two tetrahedral sheets. Several reports have shown that 2:1 clay represents one of the most powerful prevention of aflatoxicosis in livestock [20,21,23,31–33]. This could be because the planar molecule of aflatoxin can easily penetrate and bind into the interlayer of the planar 2:1 phyllosilicate clay structure.

Our results have shown clearly that the phyllosilicate clay minerals in AFB1 adsorption are highly efficient. Nevertheless, low-to-moderate adsorption of DON and ZEN was characteristic of the clays. These findings were in agreement with Avantaggiato et al. [17], who concluded that clay would not prevent and reduce *Fusarium* toxicity, including fumonisins, trichothecenes, and zearalenone. One possible reason that has been previously discussed is that DON and ZEN have lower polarity than AFB_1 [16]. Size and shape of the mycotoxin are another important characteristics that affect accessibility to the internal surface of the binding agent.

ZEN is a non-polar lipophilic compound with resorcylic acid lactone [15]. The spherical molecular geometry of ZEN is also greater than the planar structure of AFB1 [34], while DON is polar and has a complex molecular structure [5,16–19,34]. Thus, the extent of ZEN adsorption by clay is usually lower than aflatoxins. A large difference in ZEN adsorption was previously reported among different binding materials [17,35–37].

Notably, ZEN adsorption was significantly ($p < 0.05$) affected by pH condition. As shown in the monogastric experiment, desorption of ZEN-binder complexes occurred in alkaline conditions, particularly in unmodified clays. However, because of the acidic pH condition, this phenomenon was not present in the avian model. De Mil et al. [34] recently suggested that pH values may affect the ZEN phenolic hydroxyl group of ZEN, while a previous report stated that ZEN may be desorbed by clay under alkaline conditions [15].

By comparison, acidic and neutral conditions promote interaction between ZEN and yeast cell walls with more stable complexes than in alkaline conditions [38]. Consequently, modified clays with organic cations have been developed to improve ZEN adsorption, which provide sufficient space between the layers to react with mycotoxin with a relatively less polarity with the appropriate electrical charging [6,39–41]. These modified surface properties lead to greater hydrophobicity by exchanging the structural load balance cations with high molecular weight quaternary amines [10]. Feng et al. [42] have concluded that these modified clays have led to low desorption rates, with higher ZEN adsorption than non-modified clays.

Our findings confirmed a better capacity for ZEN than unprocessed clays in both imitative models in modified clays (group C). Furthermore, yeast cell walls can improve the binding activity in the materials. The yeast cell walls have been shown to improve ZEN adsorption capacity in accordance with previous studies [6,34,43].

Previous research has shown that DON can be rapidly absorbed into the blood circulation within 30–60 min [44]. Therefore, several strategies and products to prevent DON absorption such as the use of detoxifying agents have been developed. Our investigation indicated that all of the claimed mycotoxin-binders failed to bind DON at a satisfactory level. This finding was based on earlier binding tests showing limited inorganic clay activity to alleviate DON [6,35,45,46]. In inorganic clay, activated carbon has been shown to be extremely effective against DON and ZEN in *in vitro* experiments [6,36,46]. However, activated carbon is an unspecified adsorbent saturated by the feed matrix [47].

Our study was performed with three simultaneous mycotoxins and contrasted with most previous experiments conducted on the *in vitro* interaction of clay with only a single mycotoxin. The extent of adsorption in *in vitro* experiments could be affected by the simultaneous multi-toxin assay. This possibility concurs with Avantaggiato et al. [17]. They concluded that the percentage of multi-toxin adsorption may be less than experiments performed with a single toxin.

Although mycotoxin binders are recommended to be performed in *in vivo* experiments to demonstrate their ability to reduce mycotoxins, the *in vitro* model trial could, however, provide rapid advance information that shows their potential adsorption–desorption ability under various imitation conditions. In addition, our results do not support meaningful conclusions as to which kind of clay has the highest efficacy to sequester mycotoxins. All clays have variable abilities to adsorb toxins depending on their individual chemical structures which affect the electrochemical properties of each binding agent.

Labeling of mycotoxin adsorbent is another issue that should be clarified. Some countries or regions have no legislation that stipulates the use of mycotoxin adsorbent as a feed additive. We suggest that the labels of mycotoxin

adsorbent products should clearly provide the detailed information regarding the harmonized clay category (as an international agreement on class and subclass of clay category as feed additive), including additional substances.

Taking all these aspects into account, no perfect characteristic of adsorbent agents exists to counteract simultaneous multi-mycotoxins that are prevalent in the feed as there are wide variations in structure and electrochemical properties of mycotoxin adsorbents. In addition, inorganic clay alone provides only bio-availability data but this does not necessarily reduce toxicity. Adsorbent products may require the addition of biological agents to bio-remediate and reduce bio-potency and toxicity in the cell. Therefore, multi-strategies are required to alleviate the effect of mycotoxins on livestock production.

Conclusion

All mycotoxin adsorbent products have high efficacy for AFB1 adsorption. Comparably, adsorption–desorption rate experiment showed that pH condition in GI tract might influence on ZEN desorption rate. All evaluated agents have limited capacity to mitigate DON.

Acknowledgment

The authors wish to thank the Graduate School of Kasetsart University and Huvepharma (Thailand) for their financial support. We also thank Assist. Prof. Dr. Chalermpol Lekcharoensuk and Assist Prof. Suwicha Kasemsuwan for assistance with the statistical analyses.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Authors' Contribution

Jutamas Prapapanpong performed the measurements with support from Wiratchanee Mahavorasirikul and Sasiprapa Choochuay. Natthasit Tansakul designed and directed the project. Pareeya Udomkusonsri aided in interpreting the results. Primary draft of this manuscript was written by Natthasit Tansakul and Jutamas Prapapanpong. All authors critically reviewed and discussed the results on the manuscript.

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