Assessment of H-β zeolite as an ochratoxin binder for poultry

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ABSTRACT Most of the cereal-based ingredients used in poultry feed are contaminated with ochratoxin-A (OTA). We have investigated H-β zeolite (HBZ) as a new OTA binder for poultry, along with widely used clay mineral-based product (CM), using in vitro and in vivo methods. In vitro binding experiment was carried out using a biphasic assay, consisting of adsorption at pH 3.2 and desorption at pH 6.8. High adsorption (>98%) with less desorption (<5%) was observed for HBZ, whereas CM showed high binding (>98%) and moderate desorption (48%). In the in vitro experiments with the different simulated gastro-intestinal pH buffers, HBZ did not desorb OTA at any of the pH. Desorption of OTA was observed with CM, as the pH increases. From the in vitro kinetic and chemisorption studies, faster, stronger, and higher adsorption was observed for HBZ. Thermodynamic studies showed positive entropy (22.76 KJ/mol K) for HBZ, signifying predominant hydrophobic interactions towards OTA, whereas CM exhibited negative entropy (–3.67 KJ/mol K). The in vivo binding efficacy of HBZ and CM was tested in 5-wk-old broiler chickens. The study consisted of 4 experimental groups, each with 6 replicates having 2 birds per replicate. The groups were control, negative control (no toxin binder), T1 (HBZ at 1 kg/ton of feed), and T2(CM at 1 kg/ton of feed). Except control, all the replicates received 20 µg of OTA in the feed. Excreta samples of T1, T2, and NC contained 11.57, 7.16, and 2.78 µg of OTA respectively, which was significantly different from each other ($P < 0.05$). A growth performance trial was conducted in broiler chickens for 35 D. A total of 288 one-day-old birds were randomly segregated to 3 treatment groups, each with 8 replicates of 12 birds each. Treatment groups consisted of control, T1, and T2, treated with no toxin binder, HBZ, and CM at 1 kg/ton of feed, respectively. None of the treatment groups including control, affected BW gain, and feed conversion ratio ($P > 0.05$).

Key words: ochratoxin-A, mycotoxin binder, broiler chickens, H-β zeolite

2020 Poultry Science 99:76–88
http://dx.doi.org/10.3382/ps/pez535

INTRODUCTION

Cereals, such as corn, soya, wheat and their by-products are commonly used feed ingredients in the poultry diets across the world. These ingredients are often contaminated with various levels of ochratoxin-A (**OTA**) (Binder et al., 2007). Sherazi et al. (2015) have reported OTA contamination level as high as 190 µg/kg in the poultry diets. In a worldwide survey on prevalence of mycotoxins in animal feed and feedstuffs, OTA was seen in all the regions (Rodrigues and Naehrer, 2012). Ochratoxins are secondary metabolites produced by toxigenic fungi of the genera *Aspergillus* and *Penicillium*. Among the mycotoxins, OTA also known to have high toxicity and is classified as a group 2b potential human carcinogen by the International Agency for Research on Cancer (Reddy and Bhoola, 2010). Studies have shown that OTA damages the integrity of intestinal epithelial cells and is considered as a serious health hazard due to its nephrotoxic, teratogenic, hepatotoxic, and carcinogenic properties (McLaughlin et al., 2004; Peteri et al., 2007). Very often, OTA co-occur with other mycotoxins and can lead to synergistic effects, resulting in pronounced toxicological interactions (Grenier and Oswald, 2011; Murugesan et al., 2015). Reduced rates of weight gain, decreased egg production, immunosuppressive effects, and increased mortality were reported in chicken fed with OTA contaminated feed (Stoev, 2010). Pozzo et al. (2013) studied the effect of OTA in chickens using the maximum allowed level (0.1 mg/kg), suggested by the European Commission Recommendation 2006/576/EC in the feed (European commission, 2006). Even at this level, reductions in thymus weight and in total serum protein, albumin, and alpha-, beta-, and gamma-globulin levels were noticed.

Various approaches are being used to mitigate the harmful effects of mycotoxins in the animals. The most common method is the addition of mycotoxin binders, such as activated carbon and natural clay materials in

Received February 22, 2019.
Accepted September 16, 2019.
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the animal feed. Natural silicate-based clay materials are used extensively in the animal feed and are effective in binding to aflatoxin B1 (**AFB1**) (Avantaggiato et al., 2007; Kolosova and Stroka, 2011). However, these materials did not show good binding activity towards structurally different mycotoxins, such as OTA. Activated carbon has been very effective in binding most of the mycotoxins, but the higher binding of nutrients and antibiotics limits its usage in the animal feed (Avantaggiato et al., 2007; De Mil et al., 2015).

Different approaches have been adopted for developing improved versions of mycotoxin binders. Molecularly imprinted polymers (**MIP**) have been found to be effective in removing >84% of 17 β-estradiol and OTA from complex matrices and wines, respectively (Giovannoli et al., 2014; Ning et al., 2014). Though MIP offers high selectivity towards contaminant, the cost was quite high which limits its usage. Binding studies with mycotoxins showed that grape pomace could adsorb most of the mycotoxins (Avantaggiato et al., 2014). It was also found that hydrophobicity in grape pomace was one of the important parameters responsible for binding AFB1 and zearalenone (**ZEA**). Another category of mycotoxin binder is porous materials, wherein the toxins interact with the surface of the material as well as the inside of the pores (Boudergue et al., 2009). In this category, natural zeolites are widely used, but were reported to be less effective in binding multiple mycotoxins (EFSA, 2010). Clinoptilolite-heulandite (**CH**) rich materials possessed <40% binding to OTA at different pH values (Dakovic et al., 2003). However, when CH was modified with octadecyl-dimethyl benzyl ammonium ion, the efficacy increased to >95%. The increase in efficacy was due to the organic modifier contributing hydrophobicity to CH. In another study, natural zeolite modified organically was found to have higher binding to OTA and ZEA than the unmodified natural zeolite (Tomasevic-Canovic et al., 2003). These studies indicate that porous materials can be fine-tuned to achieve better binding towards mycotoxins.

Synthetic porous materials offer flexibility to modify the parameters such as particle size, pore size, surface area, and silica alumina ratio based on our needs (Kulprathipanja, 2010). Though several synthetic zeolites exist, H-β zeolite (**HBZ**) possesses certain unique properties, such as silica alumina ratio from 22 to 30, hydrophobicity, and high acidity (Kulprathipanja, 2010; Khalil et al., 2016). In addition, HBZ has coexistence of ordered and disordered frameworks and 3 mutually intersecting channels. Its framework structure has 12 membered rings with 2 types of pores: 5.6 × 5.6 A^0 and 7.7 × 6.6 A^0. These peculiar attributes make HBZ distinct from other synthetic zeolites (Zhang et al., 2013). Though HBZ has been widely used in the chemical industry as a catalyst, its application to detoxify mycotoxins in animal feed is not explored yet. The clay mineral-based product (**CM**) used widely in the poultry diets was used for comparing the efficacy of HBZ.

The objective of the study was to evaluate the OTA-binding efficacy of HBZ on comparison with other mycotoxin binders and investigate the adsorptive process using in vitro and in vivo methods in broiler chickens.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile (HPLC grade), methanol (HPLC grade), OTA, triethyl amine, and vitamins (B9, E, and C) were procured from Sigma-Aldrich, St. Louis, MO. Sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, ammonium acetate, sodium acetate, trisodium citrate dihydrate, glacial acetic acid (HPLC grade), hydrochloric acid, potassium chloride, titanium dioxide, hexane, chloroform, and HPLC grade water were purchased from Merck Life Science Pvt. Ltd., Mumbai, India. Citric acid monohydrate was obtained from Thermo-Fischer Scientific India Pvt. Ltd., Mumbai, India. Synthetic zeolites such as HBZ, Zeolite Socony Mobil (**ZSM**-5), H-ZSM, and HY-zeolite used for the studies were received from Wish Chemicals Co., Ltd., Yueyang, China. Zeolite-3, zeolite-4, and zeolite-5 were received from Zeolite and Allied Products Pvt. Ltd., Mumbai, India. Sodium-Y zeolite, Zeolite-AS, and Zeolite-AD were received from Astraa Chemicals, Chennai, India. CM used in this study contains a mixture of clay mineral-based mycotoxin binder used widely in the poultry diets. The feed ingredients used in the in vivo trial were purchased from Muthu Feeds, Namakkal, India. The feed additives (DL-methionine, L-lysine, choline chloride, BROVIT PLUS® PREMIX, dicalcium phosphate, calcite, and sodium bicarbonate) were purchased from Pooja Agencies, Namakkal, India. Kemtrace® Broiler was received from Kemin Industries South Asia Pvt. Ltd., Chennai, India.

Physical Characterization of HBZ

Powder X-ray diffraction studies were performed with Rigaku diffractometer using Cu-Kα radiation for characterizing HBZ as described by Lima et al. (2011). The diffractograms were recorded in the 2θ range of 0.2 to 80° with a 2θ step size of 0.01° and a step time of 10 s. The specific surface area and pore size measurement were carried out with Quantachrome Autosorb model using the Brunauer–Emmett–Teller (**BET**) method (Gonzalez et al., 2012). The pore size distribution was obtained from the adsorption branch of nitrogen isotherms by the Barrett–Joyner–Halenda (**BJH**) method (Sahooli et al., 2017). The morphology of the particles was determined by Hitachi S-4800 field emission scanning electron microscope (**HRSEM**) using an accelerating voltage of 5.0 to 20 kV (Sahooli et al., 2017).

Preparation of Standard Solution

All the standards were prepared in acetonitrile and stored at 8 to 10°C before use. The working

concentrations of OTA used for the in vitro assay was 1 µg/mL, which was based on the frequent contamination level seen in the Indian poultry feed (data not published). The citrate (pH 3.2), acetate (pH 5.5 and 5), and phosphate buffer (pH 6.8) of 0.1 M concentration used in this study were prepared based on the published reports (Conover, 1998). The OTA of 1 µg/mL was prepared using citrate buffer (pH 3.2, 0.1 M) after removal of organic solvent from stock solution by purging with nitrogen gas. The concentrations of the vitamin-E (30 µg/mL) and B9 (1 µg/mL) used for the binding studies were based on the commercial vitamin premixes used for the poultry feed. Vitamin-C was prepared at 100 µg/mL, which was based on the published reports (Ghazi et al., 2015).

Chromatographic Method

The HPLC apparatus consisted of Shimadzu LC-20AD coupled to a fluorescence detector (RF-20A) and diode array detector (SPD-M20A) interfaced with LC solutions (ver. 1.25) software. A reverse phase, Kinetex C18 chromatography column (Phenomenex, 250 × 4.6 mm i.d., 5 μm) was used for quantification of OTA and vitamin-E. The mobile phase composition and detection wavelength used for quantification of OTA were based on the report published by Aboul-Enein et al. (2002). A hydrophilic chromatography column (Phenomenex, HILIC, 150 × 4.6 mm i.d., 5 μm) was used for the quantification of water-soluble vitamins. The mobile phase composition, quantification wavelength, and flow rate were based on the published reports (Karatapanis et al., 2009). The chromatographic method for quantifying vitamin-E was based on the procedure reported by Brabcova et al. (2013). All chromatographic solvents were degassed for 15 min in an ultrasonic bath prior to chromatographic analyses.

General Method for OTA Extraction From Feed and Excreta

Solvent-assisted extraction method was used for extracting OTA from feed and excreta with modifications (Al-Hadithi et al., 2015). Briefly, 25 g of grinded feed was added to 100 mL of solvent mixture (acetonitrile, 85% and 5 N hydrochloric acid containing 4% potassium chloride, 15%) followed by mixing for 30 min. Solvent was removed by filtration, defatted with 50 to 100 mL of hexane twice, and extracted with 50 mL of chloroform twice. The chloroform layers were pooled together. The chloroform was evaporated using rotary evaporator (Heidoph, Hei-VAP advantage, 600 mbar, 60°C, and 100 rpm). It was then reconstituted with 1 mL of acetonitrile and quantified by HPLC as described before. For excreta, the sample size was 15 g and rest of the procedure remained the same.

In Vitro Experiments

In Vitro Biphasic Binding Assay This biphasic method was used to assess the binding efficacy of HBZ and CM towards OTA and vitamins. The biphasic method consisted of adsorption followed by desorption step, which was based on the published report with minor modifications (Malysheva et al., 2013). Briefly, the method involved the incubation of adsorbent (10 mg) with 1 mL of OTA solution prepared in 0.1 M citrate buffer, pH 3.2 (simulates gizzard pH) in the adsorption step. After centrifugation at 10,000 g for 10 min, the desorption step was carried out with 1 mL of 0.1 M phosphate buffer, pH 6.8 (simulates intestinal pH). The assay was carried out at 40°C, and the incubation time was 1 h for each step. The samples from each step were analyzed for OTA by HPLC. The adsorption percentage, desorption percentage, and net binding were calculated as per the equation reported by Malysheva et al. (2013). Similarly, binding test with vitamins was carried out and the net binding percentage was calculated.

Gastrointestinal pH Simulation Studies With OTA The adsorption of OTA with HBZ and CM was evaluated at different pH values simulating different parts of poultry gastrointestinal (**GI**) tract. Adsorbent (10 mg) was weighed into a 2 mL centrifuge tube and mixed with 1 mL of solution containing 1 μg/mL of OTA prepared in pH 6.8 buffer. Samples were incubated at 40°C for 1 h. Then, the samples were centrifuged (10,000 g, 10 min), and the supernatants were completely removed and analyzed for OTA content to calculate mycotoxin adsorption by HPLC (Equation 1). Then, 1 mL of acetate buffer (pH 5.5) was added to the pellet obtained in the previous step, mixed, and incubated at 40°C for 60 min. Then, adsorption and desorption percentage for this step was calculated as per Equations 2 and 3, respectively. Similarly, the pellets were incubated sequentially in buffers at pH 3.2, 5.0, and 6.8. The percentages of OTA that remained adsorbed at each pH 3.2, 5.0, and 6.8 were calculated based on Equation 4, Equation 5, and Equation 6, respectively.

Equation 1. Calculation of OTA adsorbed at pH 6.8

$$\text{Percentage OTA adsorbed at pH 6.8} = \frac{(\text{HPLC area of control in buffer pH 6.8}) - (\text{HPLC area of supernatant sample in pH 6.8})}{\text{HPLC area of control in buffer pH 6.8}} \times 100$$

Equation 2. Calculation of percentage OTA desorbed. pH refers to 5.5, 3.0, 5.0, and 6.8 individually as per the experimental conditions.

$$\text{Percentage desorption} = \frac{\text{HPLC area of supernatant sample in respective pH}}{\text{HPLC area of control in respective pH}} \times 100$$

Equation 3. Calculation of adsorption percentage at pH 5.5

$$\text{Percentage OTA adsorbed at pH 5.5} = (\%\ \text{OTA adsorbed at pH 6.8}) - (\%\ \text{OTA desorbed at pH 5.5})$$

Equation 4. Calculation of adsorption percentage at pH 3.2

$$\text{Percentage OTA adsorbed at pH 3.2} = (\%\ \text{OTA adsorbed at pH 5.5}) - (\%\ \text{OTA desorbed at pH 3.2})$$

Equation 5. Calculation of adsorption percentage at pH 5.0

$$\text{Percentage OTA adsorbed at pH 5.0} = (\%\ \text{OTA adsorbed at pH 3.2}) - (\%\ \text{OTA desorbed at pH 5.0})$$

Equation 6. Calculation of adsorption percentage at pH 6.8

$$\text{Percentage OTA adsorbed at pH 6.8} = (\%\ \text{OTA adsorbed at pH 5.0}) - (\%\ \text{OTA desorbed at pH 6.8})$$

Adsorption Kinetics Adsorption kinetics for OTA was carried out using contact time studies described by Avantaggiato et al. (2014). The effect of contact time on the adsorption of OTA was investigated with HBZ and CM at pH 3.2 and pH 6.8, respectively. Dosage of 10 mg/mL in triplicate independent experiments at 1 µg/mL of OTA was used for the studies. Samples were withdrawn at appropriate time (1, 2, 15, 30, and 60 min), centrifuged (10,000 g for 10 min), and the supernatant liquid portions were analyzed for residual mycotoxin content by HPLC and expressed in percentage.

Bonding Strength Bonding strength between OTA and adsorbents was carried out using chemisorption index (**CI**) studies as per the published reports with minor modifications (Dwyer et al., 1997; Ringot et al., 2005). Briefly, 10 mg of adsorbents was added to 1 mL of buffer (pH 6.8) containing 1 µg/mL of OTA (C-Initial) and incubated at 40°C for 45 min. Then, the contents were centrifuged (10,000 g, 10 min), and adsorption percentage was measured as described earlier with the supernatants (C-Bound). The pellets were then treated with 1 mL of 20% aqueous methanol, mixed well, centrifuged, and the amount of OTA in the supernatants was analyzed (C-Unbound). CI was calculated by using the following Equation 7.

Equation 7. Calculation of chemisorption index (CI)

$$\text{CI} = \frac{(\text{C} - \text{Bound}) - (\text{C} - \text{Unbound})}{(\text{C} - \text{Initial})}$$

Thermodynamic Studies Thermodynamic parameters were determined based on the reported procedure to identify the interactions associated with the mycotoxins (Avantaggiato et al., 2014). Various temperature conditions such as 278, 288, 298, 308, and 318 K were used to construct the adsorption isotherm. Adsorption isotherm was obtained by plotting the amount of mycotoxin bound per unit of material against the quantity of toxin. The data were fitted to Langmuir equation, and then the thermodynamic parameters such as Gibbs free energy change ($\Delta G°$, kJ/mol, the fundamental criterion of spontaneity), equilibrium constant (K_0) for the adsorption reaction, enthalpy ($\Delta H°$, KJ/mol), and entropy ($\Delta S°$, KJ/mol K) were calculated based on the reports published by Avantaggiato et al. (2014) and Ringot et al. (2005).

In Vivo Trials

All the animal studies were carried out in accordance to guidelines proposed and approval by the Committee for Control and Supervision of Experiments on Animals (CPCSEA, Registration number: 1784/PO/RcBi/S/14/CPCSEA).

In Vivo OTA Binding Study The in vivo binding assessment of OTA was done with Vencobb-400 broiler chicken, using 1-time feeding method with minor modifications (Sibbald, 1976; Lopez and Leeson, 2007). One-time feeding method involved administration of known quantity of experimental diets, after starving the birds.

Preparation of OTA Contaminated Feed. Broiler finisher mash feed was prepared as per ingredients listed in Table 2. The feed was tested for OTA and found to be less than the detectable limit of 1 ng/g of feed. Then, the feed was artificially contaminated with 100 ppb of OTA (100 ng/g of feed). Briefly, 10 µg/mL of OTA prepared in water (corresponds to 100 ng/g of OTA) was added to 100 g of the feed, mixed, and kept in dark for 7 D. Afterwards, 0.1 g of respective toxin binder was added to each of the treatment group, mixed, and kept for 1 D in the dark place. To confirm OTA in the

Table 1. Details of the groups used for the in vivo binding studies.

Groups	Feed	Ochratoxin A	Toxin binder	Dose
Control	Broiler mash finisher feed	–	–	–
Negative control	Broiler mash finisher feed	100 ppb	–	–
T1	Broiler mash finisher feed	100 ppb	HBZ	1 kg/ton
T2	Broiler mash finisher feed	100 ppb	CM	1 kg/ton

HBZ, H-β zeolite; CM, clay mineral-based product.

prepared feed, samples were subjected to extraction as described before and the extracted samples were quantified for OTA content by HPLC. Such artificially contaminated feeds were fed to the birds for evaluating the in vivo efficacy of HBZ and CM.

Trial Design. The study consisted of 4 groups with 6 replicates per group. Each replicate had 2 birds (1 male and 1 female), each provided with 100 g of feed. The groups were control, negative control (**NC**), T1, and T2. Feed containing no toxin binder was given to control and NC groups. Treatment groups, T1 and T2, were treated with HBZ and CM at 1 kg/ton feed, respectively. Except control, all the groups received 100 ng/g of OTA in the feed. Four-week-old broiler chickens were adapted for 5 D with ad libitum broiler mash finisher feed listed in Table 2 and water. The birds were then starved for 24 h to empty gut contents (Sibbald, 1976). After the starvation period, each bird was given 100 g of feed of the respective treatment group as described in Table 1. Water was given ad libitum throughout the trial. The excreta samples of each replicate was collected for 72 h, and dried at 50°C for 48 h.

Broiler Growth Performance Trial A growth performance trial was conducted in broiler chickens (Vencobb-400) using a complete randomized design for a period of 35 D. The trial was conducted at the Kemin Research and Development Poultry Farm, Chennai, India, using deep litter system described in Broiler management guide (2012). Broiler chickens were purchased from Komarla Hatcheries, Coimbatore, India. A total of 288 one-day-old chicks were divided into 3 groups with 96 birds in each group. Each treatment group was divided into 8 replicates, and each replicate had 12 birds (6 male and 6 female birds). The 3 groups were control, treatment 1 (T1), and treatment 2 (T2) treated with titanium dioxide, HBZ, and CM at 1 kg/ton of feed, respectively. The trial was conducted to test the effect of HBZ and CM on performance of the broiler chickens. The feed was given in the mash form in 3 phases: pre-starter (0 to 14 D), starter (15 to 28 D), and finisher (29 to 35 D). Feed composition and nutritional value are shown in Table 2 and Table 3, respectively. The feed was analyzed for toxin content as described before, and the toxin levels were found to be between 1 and 2 ng/g of feed. All the experimental birds were provided with respective toxin free feed and water ad libitum throughout the experimental period. The prepared feed was analyzed for microbial counts (Enterobacteriaceae, *Salmonella*, *Clostridium perfringens*, and mould) as described by AOAC International and Latimer (2012). The feed had a satisfactory level of less than 100 cfu/mL. The temperature of the shed ranged between 24 and 35°C, and relative humidity ranged between 65 and 90% throughout the trial period. As per the standard practice, all the birds were vaccinated against the diseases (Broiler management guide, 2012). The growth performance parameters such as BW, feed intake (**FI**), and feed conversion ratio (**FCR**) were measured for all the groups (Yegani and Korver, 2013).

Table 2. Feed composition used for the broiler performance trial.

Ingredients	Pre-starter	Starter	Finisher
	(kg/ton of feed)		
Maize	535.04	580.53	612.53
Soya meal 45%	346.28	285.46	244.68
Meat and bone meal	26.99	40.04	40.04
Rice bran oil	32.73	40.34	51.55
Rapeseed meal	30.03	30.03	28.64
Calcite	12.97	12.06	11.82
Dicalcium phosphate	4.63	0.93	0.13
DL-methionine	2.92	2.73	2.62
Sodium chloride	2.50	2.50	2.50
L-lysine	2.77	2.25	2.37
Sodium bicarbonate	1.00	1.00	1.00
Choline chloride	0.50	0.50	0.50
BROVIT PLUS® PREMIX[1]	0.50	0.50	0.50
Kemtrace® broiler[2]	0.50	0.50	0.50

[1]Each 500 g of the vitamin (VIT) premix has VIT A-12.5 MIU; VIT D3–2.8 MIU; VIT E-30 MIU; VIT K-2 g; VIT B1–2 g; VIT B2–5 g; VIT B6–3 g; VIT B12–0.015 g; Niacin-40 g; calcium pantothenate-15 g; Folic acid-1 g; biotin-0.08 g.

[2]Each 500 g of the product has at least zinc, 4.0%; copper, 0.8%; cobalt, 0.04%; manganese, 6.0%; chromium, 0.04%; iron, 6.0%; iodine, 0.40%; selenium: 0.06%.

Table 3. Nutritional value of the diets.[1]

Ingredients	Pre-starter	Starter	Finisher
Metabolizable energy (kcal/kg)	3,000	3,100	3,200
Crude protein (%)	22.500	20.800	19.200
Crude fiber (%)	3.986	3.753	3.585
Fat (%)	5.801	6.749	7.926
Linoleic acid (%)	2.287	2.612	3.037
Calcium (%)	0.940	0.920	0.880
Available phosphorus (%)	0.460	0.440	0.420
Lysine (%)	1.420	1.250	1.150
Methionine (%)	0.620	0.580	0.550

[1]Calculated value as-fed basis.

Statistical Analysis

Statistical analyses were performed using STATGRAPHICS plus 5.1 software. Data were analyzed using 1-way analysis of variance (1-way ANOVA).

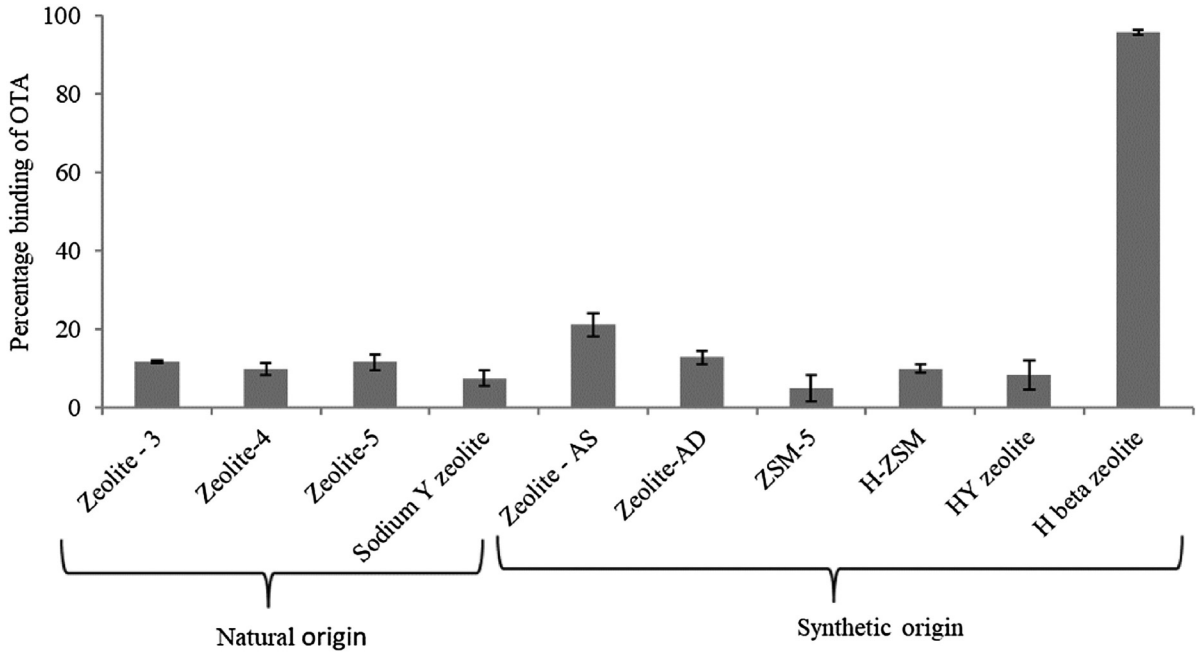

Figure 1. Binding efficacy of porous materials of natural and synthetic origin towards ochratoxin A (OTA). The results are reported as mean ± SE, n = 3. ZSM, Zeolite Socony Mobil.

A P value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Efficacy of Porous Materials With OTA

Since porous materials offer flexibility to fine-tune based on our requirements, materials of natural and synthetic origin were tested for binding with OTA. None of the natural zeolite and synthetic zeolite exhibited more than 25% binding (Figure 1). Among the tested materials, HBZ showed a higher binding to OTA. Avantaggiato et al. (2007) also studied the OTA binding efficacy of alumino silicate-based products and found to be around 20% in the GI-simulated conditions. Similarly, differences in OTA binding for various toxin binders including porous materials were also reported by Boudergue et al. (2009). Such difference in binding observed in this study could be due to the differences in chemical composition, pore size, surface area, SAR, and surface acidity of each material (Boudergue et al., 2009). Since HBZ showed higher binding among the tested materials, further experiments were carried out with HBZ.

Physical Characteristics of HBZ

Powder X-ray diffraction analysis was carried out for HBZ, and the reflections are shown in Figure 2a. The reflections of HBZ at higher 2θ values after 20° were noticed and indicate the formation of microporosity (Li et al., 2003; Li, 2004; Kantam et al., 2005; Zhang et al., 2013). Surface area analysis by the BET method showed 333 m^2/g. Li et al. (2003) obtained 434 m^2/g surface area for pure silica beta zeolite. In another study, Kantam et al. (2005) reported the surface area of 350 m^2/g for HBZ. It can be concluded that the obtained result (333 m^2/g) was in the acceptable range. Pore size analysis by the BJH method was done using N_2 sorption analyzer (Figure 2b and c). A narrow loop of type II isotherm was observed with HBZ, which confirms the formation of a well-ordered microporous structure with uniform pore size distribution (Barata-Rodrigues et al., 2003). The significant reduction in the amount of nitrogen adsorbed in both monolayer and the multilayer region confirms the formation of small pore size and lesser surface area (Sahooli et al., 2017). The capillary condensation step, which gives the direct measure of the pore diameter of the materials, has shifted towards the low relative pressure for HBZ revealing a reduction in the pore diameter of around 5 A^0 (Figure 2c; Sahooli et al., 2017). Zhang et al. (2013) also reported similar physical characteristics for HBZ.

The morphology of HBZ was studied through HRSEM, and the result is shown in Figure 2d. Spherical shaped particles were seen in the images indicating that the morphology of the material was completely retained. Loiha et al. (2009) also reported similar type of spherical images for HBZ. All these results confirm the characteristics of HBZ.

In Vitro Biphasic Study

The binding efficacy of HBZ and CM towards OTA is shown in Figure 3. It was observed that HBZ showed

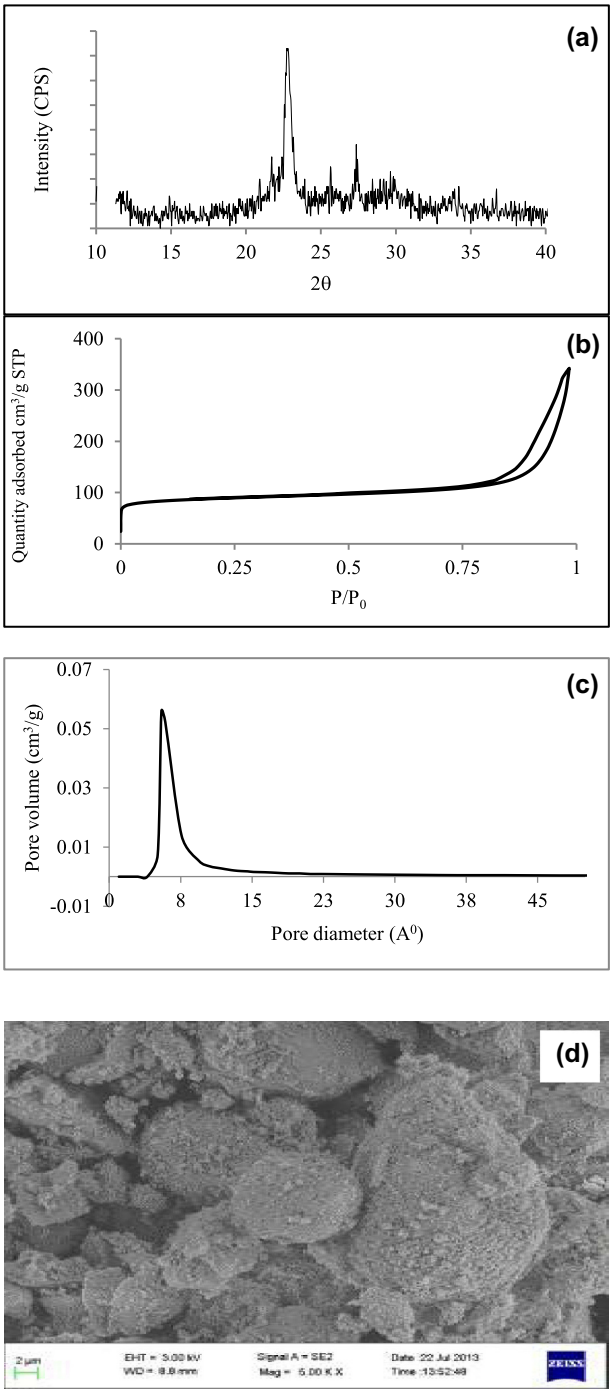

Figure 2. Characterization of H-β zeolite (HBZ). (a) Powder X-ray diffraction analysis of HBZ. (b) Isotherm linear plot of HBZ using nitrogen adsorption and desorption isotherm. (c) Pore size analysis of HBZ by Barrett–Joyner–Halenda method. (d) Scanning electron microscopy analysis of HBZ.

high adsorption (>95%) at pH 3.2 and minimal desorption (4.62%) at pH 6.8. In case of CM, high adsorption (>95%) with moderate desorption (48%) was seen in pH 6.8 (Figure 3). The adsorption efficiency of the materials depends on the pH of the solution in which the target molecule (OTA) was solubilized (Dwyer et al., 1997; EFSA, 2010). Since the end use of the material is primarily for chicken (*Gallus domesticus*), the intestinal pH condition of chicken was considered for the binding studies. Since the CM is widely used in the poultry industry as a mycotoxin binder product, it was used for comparing the efficacy with HBZ. Boudergue et al. (2009) have reported that clay materials show high adsorption for OTA at acidic pH and low adsorption at both neutral and alkaline pH, because of the acidic nature of OTA. Our results are in agreement with these findings. Boudergue et al. (2009) findings were also similar to the results observed for clay materials. Such high desorption must be attributed to weaker interactions which are susceptible to pH.

Poultry GI pH Simulation Studies With OTA

The in vitro biphasic experiments studied in the previous experiment had pH 3.2 and 6.8. For better understanding, pH simulation studies representing different parts of chicken intestine were carried out. The pH values of the crop, gizzard, early part of the intestine, and distal part of the intestine were found to be 5.5, 3.2, 5.0, and 7.0, respectively (Tomasevic-Canovic et al., 2003). Similar pH conditions were simulated in this experiment, and the adsorption efficiency was evaluated for HBZ and CM (Figure 4). It was observed that OTA-HBZ interactions were stable, after subjecting to pH change. In CM, desorption was observed at every change in pH, except at pH 3.2. Such findings agree with the published report (Boudergue et al., 2009). Interestingly, the desorption of OTA from CM started from pH 5 onwards (Figure 4).

Generally, pH affects the surface charge of the adsorbents and the degree of ionization of toxin (Boudergue et al., 2009; Avantaggiato et al., 2014). The extent of ionization of toxin depends on pH and its pKa value. If pH<pKa, the toxins exists in protonated-uncharged form, whereas at pH>pKa toxin exists in deprotonated-charged form. OTA is an acidic mycotoxin, containing phenyl alanine and iso-coumarin parts (EFSA., 2010). The pK_a values of OTA are in the range of 4.2 to 4.4 for the carboxyl group of phenylalanine part and 7.0–7.3 for the phenolic hydroxyl group of the iso-coumarin part (Avantaggiato et al., 2014). Because of this, OTA exists as a protonated uncharged form at pH<4 (acidic pH). At pH 6.8, OTA exists as a deprotonated charged (monoanion, OTA^- and dianion, OTA^{2-}) form (Ringot et al., 2005; Avantaggiato et al., 2014). These results suggest that the interaction associated with the CM and OTA could be ionic interactions, as ionic interactions were reported to be weak and susceptible for pH changes (Bhagavan and Ha, 2015). In HBZ, no significant desorption occurred on changing the pH and indicates the association of non-ionic interactions with HBZ (Figure 4). This experiment indicates that HBZ adsorbed OTA irrespective of its form, and the HBZ-OTA interactions were almost stable in the entire pH range of the chicken.

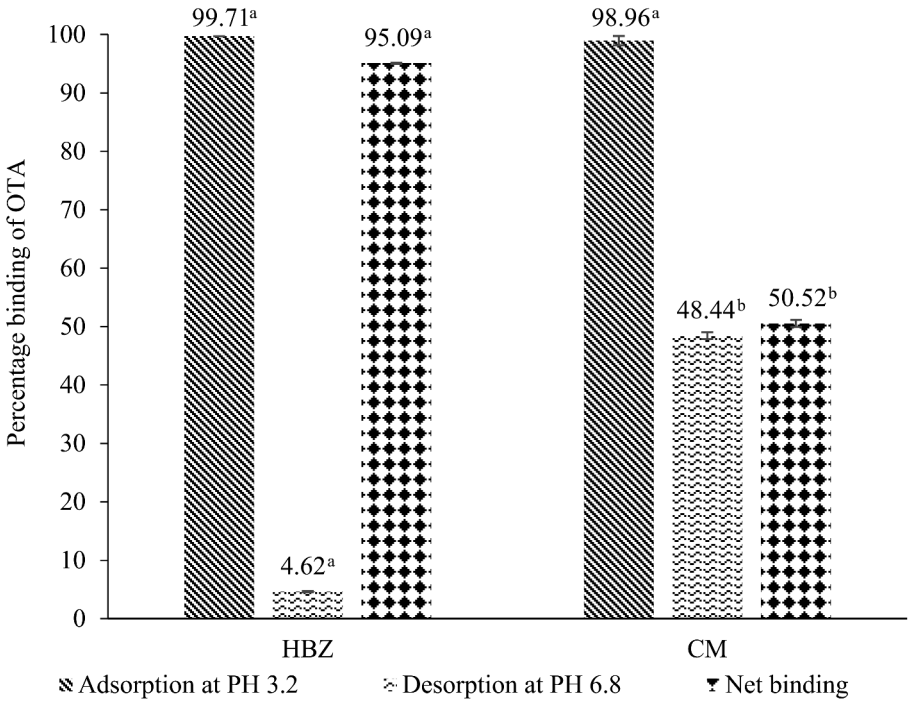

Figure 3. Binding efficacy of H-β zeolite (HBZ) and clay mineral-based product (CM) towards ochratoxin A. The results are reported as mean ± SE, n = 3. [a,b]Groups that are significantly different from each other at $P < 0.05$ are indicated by different superscripts.

Adsorption Kinetics and Bonding Strength

Mycotoxins are known to be absorbed rapidly from the GI tract by passive absorption, a concentration-dependent phenomenon (Ringot et al., 2005; Avantaggiato et al., 2014). Hence, it is essential for adsorbents to bind faster with mycotoxins at high bonding strength, so that rapid absorption of OTA into systemic circulation is prevented. Such rate-dependent binding efficacy was assessed through contact time studies and the bonding strength through CI experiment. The contact time analysis was assessed for HBZ, CM at pH 6.8, and the results are shown in Figure 5. Rapid adsorption and establishment of equilibrium in a short time was observed for both the materials. However, HBZ exhibited higher binding (>95%) in shorter time compared to CM. No difference between HBZ and CM was observed at pH 3.2 (data not shown). Avantaggiato et al. (2014) studied the contact time analysis for OTA with grape pomace and reported that the grape pomace adsorbents attained equilibrium on approaching 15 min. However, adsorption was fast in the initial stages and half of the total adsorption happened in <3 min. Similarly, in this experiment, most of OTA was adsorbed in <3 min for HBZ and CM. As observed by Avantaggiato et al. (2014), no change in adsorption was noticed for HBZ and CM, after attainment of equilibrium.

The results of the bonding strength of OTA with HBZ and CM studied through CI experiments are shown in Table 4. HBZ showed 0.80, which was significantly higher than the CM having 0.25 ($P < 0.05$). In the study carried out by Dwyer et al. (1997), clay which showed higher CI exhibited high binding to mycotoxin. Similar results were observed in the current study for HBZ and CM.

Thermodynamic Studies

Thermodynamic studies were carried out to elucidate the nature of adsorption process and the interactions associated between adsorbent (HBZ, CM) and adsorbate (OTA). The results of the thermodynamic studies, calculated at different temperatures (278, 288, 298, 308, and 318 K), are given in Table 5.

The results showed a decrease in equilibrium constant (K_0) for both HBZ and CM, as temperature increases. This could be attributed to the escape of OTA from HBZ and CM, with an increase in temperature of the solution (Avantaggiato et al., 2014). The Gibb's free energy, $\Delta G°$ (KJ/mol), for both HBZ and CM was found to be negative, indicating the adsorption process was spontaneous (Ringot et al., 2005; Avantaggiato et al., 2014). The spontaneous adsorption of OTA to HBZ and CM was also noticed in the contact time experiments (Figure 5). The results of contact time and $\Delta G°$ confirm that the adsorption was spontaneous and rapid. Avantaggiato et al. (2014) also found that the adsorbents which had negative $\Delta G°$ adsorbed faster. The current findings are in line with the published reports.

The slope and intercept of the plots of ln K_0 vs. 1/T were used to determine the thermodynamic parameters ($\Delta H°$ and $\Delta S°$) according to the Van't Hoff equation described by Avantaggiato et al. (2014), and the results

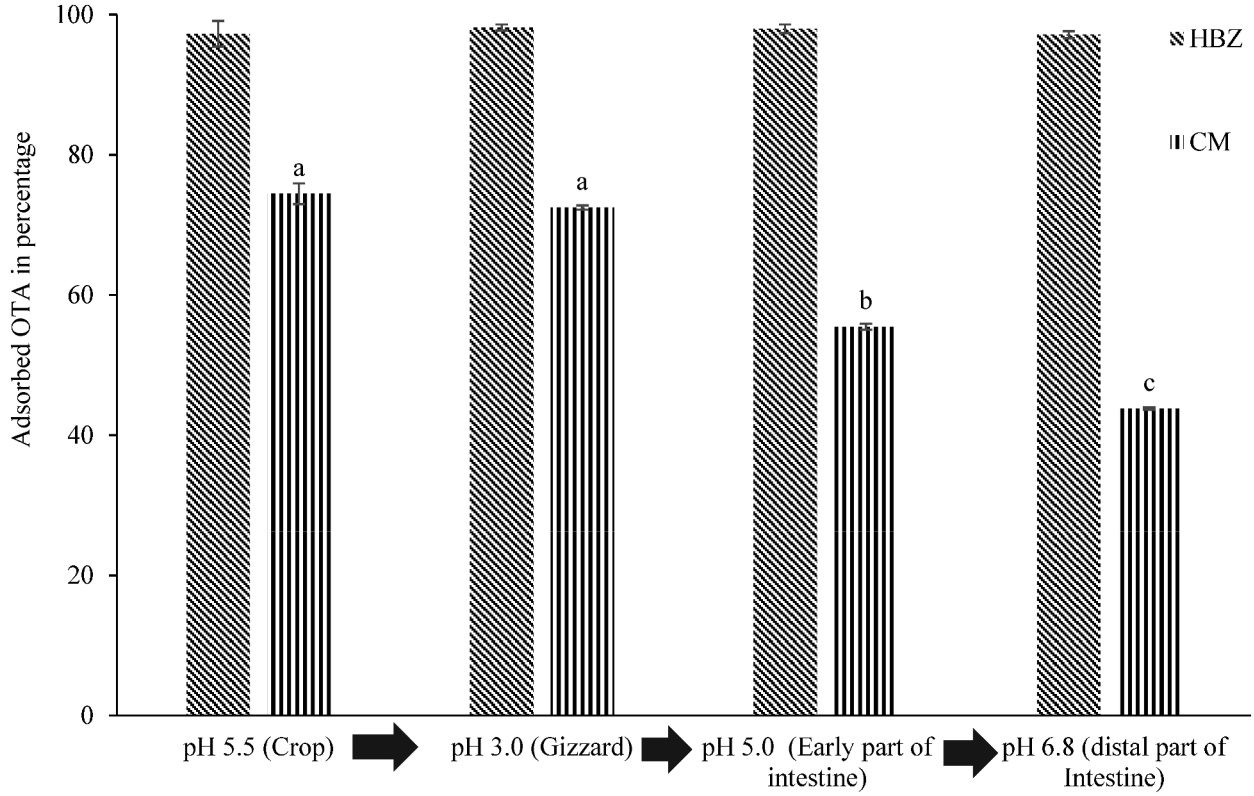

Figure 4. Stability of ochratoxin-A (OTA) adsorption to H-β zeolite (HBZ) and natural clay mineral-based product (CM). The results are reported as mean ± SE, n = 3. [a,b,c]Groups that are significantly different from each other at $P < 0.05$ are indicated by different superscripts.

are shown in Table 5. In the current study, the enthalpy values were found to be less than 20 KJ/mol for HBZ and CM. This suggests that the adsorption process occurs by physisorption phenomenon, allowing attaining of the equilibrium rapidly (Ringot et al., 2005; Avantaggiato et al., 2014).

The nature of interactions between adsorbate and adsorbent could be inferred from ΔS° values. In general, the negative values of ΔS° indicate the non-covalent interactions, whereas the positive values indicate the hydrophobic interactions (Avantaggiato et al., 2014). The negative value of ΔS° observed for CM was entropically driven, and this situation corresponds to the polar non-covalent interaction (Avantaggiato et al., 2014). On contrary, the positive ΔS° value was observed for HBZ. This suggests the hydrophobic interaction between adsorbent (HBZ) and adsorbate (OTA) must have occurred for HBZ (Avantaggiato et al., 2014).

Ringot et al. (2005) also reported negative enthalpy and positive entropy value for OTA and dry yeast cell wall, whereas Avantaggiato et al., (2014) observed for grape pomace and aflatoxin B1, ZEA. Authors also confirmed the presence of hydrophobic interactions in this scenario (Ringot et al., 2005; Avantaggiato et al., 2014). Thus, the binding of OTA on HBZ involves primarily hydrophobic, whereas on CM, predominantly polar non-covalent interaction exists.

Binding Efficacy Towards Vitamins

To evaluate the usefulness of HBZ as a mycotoxin binder product for animal feed application, its binding ability with vitamins was tested in comparison to CM. Randomly, a fat soluble (vitamin-E) and a water soluble vitamin (C, B9) were tested, and the results are shown in Table 6. The results showed that both HBZ and CM have low binding efficacy (<9%) to the tested vitamins.

In a study reported by Vekiru et al. (2007), clay showed 2 to 3% binding for vitamin-H, 7 to 25% for vitamin-B12, 0% for vitamin-B5, and >90% for AFB1. In the same study, higher binding (>90%) for vitamin and AFB1 was observed for activated carbon. This study also showed that clay had relative selectivity for AFB1, whereas activated carbon does not. This confirms the very low selectivity of activated carbon and the relative selectivity of clays for AFB1. Boudergue et al., (2009) also reported similar findings for activated carbon and clays. On comparing the results of HBZ with the published reports, it can be inferred that HBZ offers selectivity towards OTA.

Such high binding to OTA and low binding to vitamins suggest that HBZ could be used preferably during viral and bacterial infections.

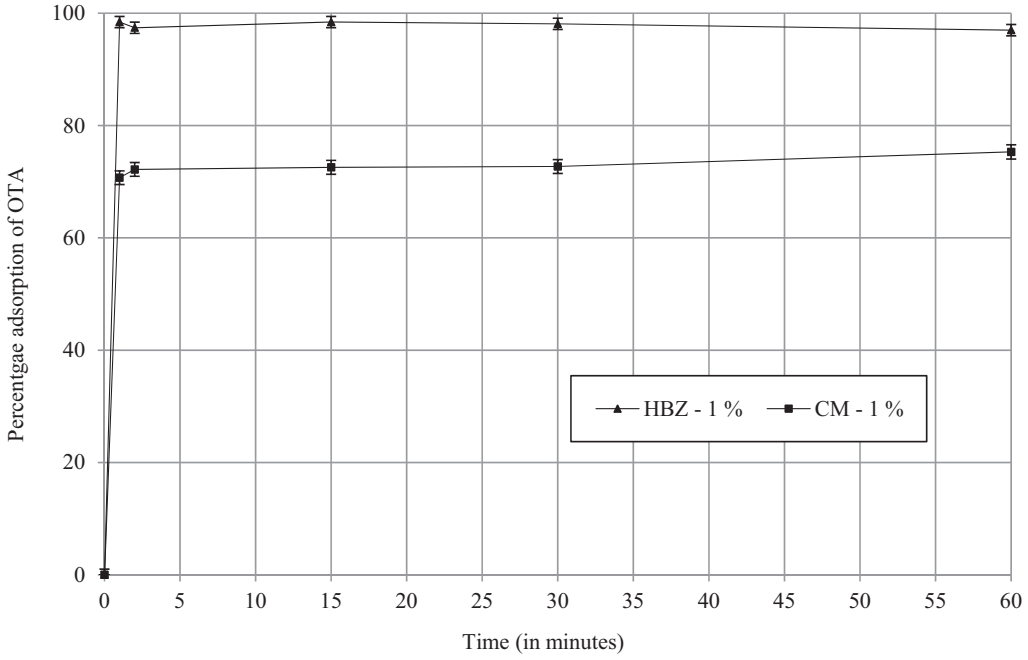

Figure 5. Adsorption kinetics of ochratoxin A (OTA) with H-β zeolite (HBZ) and clay mineral-based product (CM) through contact time studies at pH 6.8. The results are reported as mean ± SE, n = 3.

Table 4. Chemisorption index (CI) values for materials.[1]

Material	CI[1]
H-β zeolite	0.80^{a} ± 0.01
Clay mineral-based product	0.25^{b} ± 0.02

[1]The results are reported as mean ± SE.
[a,b]Groups that are significantly different from each other within a row at $P < 0.05$ are indicated by different superscripts (n = 3).

In Vivo Binding Evaluation

The in vitro studies showed that HBZ can bind effectively with OTA. This was further confirmed by in vivo studies in broiler chickens. One of the approaches for estimating the in vivo binding potential of the adsorbents is by analyzing the toxin content in the excreta of the birds supplemented with toxin binder and comparing it with the control group receiving no adsorbents (Table 1).

The solvent-assisted extraction was used for extracting OTA from feed and excreta samples. Before extracting the samples from the trial, OTA extraction efficiency of this method was tested using feed and excreta. The results showed that the solvent-assisted extraction method could recover 95% and 93% of the added toxin from the feed and excreta, respectively (Table 7).

The excreta collected from the trial was subjected to OTA extraction, quantified by HPLC, and the amount of toxin eliminated through excreta of the respective groups is shown in Figure 6. Except control, all the groups were fed with 100 ng/g of OTA mixed in the feed, which contributes 20 µg/replicate. The OTA level was found to be below detectable limit in the control group. It was observed that all the treatment groups excreted significantly higher OTA compared to NC (Figure 6, $P < 0.05$). However, T1 treated with HBZ excreted 11 µg of OTA compared to CM, which excreted 7.16 µg of OTA ($P < 0.05$). The difference of OTA intake and excretion could be considered as either absorbed in the GI or transformed into other metabolites (Boudergue et al., 2009). The higher amount of OTA excreted in HBZ-treated group could be correlated to very less desorption at pH 6.8 (Figures 3 and 4). Schoeters et al. (2014) also tested the binding efficacy of humic acid towards ZEA in the animals. Humic acid, which showed high binding to ZEA in vitro, resulted in higher excretion of ZEA in the animals. Such findings were similar to the results observed in this study. In the in vitro study, Schoeters et al. (2014) also observed that humic acid had less desorption at intestinal pH conditions. This result substantiates that the OTA-HBZ interactions were much more stable across the GIT, which resulted in higher excretion of OTA.

Broiler Growth Performance Trial

Further to the in vivo binding study, a growth performance trial was carried out in broiler chickens. The pre-starter, starter, and finisher feeds used for the performance trial were tested for OTA content by HPLC, and OTA was found to be less than 2 ng/g of feed. (Table 8). This ensures that any effect arising would be because of inclusion of mycotoxin binder in the feed. Titanium dioxide (TiO_2), inert filler, was used in the feed to normalize the composition of feed (Short et al., 1996). The performance parameters such as, BW gain,

Table 5. Thermodynamic parameters of H-β zeolite (HBZ) and clay mineral-based product (CM) assessed for ochratoxin A.[1]

Adsorbents	Temperature (K)	Equilibrium constant (K_0)	Gibbs's free energy ($\Delta G°$, KJ/mol)	Enthalpy ($\Delta H°$, KJ/mol)	Entropy ($\Delta S°$, KJ/mol K)
HBZ	278	7592.3	−20.65	−14.22	22.76
	288	5424.86	−20.58		
	298	4754.36	−20.97		
	308	4305.28	−21.42		
	318	3244.48	−21.37		
CM	278	506.06	−14.39	−15.37	−3.67
	288	378.46	−14.21		
	298	323.9	−14.32		
	308	259.84	−14.23		
	318	250.75	−14.6		

[1]The results are reported as mean (n = 3).

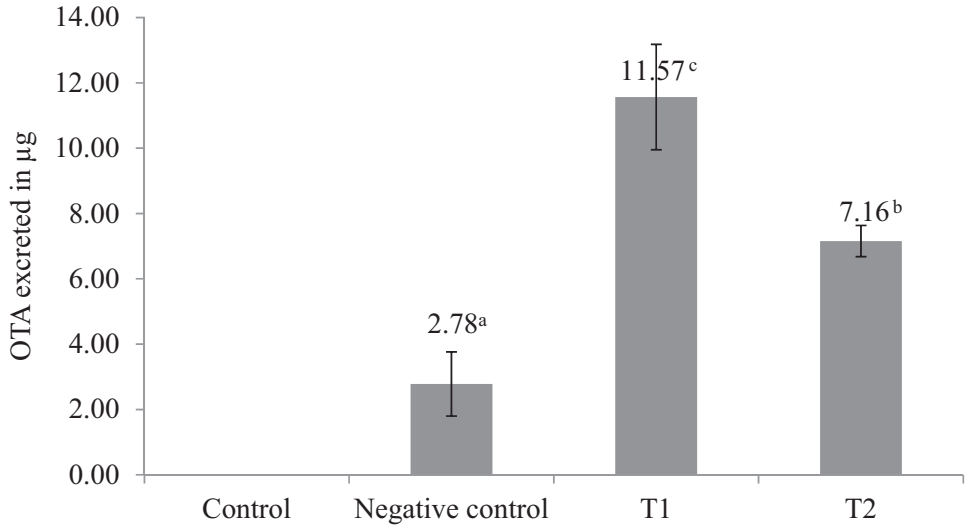

Figure 6. In vivo ochratoxin A (OTA) binding assessment of toxin adsorbents. The results are reported as mean ± SE, n = 6. Control and negative control refer to group treated with no toxin binder; T1 refers to group treated with H-β zeolite at 1 kg/ton; T2 refers to group treated with clay mineral-based product; Except control, all the groups were fed with OTA contaminated feed. [a,b]Groups that are significantly different from each other at $P < 0.05$ are indicated by different superscripts.

Table 6. In vitro binding of H-β zeolite (HBZ) and clay mineral-based product (CM) with vitamins[1]

	Net binding (%)	
Vitamins	HBZ	CM
Vitamin-E	5.86 ± 0.36	6.87 ± 1.42
Vitamin-C	3.53[a] ± 0.35	7.32[b] ± 0.62
Vitamin-B9	6.12[a] ± 0.91	8.64[b] ± 0.67

[1]The results are reported as mean ± SE.
[a,b]Groups that are significantly different from each other within a row at $P < 0.05$ are indicated by different superscripts (n = 3).

FI, and FCR were monitored in the trial, and the results are shown in Table 9. No mortality was observed during the trial.

At the end of 35 D, no significant difference in BW gain and FCR was observed for control, T1, and T2 ($P > 0.05$). Dwyer et al. (1997) observed that addition of 1% of clay in the diet did not affect BW gain and FCR. Also, no toxic effects were reported in the broiler chickens fed with 1% clay in the diet. Shi et al. (2006) found that addition of 0.3% modified montmorillonite in the diet did not affect BW gain and FCR in broiler chicken. In another study reported by Miazzo et al. (2000), no significant difference in BW gain and FCR was observed for control and a group treated with 1% zeolite in the diet. All these results are similar to the performance results of CM observed in this study (Table 9). Based on these reports, we infer that HBZ can be administered safely without causing a negative impact on broiler birds.

In summary, we have identified that HBZ is capable of adsorbing OTA very efficiently, and demonstrated its efficacy through in vitro and in vivo studies. HBZ offers additional advantage of having less interaction to vitamins also. Such findings of HBZ with OTA demonstrate the potential usage in poultry feed for OTA detoxification.

Table 7. Extraction efficiency of ochratoxin A (OTA) from feed and excreta samples.

Details of the sample	OTA (ng/g) measured by HPLC[1]	Extraction efficiency[2]
Broiler finisher feed	< BDL[3]	–
Broiler finisher feed + 100 ng/g OTA	95.31 ± 2.59	95%
Broiler excreta	< BDL	–
Broiler excreta + 100 ng/g OTA	93.29 ± 4.22	93%

[1]The results are reported as mean ± SE, n = 6.
[2]Rounded off to the nearest whole number.
[3]BDL refers below detection limit.

Table 8. Ochratoxin A (OTA) content in the feed used for the performance trial.[1]

Details of the sample	OTA (ng/g) measured by HPLC
Pre-starter feed	1.85 ± 0.29
Starter feed	1.09 ± 0.32
Finisher feed	1.98 ± 0.51

[1]The results are reported as mean ± SE, n = 6.

Table 9. Performance parameters of broiler chickens at the end of 35 D trial.[1]

Groups	BW gain (g)	Feed conversion ratio
Control	1696.79 ± 23.11	1.44 ± 0.017
T1	1707.00 ± 25.92	1.43 ± 0.020
T2	1697.81 ± 27.14	1.44 ± 0.022

[1]The results are reported as mean ± SE.
Control refers to group treated treated with no toxin binder; T1 refers to group treated with H-β zeolite at 1 kg/ton; T2 refers to group treated with clay mineral-based product at 1 kg/ton.
[a,b]Groups that are significantly different from each other within a column at $P < 0.05$ are indicated by different superscripts (n = 96).

ACKNOWLEDGMENT

The authors are grateful to Ravichandran and his team members (R&D Poultry Farm, Kemin Industries South Asia Pvt. Ltd.) for their help in conducting the in vivo studies.

CONFLICTS OF INTEREST

No conflict of interest.

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