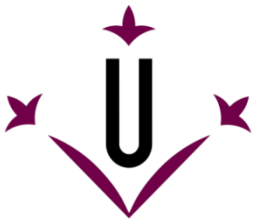

Essential title page information

Title: New mycotoxin adsorbents based on tri-octahedral bentonites for animal feed

Author names and affiliations:

Pilar Vila-Donat. Email: pilar.viladonat@tecal.udl.cat

Sonia Marín. Email: smarin@tecal.udl.cat

Vicente Sanchis. Email: vsanchis@tecal.udl.cat

Antonio J. Ramos. Email: ajramos@tecal.udl.cat

Affiliations of all authors: Applied Mycology Unit, Food Technology Department, UTPV-XaRTA, Agrotecnio Center, University of Lleida, Rovira Roure 191, 25198, Lleida, Spain.

Corresponding author: Antonio J. Ramos. Email: ajramos@tecal.udl.cat

Abstract

In the present work different clays have been characterized according to their mycotoxins adsorbent ability. Firstly, 27 bentonite clays from different geographical origins were evaluated, at 0.02% w/v, using an *in vitro* screening method versus five mycotoxins (aflatoxin B_1, AFB1; deoxynivalenol, DON; ochratoxin A, OTA; fumonisin B_1, FB1; and, zearalenone, ZEN) by Enzyme-Linked Immunosorbent Assay (ELISA). Subsequently, 7 bentonite clays (6 of which were tri-octahedral bentonites) selected from the preliminary test, and 7 commercial adsorbent products were subjected to an *in vitro* equilibrium adsorption experiment (at 0.02% w/v) against six concentrations of AFB1 (0.02-4 mg/L), and OTA (0.05-1 mg/L) by using simulated gastrointestinal (GI) juices, and successively analysed by HPLC-FD. Equilibrium isotherm functions were fitted to the data by nonlinear regression analysis.

In vitro adsorption equilibrium experiments showed that AFB1 adsorption was very high with all the adsorbents tested. In particular, the seven pre-selected bentonites adsorbed most of the AFB1 present at the lower level tested, while only three of these reached more than 50% of OTA adsorption. Adsorption increased inversely to the toxin concentration and both Langmuir and Freundlich isotherm models fitted well to the data. Generally, the pre-selected bentonites (B1-B7) showed better mycotoxin adsorption than commercial products (C1-C7) at all levels of mycotoxins tested. The 10-fold dose increase of the best tri-octahedral bentonite (B4) rendered a more effective adsorption of OTA, reaching almost 75% of adsorption (at pH 5).

Keywords: aflatoxin B_1, ochratoxin A, animal feed, bentonite, decontamination, adsorption isotherms

Abbreviations

Aflatoxin B_1 (AFB1), Aflatoxin B_2 (AFB2), Aflatoxin G_1 (AFG1), Aflatoxin G_2 (AFG2), Aflatoxins (AFs), Deoxynivalenol (DON), Enzyme-Linked Immunosorbent Assay (ELISA), European Union (EU), Fluorescence Detector (FD), Fumonisins (FBs), Fumonisin B_1 (FB1), Gastrointestinal (GI), High Performance Liquid Chromatography (HPLC), International Agency for Research on Cancer (IARC), Maximum Levels (MLs),

Ochratoxin A (OTA), Rapid Alert System for Food and Feed (RASFF), Residual Root Mean Square Error (RMSE), Zearalenone (ZEN).

Highlights

- Different bentonites have been *in vitro* tested as mycotoxin detoxifiers.
- *In vitro* adsorption experiments showed that AFB1 was the most adsorbed mycotoxin.
- *Fusarium* mycotoxins showed low levels of adsorption, especially for DON, whose adsorption was negligible.
- Tri-octahedral bentonites showed higher adsorption than di-octahedral bentonites.
- One of the tri-octahedral bentonites assayed proved to be an *in vitro* efficient AFB1 and OTA binder.

1. Introduction

Animal feed plays an important role in the food chain and has implications regarding the composition and quality of the livestock products (milk, meat, and eggs) for human consumption. Cereals, especially maize and wheat, constitute most of the daily diet of animals and are important ingredients in compound feeds (Pinotti et al., 2016). Fungal contamination of grains is a worldwide problem, especially because cereals used for livestock feed are often imported and exported across the world (Zulkifli and Zakaria, 2017). Contamination of cereals by fungi can lead to mycotoxin production which could occur both, at field or because of unsuitable moisture content or temperature, during storage. In terms of agricultural and animal production, the most important relevant mycotoxins found in feed are AFs, especially AFB_1, mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, and OTA produced by *Aspergillus ochraceus* and *Penicillium verrucosum*, whereas ZEN, DON and FB1 are produced by numerous *Fusarium* species (Faucet-Marquis et al., 2014; Marin et al., 2013).

Climate change may affect growth of these toxigenic fungi and hence mycotoxin production, as variations in environmental conditions, including temperature, relative humidity, and CO_2 levels, affect their development. Interactions between these three factors have shown to stimulate *Aspergillus* mycotoxins production (Medina et al., 2014). Lately in Europe, AFs contamination, previously uncommon, has become increasingly significant as a consequence of rising average temperatures due to climate change, and is expected to increase further (Medina et al., 2015; Miraglia et al., 2009). According to the annual report of the RASFF, mycotoxins were the main hazard in border rejection notifications in the EU in 2017. AFs were the primary mycotoxins associated to the notifications, and nuts, nut products and seeds were the most affected categories. Apart from AFs, OTA, FBs and DON were often reported in cereal-based products (Juan et al., 2013; RASFF, 2017). Recently, Hassan et al. (2018) detected AFs and OTA in 94 and 44% of mixed cereals, 70 and 40% of maize, 40 and 60% of wheat, respectively, in samples from

a Qatar market. Moreover, the co-contamination analysis showed that 44, 40 and 50% of the mixed cereals, maize and barley samples were concurrently contaminated with AFs and OTA. Co-occurrence of AFB1 and OTA had been also previously reported in a range of commodities, including cereals and feed (Ibáñez-Vea et al., 2011; Iqbal et al., 2014; Kara et al., 2015).

Due to the evidence of carcinogenic effects in humans of these two toxins, the IARC placed AFB1 in the most toxic group, i.e. group 1, while OTA was placed in group 2B, as a potential carcinogen (IARC, 1993). AFB1 and OTA, due to their fast absorption at the GI tract, high carcinogenicity for animals and humans, and high risk of carry-over to derived animal products (edible tissues, eggs or milk), have very low limits in the EU Regulations and Recommendations in animal feed, i.e, in the order of µg/kg. AFB1 is the only mycotoxin with EU- MLs in animal feed (5-20 µg/kg), while OTA is regulated only by guidance values (50-250 µg/kg) (Commission Recommendation 2006/576 EC; Directive 2003/100 EC).

Generally, the levels of mycotoxins found in animal feed are low enough to ensure compliance with the regulated/recommended levels. However, farm animals often show symptoms of chronic mycotoxicosis, even when the concentrations of individual mycotoxins in feeds do not exceed legal guidance values (Grenier and Oswald, 2011; Streit et al., 2012; Wielogórska et al., 2016).

In view of this, a new functional group was added in the EU in the category of technological feed additives defined as '*substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action*' (Commission Regulation 386/2009, EC). A variety of adsorbents have been tested for their ability to sequester mycotoxins from the GI tract (Avantaggiato et al., 2003, 2005, 2007; Boudergue et al., 2009; Di Gregorio et al., 2014; Kolosova and Stroka, 2011; Ramos et al., 1996 a, b; Wielogórska et al., 2016). Bentonites, clay materials composed largely of smectite, have demonstrated a great efficacy on mycotoxins adsorption, specifically of AFs (Deng et al., 2010; Jaynes et al., 2007; Kong et al., 2014; Magnoli et al., 2011;

Miazzo et al., 2005; Neeff et al., 2013; Pappas et al., 2014; Phillips et al., 2008; Ramos and Hernández, 1996; Thieu and Peterssson, 2008; Vekiru et al., 2007), and in lesser manner other mycotoxins (ZEN, and FBs) by *in vitro* and *in vivo* studies (Avantaggiato et al., 2005; Feng et al., 2008; Ramos et al., 1996 b; Wang et al., 2012).

It should be noted that, many studies done regarding mycotoxin adsorption have only investigated the efficacy in the adsorption of a single mycotoxin, tested at levels exceeding EU limits or guidelines. As mentioned previously, in real situations this is rarely the case (Pappas et al 2014; Streit et al., 2012). In addition, most of the assays were performed *in vitro* by using water/buffer solutions, and by testing adsorbents at doses far above those actually used as feed additives (Vila-Donat et al., 2018). Among the investigated adsorbents, only a di-octahedral bentonite (1m558) has been authorized as technological additive for reduction of the contamination of feed with AFB1 for ruminants, poultry and pigs (EC, 2013).

The present study was designed to, firstly, screen 27 bentonite clays from different geographical origins for their *in vitro* ability to bind different mycotoxins (AFB1, ZEN, DON, OTA and FB1) when tested at 0.02% w/v. Secondly, to calculate the *in vitro* equilibrium adsorption percentage and adsorption isotherms (by using simulated GI juices) of 7 bentonites (selected from the preliminary test, for their adsorbent properties) versus AFB1 and OTA (including levels below EU limits or guidelines), and compare them to 7 commercial products. And finally, to test the effect of increasing the dosage of the 2 best resulting bentonites up to 0.2% w/v.

2. Materials and methods

2.1 Reagent and materials

All chemicals used were of analytical grade. Methanol, acetonitrile and acetic acid (HPLC grade) were purchased from Scharlab S.L (Barcelona, Spain). Ultrapure water was produced by a

Milli-Q® system at 22 µm (Millipore, Bedford, MA, USA). For the initial *in vitro* screening test, the test medium was acetate buffer (pH 5). It was prepared by combining 0.1 M acetic acid (148 mL) (Panreac, Barcelona, Spain) and 0.1 M sodium acetate (352 mL) (Sigma Aldrich, Misuri, EEUU) in 500 mL of water. For the equilibrium adsorption experiments and adsorption isotherms, simulated gastric and intestinal juices were prepared according to The United States Pharmacopeia/The National Formulary (USP23/NF18, 1995). Simulated gastric juice was prepared by adding 2 g of sodium chloride (Fischer Scientific, UK), 3.2 g of pepsin from porcine gastric mucosa, ≥400 units/mg protein (Sigma Aldrich, Misuri, EEUU) and 7 mL of hydrochloric acid (37 %) (Panreac, Barcelona, Spain) to 1L of water (pH 1.3). Intestinal juice was prepared by adding 6.8 g of potassium dihydrogen phosphate (Panreac, Barcelona, Spain), 77 mL of sodium hydroxide 0.2 M, 1.25 g of pancreatin from porcine pancreas, 8 x USP specifications (Sigma Aldrich, Misuri, EEUU) and 6.75 g of lactose (Probus, S.A, Badalona, Spain) to 1 L of water (pH 6.8).

2.2 Analytical standards

Five mycotoxin standards were used: DON, FB1, ZEN, AFB1, and OTA, supplied by Biopure Romer Labs Diagnostic Gmbh (Tulln, Austria). Declared purity of all standards was in the range of 97.6 to 99.5%. Stock solutions (1000-1250 mg/L) were prepared by dissolving 5 mg of dried mycotoxin in 4-5 mL of methanol (HPLC grade). These solutions were properly diluted with buffers/simulated juices to prepare the mycotoxin working solutions for adsorption experiments. Standard solutions were prepared in the mobile phase for HPLC calibration. Stock solutions were stored in amber vials at −20 °C and brought to room temperature before use.

2.3 Samples

27 bentonite clays kindly provided by a mining company, selected by their mineralogical composition (>70% smectite), and that differed by their di- and tri-octahedral structure, were tested. From the total, 16 samples were tri-octahedral bentonites, whereas the remaining 11 were

di-octahedral bentonites (Table 2). The tri-octahedral ones are based on a structure where the octahedral layers are similar to brucite; in the tri-octahedral sheet silicates each O or OH ion is surrounded by 3 divalent cations, like Mg^{+2} or Fe^{+2}. Otherwise, the di-octahedral bentonites are based on a structure where the octahedral layers are similar to gibbsite, since the di-octahedral sheet silicates is surrounded by 2 trivalent cations, usually Al^{+3}. All bentonites (n=27) were tested at 0.02% (w/v) using an *in vitro* screening study (see section 2.4).

From the determination of adsorption percentages obtained from the screening study, 7 bentonite clays (A2, A8, A9, A12, A15, A18, and A22) were selected to be studied in depth by means of equilibrium adsorption experiments. Selected bentonites (mostly tri-octahedral bentonites) were recoded as B1, B2, B3, B3, B4, B5, B6 and B7, respectively, and were studied, along with 7 adsorbents commercially available: C1, C2, C3, C4, C5, C6, and C7 (Table 1) at 0.02 % (w/v) (see section 2.5). Finally, two tri-octahedral bentonites (B2 and B4) were tested at higher doses, up to 0.2 % (w/v).

2.4 In vitro adsorption screening tests

27 bentonite clays were *in vitro* tested at 0.02% (w/v) versus 5 mycotoxins (at a single concentration) tested separately (AFB1: 4 mg/L: DON: 10 mg/L, ZEN: 5 mg/L, OTA: 1 mg/L, FB1: 10 mg/L). Due to the low amounts of adsorbent to be used, a suspension of clay was prepared by weighing 100 mg of each adsorbent into a flask with 10 mL of H_2O and mixed on a magnetic stirrer. While stirring, 80 µL of the suspension were pipetted (to make the clay concentration 0.02 g/100 mL) into 10 mL screw cap Falcon polypropylene tubes to which 4 mL of acetate buffer (pH 5) were added next along with a single mycotoxin at the specified concentration. All tubes were placed into an Orbital Shaker-Incubator (2 h, 37 ºC, 4 g). After shaking, the adsorbent materials were separated from the buffer by centrifugation (8800 g, at 4 ºC for 10 min) (Avantaggiato et al., 2004, 2007; Dakovíc et al., 2008). Finally, an aliquot of the supernatants was pipetted and adapted to the proportion of extraction solvent (different for each

kit) for ELISA analysis (Ridascreen®, r-Biopharm AG, Darmstadt, Germany). Experiments were carried out in triplicate, testing each adsorbent by ELISA. Blanks (also in triplicate) were prepared by adding 4 mL of buffer along with the mycotoxin and without the addition of adsorbent. The amount of adsorbed mycotoxin was calculated as the difference between the amount of mycotoxin in the supernatant of the blank tubes and the amount found in the supernatant of the experimental tubes. This amount was related then to the quantity present in the supernatant of the blank tubes and expressed as a percentage.

Adsorption analysis was performed by ELISA *in vitro* tests using a competitive enzyme immunoassay (Ridascreen®, r-Biopharm AG, Darmstadt, Germany) following the specific procedure for the quantitative analysis of each mycotoxin (AFB1, ZEN, DON, OTA and FB1) in cereals. Lectures were performed using a microtiter plate spectrophotometer (Dasitaly, Rome, Italy). This screening study was performed to select 7 adsorbents to be tested later by using GI juices.

Concentration of adsorbents tested (0.02% w/v) and assay medium used (buffer solution pH 5.0) were as described in the authorisation of the 1m558 additive (EC 1060/2013). In the same way, for AFB1 a concentration of 4 mg/L was used. However, the two best adsorbents selected after the adsorption isotherm assay (see 2.5) were also tested at 0.12% and 0.2% w/v (testing 4 mg/L of AFB1, and 0.25 mg/L of OTA, assayed separately).

2.5 Equilibrium adsorption isotherms

14 adsorbents (7 selected from the preliminary test and 7 commercial adsorbents) were subjected to an *in vitro* equilibrium adsorption experiment, by testing a fixed amount of adsorbent (0.02 % w/v) against six increasing concentrations of AFB1 (0.02, 0.1, 0.5, 1, 2 and 4 mg/L) or OTA (0.05, 0.1, 0.25, 0.5, 0.75 and 1 mg/L), using two simulated physiological GI juices (at pH 1.3, and at pH 6.8) and at a constant temperature (37 ºC).

To this purpose, a suspension of clay was prepared by weighing 100 mg of each adsorbent into a flask with 10 mL of H_2O and shaking it on the magnetic stirrer. While stirring, 80 µL of the suspension were pipetted, to make the clay concentration 0.02% (w/v), into 10 mL screw cap Falcon polypropylene tubes to which 4 mL of simulated gastric (at pH 1.3) or intestinal juice (at pH 6.8) were added next along with the appropriate concentrations of AFB1 or OTA (previously mentioned).

All tubes were placed horizontally in an Orbital Shaker-Incubator (2 h, 37 ºC, 4 g). After the incubation period, the adsorbent materials were separated by centrifugation (8800 g, 4 ºC, 10 min). The supernatants were transferred to clean vials and analysed for the residual mycotoxin content by HPLC-FD for AFB1 and OTA, as reported below. Experiments were carried out in triplicate, testing each adsorbent, for each simulated juice and mycotoxin dose, by HPLC analysis. Blanks (also in triplicate) were prepared by adding 4 mL of simulated gastric/intestinal juices with the highest, intermediate or lowest concentration tested of mycotoxins and without the addition of adsorbent. The amount of adsorbed mycotoxin was calculated as the difference between the amount of mycotoxin in the supernatant of the blank tubes (selecting blank with the dose closest to the experimental tubes) and the amount found in the supernatant of the experimental tubes with adsorbent. This amount was related then to the quantity present in the supernatant of the blank tubes and expressed as a percentage.

2.6 Data calculation and curve fitting

The amount of adsorbed mycotoxin per unit of mass of adsorbent was calculated using the following equation (eq. 1):

$$Q_{eq} = \frac{[(C_o - C_{eq})V]}{m} \qquad \text{Equation 1}$$

where:

- Q_{eq}= quantity of mycotoxin adsorbed per milligram of adsorbent (mg/g)

- C_o = concentration of mycotoxin in the supernatants of the blank tubes, with no adsorbent added (mg/L)
- C_{eq}= residual mycotoxin concentration in the supernatant of the experimental tubes with adsorbent at equilibrium (mg/L)
- V= volume of solution (L)
- m = mass of adsorbent (g)

Adsorption equilibrium is established when the quantity of the mycotoxin being adsorbed (Q_{eq}) is equal to the quantity being desorbed. Then the equilibrium concentration in solution (C_{eq}) remains constant. The adsorption isotherm is a curve obtained by plotting the amount of adsorbed mycotoxin (Q_{eq}) per unit of mass of adsorbent (mg toxin/g adsorbent) against the equilibrium non-adsorbed mycotoxin concentration (mg/L) (C_{eq}). Several theoretical adsorption models have been developed to describe the equilibrium relationship between adsorbed and non-adsorbed amounts. Langmuir and Freundlich models are often reported in the literature to provide the best description of mycotoxin adsorption.

The Langmuir model (Langmuir, 1916) is valid for monolayer adsorption to a surface with a finite number of identical sites. The expression of the Langmuir model is given by the following equation (eq. 2):

$$Q_{eq} = \frac{Q_{max} * K_L * C_{eq}}{(1+(K_L * C_{eq}))} \qquad \text{Equation 2}$$

where:

- Q_{eq} = the amount of mycotoxin adsorbed per unit of mass of adsorbent (mg toxin/g adsorbent) (mg/g)
- C_{eq} = residual mycotoxin concentration at equilibrium or non-adsorbed toxin concentration (mg/L)
- Q_{max} = maximum mycotoxin uptake corresponding to sites saturation (mg/g)

- K_L = Langmuir constant related to the affinity of the adsorbent (L/mg)

The empirical Freundlich (Freundlich, 1906) equation based on adsorption onto a heterogeneous surface is given by equation (eq. 3):

$$Q_{eq} = K_F * C_{eq}^{(1/n)} \qquad \text{Equation 3}$$

where:

- Q_{eq}= the amount of mycotoxin adsorbed per unit of mass of adsorbent (mg toxin/g adsorbent) (mg/g)
- C_{eq}= residual mycotoxin concentration at equilibrium or non-adsorbed toxin concentration (mg/L)
- K_F = Freundlich constant related to adsorption capacity of the adsorbent for the mycotoxin (mg/L)
- n = adsorption intensity

In the present work, Langmuir (L) and Freundlich (F) equations were tested. Both adsorption isotherms were obtained by plotting the concentration of AFB1/OTA in solution after equilibrium (C_{eq}) against the amount of AFB1/OTA adsorbed per unit of weight of each adsorbent (Q_{eq}). Data obtained from the equilibrium adsorption experiment were transferred to the statistical program JMP Pro 13.1.0 and fitted to both isotherm models (L and F). The isotherm parameters were estimated by non-linear regression. Non-linear regression analysis of isotherm data is an interesting mathematical approach for describing adsorption isotherms at a constant temperature and to predict the adsorption behaviour under different operating conditions. It was applied to assess the goodness of the fits and to calculate the parameters involved in the adsorption mechanism such as maximum adsorption capacity and adsorption affinity (Tables 5 and 6).

2.7 HPLC analysis

AFB1 and OTA were analysed by HPLC-FD. Analyses were performed on an Agilent Technologies 1260 Infinity HPLC (California, EEUU) equipped with a quaternary pump (G1311B) and a fluorescence detector (G1321B). Data acquisition and instrument control were performed by Open Lab Chemstation ODS software (Agilent Technologies). AFB1 chromatographic separation was achieved on a Poroshell 120, EC-C18 2.7 µm, 4.6 x 50 mm column (Agilent Technologies®), at isocratic conditions water/methanol/acetonitrile (70/20/10) as mobile phase. Column temperature was at 40 ºC, the flow rate was 1.2 mL/min, and the injection volume 20 µL. Excitation and emission wavelengths were λ_{ex} = 365 nm, λ_{em} = 440 nm. To enhance the fluorescent activity of AFB1, a derivatization system (UVE™ Photochemical reactor, LCTech GmbH, Dorfen, Germany) was placed between the analytical column and the fluorescence detector.

For OTA, chromatographic separation was achieved on a Kinetex PFP 100 Å 5 µm, 4.6 x 150 mm column (Phenomenex®, California, EEUU), and the column temperature was at 40 ºC, the flow rate was 1 mL/min using mobile phase of A: acetonitrile, B: methanol, C: acetic acid 0.1% in gradient mode. The gradient elution program started with an initial elution at 15% A, 0% B, 85% C for 5 min, which changed to 14% A, 27% B, 59% C until 7 min, and continued 90% A, 0% B, 10% C until 12 min when the elution changed back to the initial 15% A, 0% B, 85% C for re-equilibration of the column. The injection volume was 100 µL. Excitation and emission wavelengths were λ_{ex} = 329 nm, λ_{em} = 460 nm.

Quantification of mycotoxins was based on the external standard method using calibration curves fitted by linear regression analysis. Linear regression analyses were conducted at optimized HPLC conditions. The linearity of the calibration graphs was studied by injecting seven-points of calibration curves of both mycotoxins (AFB1 and OTA) at concentrations of 31-2000 µg/L and 7.5-625 µg/L for AFB1 and OTA, respectively. Correlation coefficients obtained were ≥0.9997 and ≥0.9998 for AFB1 and OTA, respectively. Regarding the sensitivity, the limits of detection

297 (LOD) of the method for both mycotoxins were assessed. LOD was calculated to be 0.09 μg/L and
298 5 μg/L for AFB1 and OTA respectively. The repeatability and reproducibility of the method was
299 assessed by injecting five times standard on the same day (intra-day) and over 5 days (inter-day),
300 respectively.

301 *2.8 Statistical analysis*

302 Analysis of variance (ANOVA) was used to detect statistical differences among the
303 adsorbents used, concentrations and juices tested (Statgraphics Plus. Version 5.1). Differences
304 were considered to be significant at $P<0.05$. The method used to discriminate among the means
305 was Fisher's least significant difference (LSD) procedure.
306 Fitting of adsorption isotherms and estimation of suitable parameters was carried out using JMP
307 Pro 13.1.0.
308

309 **3. Results**

310 *3.1 In vitro adsorption screening tests (ELISA)*

311 In the present study a single concentration method was applied to measure the adsorption
312 of toxins in a buffer solution (pH 5), where a known amount of mycotoxin reacted with a known
313 amount of adsorbent. Table 2 shows the results of mycotoxin adsorption of the 27 bentonite clays
314 assayed. More than half of the samples adsorbed more than 70% of the AFB1 present, and eleven
315 samples adsorbed ≥90% AFB1. Of the latest, all but one (A18 sample), were tri-octahedral
316 bentonites (Table 2).

317 Regarding FB1, eleven samples adsorbed ≥30%, and more than half of the samples
318 presented an adsorption percentage lower than 1%. Only one sample reached more than 70% of
319 adsorption (A18), whereas for ZEN and OTA this adsorbent only reached 36% and 20% of
320 adsorption, respectively. The adsorption of DON was negligible (<13%) at the dose tested in this
321 assay. Adsorbents which showed good adsorbent and mineralogical properties and were available

322 geographically (A2, A8, A9, A12, A15, A18, and A22) were selected to perform a deeper
323 subsequent adsorption isotherm experiment by using simulated GI fluids (see section 3.2).

324 It is noteworthy that the present study also revealed that, among the samples tested, tri-
325 octahedral bentonites (A2, A5, A7-A10, A12, A15, A20-A27) showed higher adsorption
326 capacities than di-octahedral bentonites (A1, A3, A4, A6, A11, A13, A14, A16-A19), with the
327 exception of A18 ($P<0.05$).

328 *3.2 Equilibrium adsorption experiments*

329 Six mycotoxin concentrations (including levels below EU limits or recommendations)
330 were assayed to measure mycotoxin adsorption in simulated GI juices (at pH 1.3 and at pH 6.8),
331 where a known amount of mycotoxin reacted with a known amount of adsorbent (0.02% w/v).
332 Results obtained with the 14 adsorbents assayed showed in general similar AFB1 adsorption
333 percentages at pH 1.3 and at pH 6.8 (Table 3), being slightly higher in basic than in acid GI juice
334 ($P<0.05$).

335 From results presented in Table 3, it can be seen that AFB1 adsorption was high with all
336 the adsorbents tested (over 82% at 0.02 mg AFB1/L). Adsorption ranged from 30 to 96.6% at the
337 higher level of AFB1 tested (4 mg AFB1/L) and from 92.1 to >100% at the lower level tested
338 (0.02 mg AFB1/L) in intestinal simulated juice. So, adsorption increased inversely to toxin
339 concentration ($P < 0.05$). Bentonites B1, B2, B4-B7 showed the highest adsorption percentages in
340 both simulated GI juices, when compared to the results obtained among the 14 adsorbents at the
341 higher dose of toxin (4 mg AFB1/L): adsorption varied in the range of 72.8-96.6%, reaching all of
342 these 100% when lower levels of AFB1 (0.02 mg AFB1/L) were tested. Generally, the pre-
343 selected bentonites (B1-B7) tested showed better AFB1 adsorption percentages than commercial
344 products assayed (C1-C7). ($P<0.05$). Only two commercial products (C1 and C4) showed a similar
345 adsorption than pre-selected bentonites (Table 3).

346 Unlike AFB1, OTA adsorption was significantly affected by pH ($P < 0.05$). In fact, in
347 acidic conditions (pH 1.3), adsorbents were more effective than in basic conditions (pH 6.8)
348 (Table 4). Experimental values for OTA adsorption, considering only those obtained with
349 simulated gastric juice, range from 2.4 to 41.5% at the higher level tested (1 mg OTA/L) and in
350 the range from 8 to 59.8% at the lower level tested (0.05 OTA mg/L) (Table 4). Regarding OTA
351 adsorption, it increased as toxin concentration decreased, but it was much less pronounced than in
352 the case of AFB1 (Table 4). Among the 14 adsorbents tested, B2, B4 and B6 showed the greater
353 adsorption percentages, adsorbing more than half of the toxin (at the lower OTA level tested).
354 Again, assayed tri-octahedral bentonites proved to be different than commercial products ($P<0.05$)
355 (Table 4).

356 *3.3 Adsorption isotherms*

357 Regarding AFB1 adsorption, the maximum adsorbed amounts (Q_{max}), derived from
358 Langmuir isotherms at pH 1.3 and at pH 6.8, are summarized in Table 5. With the exception of the
359 C5 adsorbent, the adsorbents coded as B were those that generally showed higher Q_{max}, both at
360 gastric and intestinal pH (Table 5), the adsorbent B6 being the one that showed higher values at
361 both tested pH. According to the K_L constant, which is related to the affinity of the adsorbent, B5
362 at pH 6.8, showed the greater adsorption affinity for AFB1. Regarding Freundlich equation, and
363 taking into account the K_F constant, which is related to adsorption capacity of the adsorbent, the
364 AFB1 adsorption capacities ranged from 15.2±0.3 to 49.1±1.3 mg/L and from 3.4±0.1 to 26.6±1.3
365 mg/L for pre-selected bentonites (B1-B7) and commercial products (C1-C7), respectively, at pH
366 6.8 (Table 5). Generally, the samples of bentonites assayed in this study (B1-B7) showed higher
367 adsorption capacities than commercial products (C1-C7).

368 Both adsorption models (Langmuir and Freundlich) provided a good fit to experimental
369 adsorption data of AFB1. As indicated by the root mean square errors (RMSE), the Freundlich

model showed a better fit to the AFB1 adsorption data than the Langmuir isotherm. As it can be seen on Table 5, RMSE values obtained by Freundlich isotherms were generally slightly lower than those obtained by Langmuir isotherms. As an example, in Fig. 2, four curves obtained fitting the Langmuir model to AFB1 adsorption experimental data (adsorbents B2 and B4, pH 1.3 and 6.8) can be seen.

Regarding OTA adsorption isotherms, the maximum adsorption capacity (Q_{max}) at pH 1.3 by using Langmuir model was 1.6±0.2 mg/g for B4, while at pH 6.8 data did not fit (Table 6). Generally, experimental data of OTA adsorption fitted better to Freundlich equation than to Langmuir equation, considering in most cases Langmuir parameters did not converge as can be observed in Table 6. According to K_F Freundlich constant, tri- octahedral bentonite B4 showed the higher adsorption capacity at pH 1.3. Curve obtained after plotting experimental data of OTA adsorption for adsorbent B4 at pH 1.3 can be seen in Fig. 2.

3.4 Effect of adsorbent dosage

The effect of adsorbent dosage on the mycotoxin adsorption process was studied by increasing the dosage of bentonites B2 and B4 from 0.02% up to 0.2% (w/v) (Table 7).

In both cases, the adsorption was affected by this ten-fold increase even if it was more pronounced for OTA than for AFB1. In the case of AFB1, significant differences ($P<0.05$) were observed between lower and intermediate or higher adsorbent dosage (Table 7), although in all cases very high absorptions (>96%) were obtained (Table 7). Regarding OTA, the absorption was significantly affected ($P<0.05$) by the adsorbent dosage, and the percentage of toxin adsorbed, increased with increasing dosages of bentonite. Experimental values for the adsorption of OTA were in the ranges of 3.2-27.7% for B2, and 15.1-75% for B4 (Table 7).

4. Discussion

394 *In vitro* preliminary tests of mycotoxin adsorption is a powerful tool for screening potential
395 mycotoxin adsorbent agents (Boudergue et al., 2009). In the present study, a screening *in vitro* test
396 was performed by testing adsorbent clays (n=27) at a very low dose (0.02 % w/v) versus high
397 levels of mycotoxins (1-10 mg/L). In fact, the mycotoxin doses tested in the preliminary
398 experiment were well above the EU-MLs or recommendations for mycotoxins in animal feed,
399 especially for AFB1 and OTA. Regarding AFB1, the concentration tested (4 mg/L) was 200 times
400 higher than higher EU-MLs (20 μg/kg), whereas OTA was also tested at a concentration 4 times
401 higher than the higher EU-guidance level (250 μg/kg). Assayed concentrations of ZEN and DON
402 were closer to the EU-recommendations, while the concentration of FB1 tested was within the
403 range recommended, considering the high guideline levels established for this toxin, as FB1 has a
404 very low absorption in animals (Pierron et al., 2016). Anyhow, this preliminary study revealed that
405 more than half of the adsorbents showed high adsorbing capacity for AFB1 and lower for FB1,
406 ZEN and OTA, while little DON adsorption was recorded. Our results are in agreement with other
407 previous works in which several adsorbent materials, including smectites, activated carbons (AC),
408 polymers such as cholestyramine and other commercial products were tested, and no adsorbent
409 materials, with the exception of AC and cholestyramine, showed a relevant ability binding DON
410 (Avantaggiato et al., 2003, 2005, 2007; Döll et al., 2004; Ramos et al., 1996 a, b; Sabater-Vilar et
411 al., 2007). Regarding AC, DON adsorption abilities vary widely depending on the type of
412 carbonaceous substances and activation processes. Moreover, the application of AC in animal feed
413 could adsorb minerals, vitamins and other nutrients as well, and the effectiveness of AC towards
414 DON could not be confirmed *in vivo* (Avantaggiato et al., 2004; Sabater-Vilar et al., 2007).
415 Regarding cholestyramine, the high cost of this polymer would be a limiting factor for its practical
416 implementation (Avantaggiato et al., 2005; Ramos et al., 1996 b).

417 A similar experiment was performed by Pappas et al. (2014) who *in vitro* tested two
418 bentonites from Greece (assayed at 1% w/v) versus AFB1 (0.02 ppm), ZEN (2 ppm) and OTA

(0.1 ppm) by using buffered water at pH 3. Their results indicated a complete adsorption of 0.02 ppm of AFB1 (≈100%) by both bentonites at 1%, and no desorption was observed, whereas adsorption of ZEN and OTA were low (less than 5 and 18%, respectively). However, in this study a higher dosage of bentonite (50 times higher) was used compared to our experiment. As far as we know, there is no published work in which the efficiency of bentonite clays versus five different mycotoxins was tested using the dose at which the only EU authorized adsorbent has been tested (0.02%, w/v) (EC, 2013).

Of the latter, seven adsorbents (mainly tri-octahedral bentonites) were selected to perform a deeper subsequent adsorption isotherm experiment by simulating GI conditions. Six mycotoxin concentrations of AFB1 and OTA (including levels below EU limits or recommendations) were assayed to measure mycotoxin adsorption, where known amounts of mycotoxin reacted with a known amount of adsorbent (0.02% w/v). For this experiment, reasonable concentrations of mycotoxins and adsorbents were tested using simulated GI juices (at pH 1.3 and at pH 6.8), to mimic conditions close to an *in vivo* digestion. In fact, the acidic pH used in this experiment can be found in some portions of the proventriculus and gizzard of poultry while basic pH can be found in the distal two parts of the small intestine (jejunum and ileum) (Pappas et al., 2014). Also in monogastric animals, pH along the GI tract can vary from 1.2 to 4.5 in stomach, increasing from 5 to 7.5 in the intestinal lumen (Greco et al., 2019). According to experimental data, similar AFB1 adsorption percentages at pH 1.3 and at pH 6.8 were obtained, being slightly higher in basic juice than in acid juice ($P<0.05$). In fact, AFB1 adsorption by bentonites (B1-B7) at concentration as low as 0.02% (w/v) was very high (approx. 100%), independently of pH. AFB1 is a non-ionizable molecule, therefore a change of pH should not affect AFB1 adsorption. Similar results were obtained by Dakovíc et al. (2008) for *in vitro* adsorption of AFB1 by a bentonite from Bosnia and by a copper modified montmorillonite. They reported that these adsorbents were able

443 to bind almost 100% of AFB1 at pH 3, and that the binding ability did not change as the pH
444 increased from 3 to 9.

445 On the other hand, OTA adsorption was significantly affected by pH ($P<0.05$). OTA
446 consists of a dihydroisocoumarin moiety linked through its carboxyl group by an amide linkage to
447 L-phenylalanine. The decreased OTA adsorption by adsorbents at basic pH may be induced by the
448 presence of an anionic form of the toxin, which probably leads to repulsion between OTA
449 molecules and negative charges that could be found on the adsorption surface. Similar findings
450 were obtained by Dakovíc et al. (2003), who described that OTA adsorption by a
451 octadecyldimethyl benzyl ammonium exchanged-clinoptilolite-heulandite tuff was higher at pH 3
452 than pH 7. Also Santos et al., (2011) evaluated the adsorption of OTA by a humic acid polymer,
453 and described an increased OTA adsorption capacity at pH 3.0, and a partial desorption of this
454 mycotoxin at pH 8.4.

455 It should to be noted that the adsorbents (B1-B7) of this study are composed only by the
456 mineral fraction (>70% smectite), while the commercial products (C1-C7) assayed contained in
457 addition to the mineral fraction (di-octahedral bentonites), other components, such as organic
458 additives, to increase their effectiveness or to extend its spectrum of action versus different
459 mycotoxins. However, bentonites assayed (B1-B7) turned out to be more efficient ($P<0.05$).

460 In order to investigate the mechanism of AFB1/OTA adsorption by different adsorbents,
461 two adsorption models, Langmuir and Freundilich, often reported in literature, were used to
462 provide the best description of mycotoxin adsorption at pH 1.3 and at pH 6.8. In general, the
463 adsorption isotherms describe how adsorbates interact with adsorbents and, therefore, they are
464 crucial in optimizing the use of adsorbents. The Langmuir equation is valid for monolayer
465 adsorption onto a surface with a finite number of identical sites. As shown above, Langmuir
466 equation includes a maximum adsorption capacity (Q_{max}) parameter in the formula. It represents
467 the maximum adsorbed amount of mycotoxin to form a complete monolayer on the adsorbent

surface, corresponding to sites saturation (mg/g). The Freundlich model provides no information on the monolayer adsorption capacity. In contrast to the Langmuir model, it is based on adsorption onto a heterogeneous surface and does not have a finite saturation capacity. The heterogeneity index in the Freundlich model, *n*, gives information on the population of the binding sites and the adsorption intensity, and it is associated with the favourability of the binding process. In order to evaluate the fit of the isotherm to the experimental data, the optimization procedures require a statistical goodness-of-fit-measure. In this study the RMSE was employed to determine which isotherm better fitted to our results and values can be observed in Tables 5 and 6.

The fact that the Freundlich isotherm fits slightly better the experimental AFB1/OTA data may be due to the heterogeneous distribution of active sites on the adsorbent surface, since the Freundlich equation assumes that the adsorbent has a multilayer adsorption. Similar results were obtained by Ramos and Hernández (1996) who studied adsorption of AFB1, AFB2, AFG1, and AFG2 by montmorillonite, at pH 7, and reported that the Freundlich isotherm fitted the experimental data better than the Langmuir isotherm. Also, Desheng et al. (2005), found that AFB1 adsorption on calcium montmorillonite, at pH 2 and pH 8, could be determined by both isotherm models, Langmuir and Freundlich. Conversely, Dakovíc et al. (2008) determined that AFB1 adsorption by two tested montmorillonites followed a non-linear (Langmuir) type of isotherm, at pH 3. Also, Santos et al., (2011) described that Langmuir equation proved to be the best model to predict monolayer equilibrium sorption of OTA onto the humic acid polymer.

Finally, the effect of dosage on mycotoxin adsorption of B2 and B4 bentonites was studied to calculate the optimal adsorbent dosage for further experiments. The 10-fold dose increase of bentonite B4 rendered a much more effective adsorption of OTA, reaching almost 75% adsorption ($P<0.05$). This greater adsorption of OTA at this dose, together with a very high adsorption of AFB1, makes this adsorbent a good candidate for subsequent *in vivo* tests with farm animals. In

fact, the addition of 0.2% of adsorbent to the feeds falls within the amounts usually used by feed manufacturers for this type of products.

5. Conclusions

Application of good agricultural practices is not always sufficient to guarantee feed safety, since undesirable effects can occur even with very low levels of mycotoxin contamination.

In vitro preliminary tests of mycotoxin adsorption is a powerful tool for screening potential mycotoxin-adsorbent agents before testing in animals since if no adsorption occurs *in vitro*, little or no chance exists to do so *in vivo*. However, complete *in vitro* studies to determine the stability of the complex formed in the GI tract are not frequent in literature. Our preliminary study revealed a high number of adsorbents (most of them tri-octahedral bentonites) able to adsorb most of the present AFB1 and, to a lesser extent, FB1, ZEN and OTA. However, an efficient adsorbent for DON was not obtained.

The present work has determined a tri-octahedral bentonite (B4) as an efficient binder of two highly toxic toxins (AFB1 and OTA), which are increasingly found co-contaminating animal feed. Moreover, this bentonite showed better *in vitro* adsorption properties than most of the commercial products assayed ($P<0.05$). Addition of tri-octahedral bentonite at a low dosage (from 0.02 up to 0.2%) to the initial composition of feedstuffs would efficiently prevent the mycotoxicosis originated by these toxins by inducing the formation of a mycotoxin-bentonite complex which would impede the GI absorption. *In vivo* studies with different animal species are needed to study the possible interferences of the selected bentonites with minerals or essential nutrients absorption. It should be noted that EU-Regulation does not allow the use of adsorbents when the mycotoxin contamination of feed exceeds the EU-limits or guidelines.

Declaration of interest statement

The authors declare there are no conflicts of interest.

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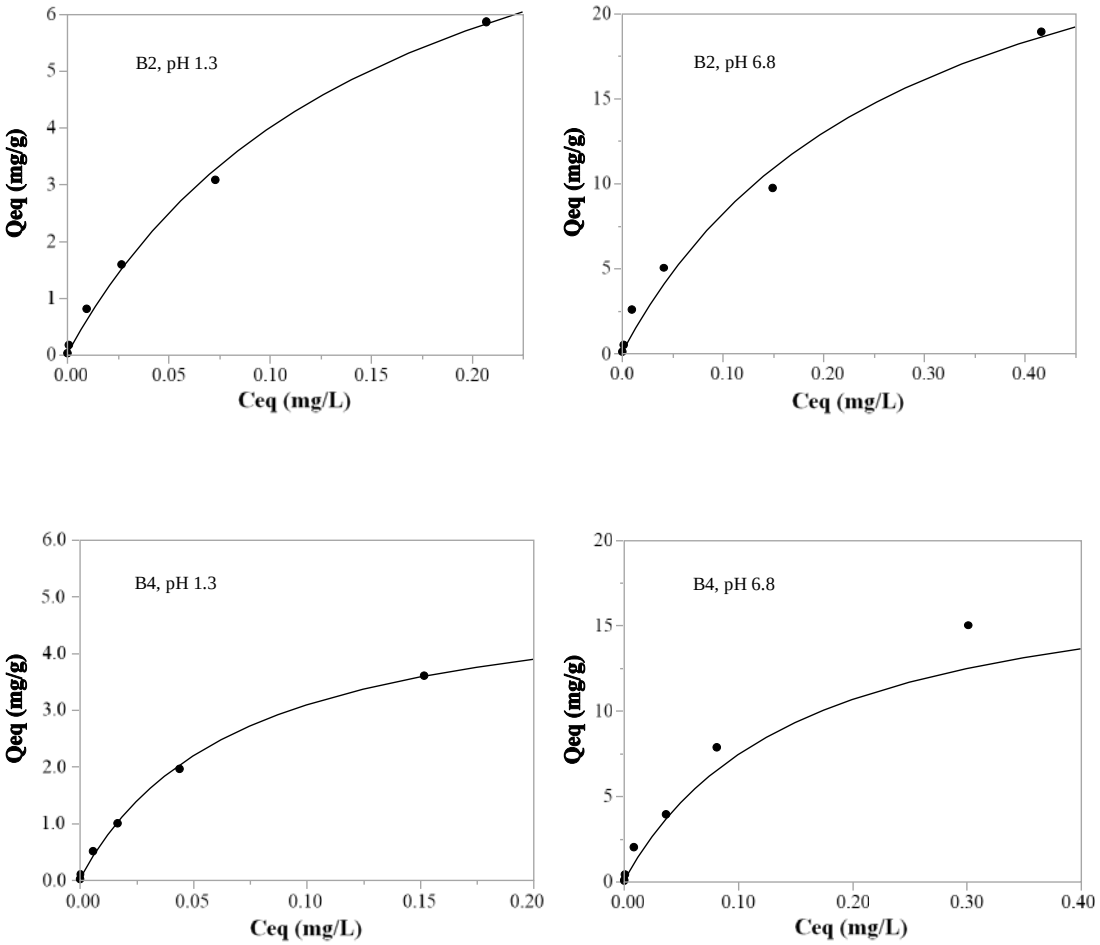
Figure legends

Figure 1. Equilibrium adsorption Langmuir isotherms obtained at 37 ºC in gastric (on the left, at pH 1.3) and intestinal (on the right, at pH 6.8) simulated juices by testing a fixed amount (0.02%, w/v) of adsorbent B2 (at the top) and B4 (in the bottom) versus increasing concentrations of AFB1 (0.02-4 mg/L).

Figure 2. Equilibrium adsorption Freundlich isotherm obtained at 37 ºC in gastric (pH 1.3) simulated juice by testing a fixed amount of adsorbent B4 (0.02% w/v) versus increasing concentrations of OTA (0.05-1 mg/L).

715 Fig. 1.



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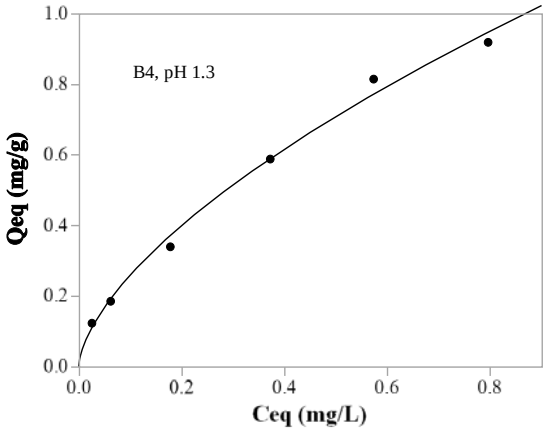
726 Fig. 2.



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738 **Table 1.** Mineralogical composition of commercial products (C1-C7).

Commercial binder	Composition
C1	Bentonite (1m558), Sepiolite (E-562)
C2	Processed montmorillonite, diatomaceous earth, yeast walls, seaweed extracts, sugar cane molasses
C3	Montmorillonite, interspersed montmorillonite, diatomaceous earth, yeast walls, seaweed extracts
C4	Bentonite (1m558), strains of microorganisms (1m01 y 1m03), diatomaceous earth, E551c, seaweed meal
C5	Bentonite (1m558), calcium carbonate, yeast
C6	Di-octahedral bentonite
C7	Activated bentonite

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741 **Table 2.** Mycotoxin adsorption, expressed as adsorption percentage (%), of 27 bentonite clays
742 tested *in vitro* at pH 5 (acetate buffer) and 0.02 % (w/v) versus AFB1 (4 mg/L), DON (10 mg/L),
743 OTA (1 mg/L), FB1 (10 mg/L), and ZEN (5 mg/L), assayed separately.

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Adsorbent	**Type of bentonite**		**Mycotoxin adsorption (%)**				
	Di-octahedral	Tri-octahedral	AFB1	DON	OTA	FB1	ZEN
A1	X		20.1^{a}	6.1	<1^{a}	<1^{a}	<1^{a}
A2		X	>95.0^{g}	3.4	<1^{a}	40.7^{fg}	<1^{a}
A3	X		26.5^{a}	<1	<1^{a}	7.4^{abc}	13.3^{d}
A4	X		28.4^{a}	5.4	<1^{a}	2.9^{abc}	<1^{a}
A5		X	69.9^{d}	<1	<1^{a}	4.1^{abc}	<1^{a}
A6	X		44.1^{b}	4.9	<1^{a}	<1^{a}	<1^{a}
A7		X	74.8^{de}	<1	<1^{a}	34.5^{def}	<1^{a}
A8		X	89.2^{fg}	12.6	<1^{a}	30.4^{de}	<1^{a}
A9		X	69.0^{d}	<1	<1^{a}	1.6^{ab}	<1^{a}
A10		X	88.9^{fg}	<1	<1^{a}	30.4^{de}	<1^{a}
A11	X		53.9^{bc}	<1	<1^{a}	<1^{a}	<1^{a}
A12		X	>95.0^{g}	<1	<1^{a}	37.3^{ef}	<1^{a}
A13	X		43.8^{b}	<1	<1^{a}	<1^{a}	<1^{a}
A14	X		54.3^{bc}	1.7	4.0^{c}	<1^{a}	<1^{a}
A15		X	95.4^{g}	2.5	6.9^{d}	44.9^{ef}	11.2^{c}
A16	X		75.9^{a}	3.8	3.9^{c}	<1^{a}	<1^{a}
A17	X		75.7^{de}	2.6	4.4^{c}	<1^{a}	<1^{a}
A18	X		100^{g}	3.4	20.3^{h}	71.8^{h}	36.4^{f}
A19	X		65.1^{cd}	3.9	3.3^{b}	<1^{a}	<1^{a}
A20		X	81.8^{ef}	5.7	4.0^{c}	8.2^{bc}	<1^{a}
A21		X	76.5^{de}	2.9	4.1^{c}	9.3^{c}	<1^{a}
A22		X	99.2^{g}	4.1	10.5^{g}	45.8^{g}	16.3^{e}
A23		X	100^{g}	4.2	9.4^{f}	33.7^{def}	14.1^{d}
A24		X	45.7^{b}	5.2	<1^{a}	<1^{a}	<1^{a}
A25		X	96.1^{g}	5.7	8.2^{e}	35.1^{def}	5.0^{b}
A26		X	90.6^{g}	4.7	5.1^{e}	4.9^{abc}	<1^{a}
A27		X	97.5^{g}	5.1	5.8^{a}	34.2^{def}	2.9^{a}

745 Values are average level of three triplicates. % RSDs in all cases were <10. Means with different superscript
746 letters are different at P<0.05.

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748 **Table 3.** AFB1 adsorption percentages of 14 adsorbents tested at 0.02% (w/v) versus six AFB1
749 concentrations in gastric (pH 1.3) and intestinal (pH 6.8) simulated juice. B: pre-selected
750 bentonites; C: commercial adsorbents.

		AFB1 adsorption (%) mg AFB1/L					
Adsorbent	**Juice**	**0.02**	**0.10**	**0.5**	**1**	**2**	**4**
B1	Gastric	>97.5^{e}	98.1^{e}	95.8^{d}	94.2^{c}	91.5^{b}	85.9^{a}
	Intestinal	>99.3^{c}	99.6^{c}	99.4^{c}	98.7^{c}	96.9^{b}	95.0^{a}
B2	Gastric	>99.1^{e}	98.1^{e}	94.5^{d}	92.2^{c}	89.4^{b}	84.9^{a}
	Intestinal	>99.7^{d}	98.5^{d}	98.1^{d}	96.0^{c}	92.8^{b}	89.9^{a}
B3	Gastric	98.3^{e}	96.3^{e}	86.1^{d}	79.1^{c}	74.7^{b}	62.0^{a}
	Intestinal	99.6^{f}	98.5^{e}	94.3^{d}	93.0^{c}	82.8^{b}	74.5^{a}
B4	Gastric	>98.0^{e}	98.9^{e}	94.7^{d}	92.4^{c}	89.9^{b}	82.6^{a}
	Intestinal	>99.4^{c}	99.1^{c}	97.8^{c}	95.5^{b}	95.0^{b}	90.7^{a}
B5	Gastric	>97.7^{c}	96.7^{c}	95.3^{c}	91.8^{b}	86.6^{a}	85.1^{a}
	Intestinal	>98.7^{c}	99.4^{c}	99.4^{c}	99.1^{c}	97.9^{b}	96.6^{a}
B6	Gastric	>96.5^{d}	96.4^{cd}	93.4^{cd}	89.3^{bc}	84.5^{b}	79.5^{a}
	Intestinal	>99.3^{c}	98.6^{c}	98.2^{bc}	97.6^{bc}	96.6^{ab}	94.9^{a}
B7	Gastric	>99.1^{d}	97.9^{cd}	97.2^{cd}	89.5^{bc}	82.2^{b}	72.8^{a}
	Intestinal	>99.3^{d}	98.4^{cd}	97.9^{c}	97.7^{c}	95.7^{b}	94.1^{a}
C1	Gastric	>98.7^{d}	92.0^{c}	91.5^{c}	91.3^{c}	82.8^{b}	73.4^{a}
	Intestinal	>99.9^{d}	99.8^{d}	99.4^{cd}	98.4^{c}	95.5^{b}	90.1^{a}
C2	Gastric	96.8^{e}	77.8^{d}	57.6^{c}	39.1^{b}	38.2^{b}	27.9^{a}
	Intestinal	92.1^{f}	80.1^{e}	60.3^{d}	42.1^{c}	37.2^{b}	32.5^{a}
C3	Gastric	82.0^{f}	71.9^{e}	62.1^{d}	49.9^{c}	35.2^{b}	26.4^{a}
	Intestinal	96.5^{f}	80.1^{e}	72.0^{d}	55.5^{c}	43.4^{b}	30.0^{a}
C4	Gastric	>98.3$_{e}$	91.6^{cd}	85.7^{c}	74.0^{b}	75.8^{b}	65.6^{a}
	Intestinal	>99.8^{e}	>99.8^{e}	99.2^{d}	98.2^{c}	97.6^{b}	90.7^{a}
C5	Gastric	95.6^{d}	94.3^{d}	87.8^{cd}	81.9^{bc}	75.2^{b}	65.5^{a}
	Intestinal	97.8^{d}	96.9^{d}	96.2^{cd}	94.4^{bc}	93.3^{b}	88.3^{a}
C6	Gastric	86.3^{c}	77.8^{bc}	70.5^{ab}	64.0^{a}	63.9^{a}	57.3^{a}
	Intestinal	98.8^{d}	98.8^{d}	96.4^{d}	90.3^{c}	81.7^{b}	72.9^{a}
C7	Gastric	92.2^{c}	87.9^{c}	83.3^{c}	71.9^{b}	63.7^{ab}	55.7^{a}
	Intestinal	98.4^{d}	97.8^{d}	94.0^{cd}	90.2^{bc}	88.3^{b}	74.6^{a}

751 Values are average level of three triplicates. % RSDs in all cases were <10. Means with different
752 superscript letters are different at P<0.05.
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Table 4. OTA adsorption percentages of 14 adsorbents tested at 0.02% (w/v) versus six OTA concentrations in gastric (pH 1.3) and intestinal (pH 6.8) simulated juice. B: pre-selected bentonites; C: commercial adsorbents.

		OTA adsorption (%) mg OTA/L					
Adsorbent	**Juice**	**0.05**	**0.10**	**0.25**	**0.50**	**0.75**	**1**
B1	Gastric	26.3^{f}	18.7^{b}	17.3^{a}	21.8^{d}	21.2^{c}	22.7^{e}
	Intestinal	6.10^{f}	2.6^{e}	1.3^{a}	1.8^{c}	1.6^{b}	2.0^{d}
B2	Gastric	59.8^{f}	22.2^{e}	12.5^{d}	8.7^{c}	8.3^{b}	7.9^{a}
	Intestinal	22.8^{e}	9.7^{d}	3.4^{c}	1.5^{b}	<1^{a}	<1^{a}
B3	Gastric	23.6^{f}	8.9^{e}	6.2^{d}	4.1^{c}	2.9^{b}	2.4^{a}
	Intestinal	7.8^{d}	4.0^{c}	2.6^{b}	<1^{a}	<1^{a}	<1^{a}
B4	Gastric	50.2^{f}	37.7^{e}	27.4^{d}	23.7^{c}	21.8^{b}	18.4^{a}
	Intestinal	18.0^{e}	6.7^{d}	3.5^{a}	3.4^{a}	3.9^{b}	4.4^{c}
B5	Gastric	25.0^{e}	20.5^{c}	20.2^{b}	21.9^{d}	19.5^{a}	20.1^{b}
	Intestinal	4.7^{c}	1.9^{b}	<1^{a}	<1^{a}	<1^{a}	<1^{a}
B6	Gastric	51.2^{f}	48.6^{e}	44.9^{d}	42.8^{c}	40.4^{a}	41.5^{b}
	Intestinal	19.8^{e}	12.3^{d}	9.7^{c}	4.9^{b}	3.8^{a}	3.8^{a}
B7	Gastric	32.1^{f}	31.6^{e}	26.5^{d}	23.8^{b}	23.1^{a}	26.3^{c}
	Intestinal	14.2^{e}	8.4^{d}	5.3^{c}	3.0^{a}	3.2^{a}	4.2b
C1	Gastric	37.3^{e}	16.9^{d}	10.8^{c}	7.5^{b}	6.3^{a}	6.2^{a}
	Intestinal	8.7^{c}	3.5^{b}	<1^{a}	<1^{a}	<1^{a}	<1^{a}
C2	Gastric	14.4^{f}	7.2^{e}	3.6^{d}	3.3^{c}	3.2^{b}	2.5^{a}
	Intestinal	5.2^{d}	3.3^{c}	1.2^{b}	<1^{a}	<1^{a}	<1^{a}
C3	Gastric	22.8^{e}	12.5^{d}	7.2^{c}	6.0^{b}	5.9^{b}	5.1^{a}
	Intestinal	11.8^{e}	5.9^{d}	2.8 ^{c}	1.5^{b}	1.2^{a}	<1^{a}
C4	Gastric	27.8^{e}	16.3^{d}	10.7^{c}	5.0^{a}	6.0^{b}	5.0^{a}
	Intestinal	12.7^{f}	4.6^{e}	2.3^{d}	1.3^{c}	1.1^{b}	0.9^{a}
C5	Gastric	38.3^{f}	33.2^{e}	27.4^{d}	23.7^{b}	22.5^{a}	24.2^{c}
	Intestinal	18.0^{f}	11.5^{e}	8.0^{d}	5.4^{c}	4.0^{a}	5.2^{b}
C6	Gastric	8.0^{f}	2.1^{d}	1.3^{a}	1.6^{c}	1.^{5b}	4.5^{e}
	Intestinal	8.2^{d}	4.4^{c}	2.3^{b}	<1^{a}	<1^{a}	<1^{a}
C7	Gastric	17.6^{e}	9.6^{d}	7.1^{c}	4.7^{a}	4.8^{a}	6.2^{b}
	Intestinal	10.3^{d}	5.9^{c}	2.1^{a}	2.1^{a}	2.1^{a}	3.1^{b}

Values are average level of three triplicates. % RSDs in all cases were <10. Means with different superscript letters are different at P<0.05.

Table 5. AFB1 adsorption (0.02-4 mg/L) Langmuir and Freundlich parameters of 14 adsorbents tested at 0.02% (w/v), in gastric (pH 1.3) and intestinal (pH 6.8) simulated juice. B: pre-selected bentonites; C: commercial adsorbents.

		Langmuir			Freundlich		
Adsorbent	**Juice**	Q_{max} **(mg/g)**	K_L **(L/mg)**	**RMSE**	K_F **(mg/L)**	N	**RMSE**
B1	Gastric	5.2± 0.3	17.5± 2.4	0.09	12.1± 0.6	1.7± 0.1	0.06
	Intestinal	20.4± 3.4	14.8± 5.9	0.97	38.4± 2.1	1.9± 0.1	0.30
B2	Gastric	10.2± 0.9	6.4± 1.1	0.16	15.8± 0.1	1.6± 0.0	0.02
	Intestinal	31.1± 5.8	3.5± 1.3	1.09	31.3± 1.1	1.7± 0.1	0.39
B3	Gastric	6.7± 0.4	3.8± 0.5	0.13	6.3± 0.2	1.8± 0.1	0.12
	Intestinal	20.4± 2.9	2.9± 1.1	1.22	15.2± 0.3	2.1± 0.1	0.44
B4	Gastric	5.2± 0.2	14.1± 1.6	0.08	10.3± 0.6	1.8± 0.1	0.08
	Intestinal	22.7± 1.7	6.4± 1.0	0.47	29.9± 1.8	1.7± 0.1	0.46
B5	Gastric	9.0± 3.3	5.2±3.0	0.21	15.2± 1.9	1.4± 0.1	0.13
	Intestinal	10.6± 1.5	42.6± 15.1	0.45	36.9± 3.4	1.8± 0.1	0.19
B6	Gastric	13.7± 2.0	3.2± 0.8	0.32	14.1± 0.2	1.6± 0.0	0.08
	Intestinal	24.5± 2.1	8.9± 1.4	0.34	49.1± 1.3	1.5± 0.0	0.12
B7	Gastric	11.9± 1.9	3.9± 1.5	0.63	10.7± 0.3	2.0± 0.1	0.23
	Intestinal	23.3± 3.7	7.8± 2.4	0.60	41.5± 2.3	1.6± 0.0	0.27
C1	Gastric	7.3± 0.7	7.0± 1.7	0.27	9.0± 0.5	1.9± 0.1	0.19
	Intestinal	20.7± 2.7	11.9± 4.7	1.35	26.2± 0.6	2.4± 0.1	0.28
C2	Gastric	2.2± 0.3	2.3± 0.7	0.08	1.6± 0.1	1.8 ± 0.1	0.06
	Intestinal	14.9± 4.2	0.3± 0.1	0.44	3.4± 0.1	1.5 ± 0.1	0.26
C3	Gastric	2.6± 0.2	3.0± 0.8	0.12	2.0± 0.1	2.1± 0.1	0.07
	Intestinal	6.3± 0.5	1.8± 0.4	0.29	3.6± 0.1	2.3 ± 0.1	0.18
C4	Gastric	7.4± 1.2	2.8± 0.8	0.19	7.1± 0.6	1.5± 0.1	0.19
	Intestinal	19.1± 0.8	20.2± 2.8	0.57	26.0± 2.0	2.5± 0.2	0.88
C5	Gastric	13.3± 1.2	2.0± 0.4	0.30	9.3± 0.1	1.7± 0.0	0.07
	Intestinal	23.6± 0.9	4.6± 0.4	0.25	26.6± 1.3	1.7± 0.1	0.46
C6	Gastric	6.3± 0.6	1.8± 0.2	0.04	5.6± 0.2	1.3± 0.1	0.05
	Intestinal	20.6± 3.4	2.3± 0.9	1.11	14.4± 0.2	2.0± 0.1	0.31
C7	Gastric	3.2± 0.5	4.7± 1.4	0.09	3.9± 0.1	1.6± 0.1	0.03
	Intestinal	15.7± 0.6	4.3± 0.4	0.30	13.7± 0.7	2.1± 0.2	0.63

Q_{max}: maximum adsorption capacity; K_L: Langmuir affinity constant; K_F: Freundlich capacity constant; n: adsorption intensity; RMSE: root-mean-square error; NC (not converge).

Table 6. OTA adsorption (0.05-1 mg/L) Langmuir and Freundlich parameters of 14 adsorbents tested at 0.02% (w/v), in gastric (pH 1.3) and intestinal (pH 6.8) simulated juice. B: pre-selected bentonites; C: commercial adsorbents.

		Langmuir			Freundlich		
Adsorbent	**Juice**	Q_{max} **(mg/g)**	K_L **(L/mg)**	**RMSE**	K_F **(mg/L)**	***N***	**RMSE**
B1	Gastric	NC	NC	NC	0.06	0.8± 0.0	0.0009
	Intestinal	NC	NC	NC	0.00	0.9± 0.1	0.0002
B2	Gastric	NC	NC	NC	0.01	2.2± 0.6	0.0011
	Intestinal	NC	NC	NC	NC	NC	NC
B3	Gastric	NC	NC	NC	0.12	3.1± 0.4	0.007
	Intestinal	NC	NC	NC	NC	NC	NC
B4	Gastric	1.6± 0.2	1.6± 0.4	0.0	1.08	1.6± 0.1	0.0309
	Intestinal	NC	NC	NC	0.24	0.8± 0.2	0.0196
B5	Gastric	NC	NC	NC	0.04	1.0± 0.0	0.0010
	Intestinal	NC	NC	NC	0.05	1.0± 0.1	0.0004
B6	Gastric	0.5± 0.4	0.3± 0.2	0.00	0.12	1.1± 0.1	0.0015
	Intestinal	NC	NC	NC	NC	2.4± 0.4	0.0005
B7	Gastric	NC	NC	NC	0.06	0.9± 0.1	0.0025
	Intestinal	NC	NC	NC	NC	1.1± 0.3	0.0009
C1	Gastric	0.4± 0.1	2.8± 1.7	0.0	0.32	2.1± 0.3	0.0231
	Intestinal	NC	NC	NC	NC	NC	NC
C2	Gastric	0.2± 0.1	1.4± 0.9	0.0	0.14	1.8± 0.3	0.0131
	Intestinal	NC	NC	NC	NC	NC	NC
C3	Gastric	0.6± 0.2	1.0± 0.6	0.0	0.28	1.6± 0.1	0.0170
	Intestinal	NC	NC	NC	0.04	6.3± 0.6	0.0016
C4	Gastric	0.3± 0.1	3.6± 2.7	0.0	0.23	2.1± 0.4	0.0244
	Intestinal	NC	NC	NC	0.04	5.3± 1.9	0.0054
C5	Gastric	NC	NC	NC	0.05	1.1± 0.1	0.0017
	Intestinal	NC	NC	NC	0.01	1.4± 0.2	0.0007
C6	Gastric	NC	NC	NC	0.02	0.2± 0.1	0.0006
	Intestinal	NC	NC	NC	NC	0.4± 0.2	0.0007
C7	Gastric	NC	NC	NC	0.01	1.0± 0.3	0.0011
	Intestinal	NC	NC	NC	NC	0.7± 0.2	0.0006

Q_{max}: maximum adsorption capacity; K_L: Langmuir affinity constant; K_F Freundlich capacity constant; *n*: adsorption intensity; RMSE: root-mean-square error; NC (not converge).

787 **Table 7**. Effect of B2 and B4 adsorbent dosage on aflatoxin B_1 (AFB1) and ochratoxin A (OTA)

788 adsorption.

		Mycotoxin adsorption (%)	
Bentonite	Dosage (%, w/v)	AFB1	OTA
B2	0.02	97.9[a]	3.2[a]
	0.12	99.7[b]	18.7[b]
	0.20	99.8[b]	27.7[c]
B4	0.02	96.3[a]	15.1[a]
	0.12	99.8[b]	55.0[b]
	0.20	99.9[b]	75.0[c]

789 Values are average level of three triplicates. % RSDs in all cases were <5. Means with
790 different superscript letters are different at P<0.05.
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