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ORIGINAL PAPER

Aflatoxin B_1 Sorption and Safety of Dietary Sodium Bentonite in Sprague-Dawley Rats

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Abstract Bentonites are readily available clays used in the livestock industry as feed additives to reduce aflatoxin (AF) exposure; their potential interaction with nutrients is the main concern limiting their use, however. The objective of the present study was to determine the safety of a dietary sodium-bentonite (Na-bentonite) supplement as a potential AF adsorbent, using juvenile Sprague Dawley (SD) rats as a research model. Animals were fed either a control diet or a diet containing Na-bentonite at 0.25% and 2% (w/w) inclusion rate. Growth, serum, and blood biochemical parameters, including selected serum vitamins (A and E) and elements such as calcium (Ca), potassium (K), iron (Fe), and zinc (Zn) were measured. The mineral characteristics and the aflatoxin B_1 sorption capacity of Na-bentonite were also determined. By the end of the study, males gained more weight than females in control and Na-bentonite groups ($p \leq 0.0001$); the interaction between treatment and sex was not significant ($p = 0.6780$), however. Some significant differences between the control group and bentonite treatments were observed in serum biochemistry and vitamin and minerals measurements; however, parameters fell within reference clinical values reported for SD rats and no evidence of dose-dependency was found. Serum Na and Na/K ratios were increased, while K levels were decreased in males and females from Na-bentonite groups. Serum Zn levels were decreased only in males from Na-bentonite treatments. Overall, results showed that inclusion of Na-bentonite at 0.25% and 2% did not cause any observable toxicity in a 3-month rodent study.

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Introduction

The inclusion of bentonite in feed by the livestock industry is a readily available method to reduce aflatoxin exposure (Harvey et al., 1991). Aflatoxin B_1 (AFB_1) is a potent hepatocarcinogen (Busby & Wogan, 1984) with genotoxic (Smela et al., 2001), immunotoxic (Hinton et al., 2003; Turner et al., 2003), and antinutritional (Pimpukdee et al., 2004) effects. AFB_1 is a group 1 carcinogen (International Agency for Research on Cancer [IARC], 2012) produced mainly by *Aspergillus flavus* and *A. parasiticus* fungi that commonly contaminate cereals and nuts (Council for Agricultural Science and Technology [CAST], 2003). Although AFB_1 is the most toxic and prevalent of the aflatoxin chemical subtypes, the mixture of total aflatoxins (AFs) is also regarded as a group 1 carcinogen (IARC, 2012). Due to their deleterious effects, the FDA established total aflatoxin action levels ranging from 20 μg/kg to 300 μg/kg for different ingredients used in animal nutrition (Food and Drug Administration [FDA], 2019).

Bentonites are montmorillonite-rich clays commonly used as anti-caking agents or flow promoters in feeds. These minerals have GRAS (generally recognized as safe) status when used at levels not exceeding 2% inclusion in feed (Code of Federal Regulations, 2010) and have many potential applications in sorption of toxic compounds, drug delivery, conferring thermal and pH resistance properties to materials, among other useful human health therapeutic applications such as diarrhea control (Uddin, 2018). Montmorillonite is regarded as the active ingredient for aflatoxin (AF) binding (Deng et al., 2010 Grant & Phillips, 1998). By adsorbing AFs from the diet, montmorillonite reduces the toxin bioavailability (Phillips, 1999; Phillips et al., 2009) thus protecting animals from toxicity. Calcium bentonites (Ca-bentonites) have been investigated extensively in vitro and in vivo and have been proved to protect animals effectively from the negative effects of AF (Ledoux et al., 1999; Smith et al., 1994; Phillips et al., 2006). In humans, the use of Ca-bentonites (i.e. NovaSil) was shown to be safe and effective in reducing the levels of blood and urine biomarkers of AF exposure (Afriyie-Gyawu, Ankrah et al., 2008a; Wang et al., 2008). Similarly, oral sodium bentonite has been shown to protect domestic animals from AF exposure. For instance, bentonite in dairy cattle feed resulted in a reduction in AFM_1 (an AFB_1 metabolite) excretion in milk (Diaz et al., 2004). Similarly, New Zealand breed rabbits were protected from aflatoxicosis when dosed at 5 g bentonite/kg of feed (Hassan et al., 2019). Due to the evident benefits observed from inclusion of these types of minerals in feeds, the European Union and the US state of Texas allow the use of some clays (e.g. bentonites) and other additives as AF adsorbents in feeds (European Food Safety Authority [EFSA], 2010; Office of the Texas State Chemist [OTSC], 2011).

Despite its beneficial effects, the potential interactions of sodium bentonite with anticoccidials, antibiotics (Gray et al., 1998; Shryock et al., 1994), and vitamin A (Briggs & Spivey-Fox, 1956; Laughland & Phillips, 1956) require further studies on the safety of this mineral. Additionally, due to their inherent natural origin and differences in composition, bentonites may contain heavy metals, dioxins, and furans as contaminants. For these reasons, in vivo safety evaluation of any bentonite for use as a toxin binding agent is essential before its inclusion in the diet. The objectives of the present study were to determine the mineral characteristics, sorption properties for AF in vitro, and safety of a selected sodium bentonite (Na-bentonite) in a 3-month rodent study using Sprague-Dawley rats.

Materials and Methods

Na-bentonite was provided by American Colloid's Wyoming facility, Reserve 2010-5113 (AMCOL International Corporation, Hoffman Estates, Illinois, USA). Ultrapure water (UP-H_2O) was obtained by processing distilled water through a PURELAB Ultra filtration system (ELGA Woodridge, Illinois, USA). AFB_1 standard (CAS 1162-65-8) was purchased from Sigma Aldrich (St. Louis, Missouri, USA). All other reagents and solvents were of analytical grade.

Measurement of Dioxins, Furans, and Heavy Metals in Na-bentonite

As bentonites are naturally occurring minerals, they may contain undesirable toxic compounds that may limit their use as dietary interventions in animals or humans. Hence, data for the 17 most important chlorinated dibenzo-p-dioxins/furans (CDDs/CDFs) found in

Na-bentonite were performed by Analytical Perspectives (Wilmington, North Carolina, USA). Procedures followed the US Environmental Protection Agency (USEPA) method 8290 for sample preparation, clean-up, and analysis with high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) (Environmental Protection Agency [EPA], 2007c). Measurements of arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) were also performed by the same laboratory. Samples were digested and prepared according to method 3051A using a PerkinElmer Multiwave 3000 Anton Paar Microwave Reaction System (Graz, Styria, Austria) (EPA, 2007a). For Hg analyses, a PerkinElmer FIMS-100 CVAA analyzer was used following EPA method 7470A (EPA, 1994). Other metals were analyzed with a PerkinElmer ELAN 6100 ICP-MS using EPA method 6020A (EPA, 2007b). Elemental composition of Na-bentonite was obtained using X-ray fluorescence (XRF) with a Phillips PW2400 XRF spectrometer. Data were run and provided by AMCOL.

Mineralogical Analyses

Mineralogical analyses were run in the clay mineralogy laboratory at Texas A&M University (TAMU). After size fractionation following the steps described by Soukup et al. (2008), the clay, silt, and sand fractions were analyzed with X-ray diffraction (XRD), Fourier-transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Most mineralogical analyses were performed following standard procedures previously reported (Soukup et al., 2008) with minor modifications. The moisture content was determined by subtracting the oven-dry weight (110°C) from the air-dry weight of 5 g of Na-bentonite. For electrical conductivity (EC) measurement, 10 g of sample was weighed into 250 mL plastic bottles; the ratio of deionized water:solid used was 25:1. The sample was shaken for 30 min on a rotary shaker at room temperature and then centrifuged at 1073×*g* for 10 min. After centrifugation, the supernatant was used to measure the EC and pH sequentially. To evaluate the presence of sulfates, 2 mL of the supernatant was collected in a glass tube followed by the addition of an equal volume of acetone (sulfate precipitation).

Cation exchange capacity (CEC) and extractable base determination in NH_4OAc were performed by the Soil Characterization Laboratory from the Soil and Crop Sciences Department at TAMU, using a mechanically controlled variable-rate leaching device as reported by Holmgren et al. (1977). Soluble bases using DI water were determined by the Soil, Water and Forage Testing Laboratory at TAMU, using a saturated paste extract method based on Rhoades and Clark (1978). 100 g of sample were used for this analysis.

X-ray diffraction (XRD) patterns were recorded using a Bruker D8 ADVANCE X-ray diffractometer (Billerica, Massachusetts, USA). Sand and silt fractions were ground in a mortar, passed through a 140 mesh (105 μm) sieve, and mounted as powder to collect the XRD patterns. Clay fractions were saturated with magnesium (Mg) and potassium (K), and then air-dried on glass discs to obtain oriented films for XRD analysis. Mineral identification was determined using *EVA* computer software (Bruker, Madison, Wisconsin, USA). Infrared patterns were recorded in a Spectrum 100 FTIR (PerkinElmer, Inc. Waltham, Massachusetts, USA) using the diffuse reflectance infrared Fourier-transform (DRIFT) method and the attenuated total reflectance (ATR) method with a scanning range of 450–7800 cm^{-1} and a resolution of 2 cm^{-1}. For the DRIFT method, 0.005 g of sample was placed in a holder, using KBr as background material. For the ATR method, 1 mg of the clay fraction was mixed with 300 mg of KBr under a lamp to maintain dryness. Clay sample and KBr were mixed in a steel capsule for 30 s and then placed in a holder for reading. Powder samples were used directly in the ATR analysis with a Universal ATR accessory (PerkinElmer, Inc., Waltham, Massachusetts, USA). Silt samples were examined using a JEOL 6400 scanning electron microscope with energy dispersive X-ray (EDS) recording capability (Akishima, Tokyo, Japan). For TEM analysis, a clay sample was mounted on a holey carbon grid, and images were recorded using a JEOL JEM-2010 microscope with EDS capability (Akishima, Tokyo, Japan).

AFB_1 Sorption Analyses

Aflatoxin adsorption isotherms were performed based on methods reported by Grant & Phillips (1998), and previously described in detail by Marroquin-Cardona et al. (2009). Briefly, a Na-bentonite suspension was prepared in an Erlenmeyer flask by adding 100 mg of clay and 50 mL of water to give a concentration of 2 mg/mL. A toxin parent solution (8 μg/mL) was

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prepared by dissolving toxin AFB_1 crystals (Sigma-Aldrich, Saint Louis, Missouri, USA) in acetonitrile. Then, 11 dilutions of AFB_1 ranging from 0.4 to 8 μg/mL were prepared by adding appropriate amounts of parent AFB_1 solution and UP-H_2O in glass tubes to complete a 5 mL volume; each dilution was done in triplicate. Na-bentonite suspension (50 μL) was added to test tubes (i.e. 0.1 mg clay in each test tube) and tubes were shaken for 2 h at 1000 rpm on an IKA-Vibrax orbital shaker (Wilmington, North Carolina, USA) at 25°C. Isotherms were conducted at pH 2 and 6.5 by adjusting UP-H_2O pH. Upon completing the 2 h shaking, tubes were centrifuged at 939×g for 20 min to collect supernatant for AFB_1 measurement at 364 nm wavelength on a Shimadzu UV-Vis spectrophotometer (Columbia, Maryland, USA). Computer-generated equilibrium isotherms were extrapolated from Na-bentonite adsorption data and fitted to the Langmuir model (based on r^2 value). The parameters Q_{max} (mol AFB_1 kg^{-1} bentonite) and K_d were estimated to determine sorption capacity and affinity onto Na-bentonite.

Rodent Study Experimental Design

Authorization for the study was obtained by Texas A&M University, according to animal use protocol (AUP) 2008-39. Sprague Dawley®™ (SD) rats were obtained from Harlan (Houston, Texas, USA). Animals in each group came from different litters to assure independence of samples. Four-week-old female and male SD rats were maintained on rodent feed meal (8604) from Teklad Harlan (Madison, Wisconsin, USA) and water ad libitum. After a brief acclimation period (5 days), the rats (1 rat per cage) were allocated to each group, including one control and two Na-bentonite treatment groups. Each group consisted of 10 male and 10 female rats, with average weights at the beginning of the study of 107–133 g and 79–105 g, respectively. The control group received basal rodent diet throughout the study while the treatment groups received formulations containing basal rodent diet with low (0.25%) and high (2%) inclusion of Na-bentonite. Dietary clay concentrations were based on the minimum (0.25% w/w) and maximum (2%) doses previously tested in similar safety studies (Afriyie-Gyawu et al., 2005).

Animals were housed in a climate-controlled environment (temperature 22–25°C) with artificial illumination (12 h dark/12 h light). General appearance, behavior, and signs of morbidity and mortality were inspected daily. Body weights were measured initially and every three days throughout the study (30 time points). Feed consumption was also recorded every three days (29 time points). Before termination, blood was drawn via cardiac puncture under isoflurane anesthesia. Following euthanasia, organs and tissues of interest were removed and evaluated for gross anatomical abnormalities. Wet weights of liver, kidneys, heart, lungs, brain, spleen, tibia, and uterus plus ovaries were recorded.

Hematological and Serum Analysis

Hematology and serum biochemical parameters were analyzed by the Clinical Pathology Lab, Texas Veterinary Medical Diagnostics Lab (TVMDL) (College Station, Texas, USA). Hematological analysis of whole blood samples was conducted using an Abbott CELL-DYN 3700 Hematology Analyzer (Abbott Laboratories, Abbott Park, Illinois, USA) and included hemoglobin (Hb) concentration, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), percent corpuscular volume (PCV), platelets, and red blood cell (RBC) and white blood cell (WBC) counts. Serum biochemical parameters were assessed using an automated analyzer Modular P (Roche Diagnostics, Indianapolis, Indiana, USA) and included albumin/globulin ratio (A/G), albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMYL), aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium (Ca), cholesterol (CHOL), creatine kinase (CK), chloride (Cl), creatinine (CRT), gamma glutamyl-transferase (GGT), globulins (GLOB), glucose (GLUC), potassium (K), sodium (Na), Na/K ratio, phosphorous (P), total bilirubin (T-BIL), and total serum protein (TSP). Serum micronutrients iron (Fe) and zinc (Zn) were measured with a Hitachi 911 chemistry analyzer (Roche Laboratories, Indianapolis, Indiana, USA) and a PerkinElmer Analyst 100 (Shelton, Connecticut, USA), respectively. Vitamin A and E were measured by HPLC according to previously reported methods by Weinmann et al. (1999) and Rupérez et al. (2004).

Statistical Analysis

To investigate the weight gained among the control group and the low dose (0.25 %) and high dose (2%) Na-bentonite groups, a linear mixed model was

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constructed with the fixed effect treatment, sex, and treatment-sex interaction. Time was included as a continuous variable and tested for interactions with treatment and sex. Differences in final body weight (FBW) were investigated with a similar model that included the fixed effect treatment and the covariates initial body weight (IBW) and total feed consumption (TFC) as potential explanatory variables. Backward selection was done by removing one term at a time, starting with non-significant interactions ($p > 0.05$). To investigate the differences in biochemical parameters, organ weights, and selected vitamins and minerals among the control and the two doses of Na-bentonite for males and females separately, a linear or a generalized linear mixed model was constructed with the fixed effect treatment as the only independent variable. The MIXED and GLIMMIX SAS procedures were used for all statistical analyses using *SAS* 9.2 with *Enterprise Guide* 4.1. In all of these analyses, subject (i.e. rat) was considered as a random effect. All post-test comparisons were adjusted by the method of Tukey-Kramer (Tukey, 1977). Total bilirubin data were analyzed with non-parametric tests, data were classified into categories before analysis with a Kruskal-Wallis test followed by the Mann-Whitney test (Mann & Whitney, 1947; Kruskal & Wallis, 1952).

Results and Discussion

Levels of Dioxins, Furans, and Heavy Metals in Na-bentonite

Detectable dioxins/furans in Na-bentonite included tetrachlorodibenzo-p-dioxin, hexachlorodibenzo-p-dioxin, heptachlorodibenzo-p-dioxin, tetrachlorodibenzo-p-furan, and pentachlorodibenzo-p-furan. The average calculated dioxin/furan toxic equivalents (TEQs) were 0.0811 pg/g. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has placed a provisional tolerable daily intake (PTDI) value for dioxins at 2.3 pg/kg body weight/day (Joint FAO/WHO Expert Committee on Food Additives [JECFA], 2002) for humans. To reach the PTDI level, and assuming bioavailability of the dioxins in the gastrointestinal tract, an average 70 kg person would have to consume approximately 2 kg of Na-bentonite per day. Under realistic conditions, consumption of bentonite will be at much lower levels that confer a very low risk of dioxin exposure. The metal levels found in Na-bentonite were 0.01 mg/g for As (metalloid), 0.003 mg/g for Cd, <0.00003 mg/g for Hg, and 0.03 mg/g for Pb. Using the tolerable daily intake (TDI) levels reported by the JECFA (JECFA, 1989, 2007, 2011), the TDIs calculated for a 70 kg person are 0.14 mg for As, 0.07 mg for Cd, 0.01 mg for Hg, and 0.21 mg for Pb. Even ingesting 3 g of Na-bentonite, which is the maximum level of calcium clays given in capsules to humans for reduction of AF bioavailability (Afriyie-Gyawu, Ankrah, et al., 2008a), the exposure of these metals through ingestion would be very low.

Mineralogical Analyses

The mineral characteristics and elemental composition of Na-bentonite according to XRF data are summarized in Table 1. Insignificant reaction followed HCl application and trace amounts of calcite were detected by XRD (Fig. 1) suggesting that carbonate minerals were present in small amounts in Na-bentonite. Calcite could be associated with beneficial effects as some forms of calcium carbonate can act as a calcium supplement (Palacios et al., 2020). Similarly, little or no reaction following H_2O_2 treatment suggested that Na-bentonite does not contain significant amounts of oxidizable or reducible components (e.g. manganese oxides, siderite, etc.) or organic matter. The pH of the sample was 8.3, a value within ranges reported for clay samples containing smectites (Kannewischer et al., 2006; Marroquin-Cardona et al., 2009). The EC values observed suggested soluble salts were present in this sample. The presence of sulfates (i.e. gypsum) was suggested by the acetone test precipitation and was confirmed by XRD (Fig. 1). Gypsum is a mineral form of calcium sulfate, which is used commonly as a food additive (General Standard for Food Additives, [GSFA], 2019), hence its presence in Na-bentonite does not represent a risk. The percentages calculated for each fraction (sand, silt, and clay) showed that Na-bentonite was composed mainly of <2 μm clay particles (90%) (Table 1).

The major minerals identified in the powder mount XRD were montmorillonite (12.5 Å), gypsum (7.6 Å), and quartz (3.3 Å) (Fig. 1). Peaks for quartz, mica (10.0 Å), and cristobalite (4.04 Å), as well as peaks of feldspars from the plagioclase group (e.g. anorthite) and alkali feldspars (sanidine and albite) were observed in the sand and silt fractions. The presence of montmorillonite was confirmed after XRD analysis of the clay fraction exchanged with Mg and K. After treatment with

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Table 1 Mineral characteristics and XRF elemental composition of Na-bentonite

Parameter	Value	Oxides and elements	wt.%
Moisture %	6.4	SiO_2	61.247
pH	8.3	Al_2O_3	19.216
EC	751.0	CaO	1.341
HCl reaction	Little	Cl	0.047
H_2O_2 reaction	Little	Cr_2O_3	0.032
Magnetic minerals	ND	Fe_2O_3	3.957
Sulfate precipitation	Little	K_2O	0.456
Wet state fractions (%) (water + Na_2CO_3)		MgO	2.837
>53 μm	3.0	MnO	0.065
2–53 μm)	7.0	Na_2O	1.755
<2 μm	90.0	P_2O_5	0.02
CEC (mEq/100 g clay)	90.5	SO_3	0.308
Extractable cations (mEq/100 g clay)		TiO_2	0.148
Ca	32.5	O	45.18
K	1.4	Si	28.63
Mg	19.9	Al	10.17
Na	58.8	Ca	0.96
Extractable cations DI-water (mEq/100 g clay/L)[a]		Cl	0.05
Ca	2.7	Cr	0.02
K	0.5	Fe	2.77
Mg	1.6	K	0.38
Na	31.9	Mg	1.71
		Mn	0.05
		Na	1.30
Oxides and elements	wt.%	P	0.01
		S	0.12
Total oxides and elements	91.43	Ti	0.09

[a] Extractable bases were measured in DI-water vacuum extracts from a paste made with 100 g of clay according to Rhoades and Clark (1978); *ND* not detected

Mg and glycerol solvation, the *d*-spacing of the most intense montmorillonite peak increased from 15 Å to 18 Å. Samples exchanged with K and then air-dried, showed a peak of 12 Å, which is in the range expected for montmorillonites (10–14 Å) (Hawkins & Egelstaff, 1980; Oueslati et al., 2015). After heating the samples at 330°C and 550°C, the ~12 Å peak changed to ~10 Å and 9 Å, respectively. The XRD analysis also suggested the presence of quartz and opal CT in the clay fraction. The presence of montmorillonite was confirmed by the FTIR analysis. The specific identification of FTIR peaks is shown in Table 2. The SEM examination revealed quartz and feldspars with a variety of particle shapes and compositions. SEM also revealed traces of other minerals such as monazite particles containing phosphorus (P), lanthanides, and actinide elements such as cerium (Ce), neodymium (Nd), samarium (Sm), and protactinium (Pa) (Fig. 2a), and a particle composed of calcium phosphate compatible with hydroxylapatite ($Ca_5(PO_4)_3(OH)$) (Fig. 2b). Hydroxylapatite is a mineral that showed no adverse effects on mice and rabbits under acute and sub-chronic oral toxicity studies (Hanh et al., 2019). The TEM images revealed the common folding tendency of the montmorillonite particles (Fig. 2c). The particles are shown as thin films of considerable length (3–5 μm). Moiré patterns were observed in some particles, and this effect was probably due to the overlap of plates with different orientation (Fig. 2d).

AFB_1 Sorption Analyses

The parameters Q_{max} and K_d calculated for Na-bentonite demonstrated the AF adsorption effectiveness of the clay in vitro. A favorable AF sorption pattern characterized as an L-shape curve at both pH levels (Fig. 3) was depicted for Na-bentonite. Specifically, an L2-shape was documented at pH 6.5 with a calculated Q_{max} value of 0.42±0.02 mol AFB_1/kg of clay (Langmuir fit $r^2 = 0.95$). A slightly lower Q_{max} of 0.39±0.01 mol AFB_1/kg of clay (Langmuir fit $r^2 = 0.99$) was calculated at pH 2.0, for which an L1 pattern was observed. The L1 shaped pattern is indicative of sorption processes in approximation of a plateau, while an L2 pattern suggests that saturation has been reached (Giles et al., 1960; Grant & Phillips, 1998). The differences in patterns may account for the different Q_{max} values at pH 6.5 versus pH 2. The K_d values obtained at pH 6.5 and 2 were $5.45 \cdot 10^5$ and $2.86 \cdot 10^5$, respectively. According to the sorption capacity for AFB_1 ($Q_{max} > 0.3$ mol/kg of clay) and the mineral characteristics of Na-bentonite (pH of 8.3, high smectite composition, CEC value of 90.5, and FTIR evidence of framework Mg and/or Fe) this material could be considered for inclusion in feeds as an aflatoxin binder, according to the classification system proposed by Dixon et al. (2008).

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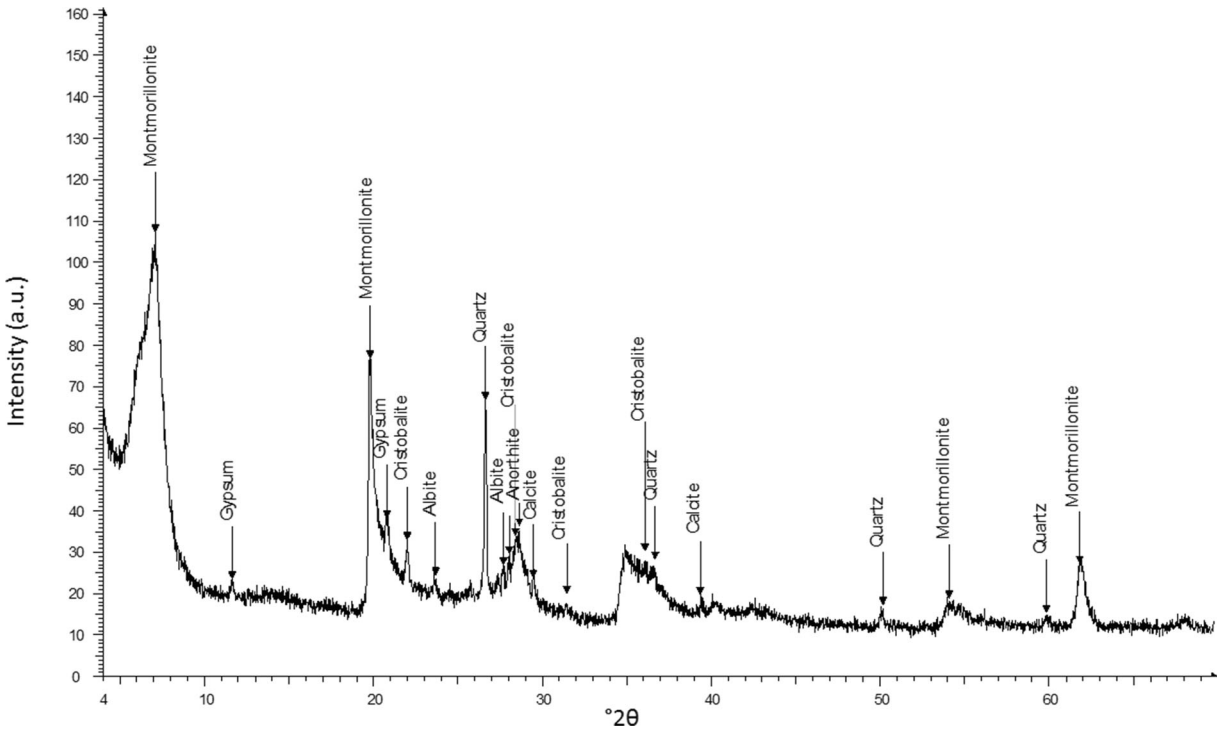

Fig. 1 Powder mount XRD patterns of Na-bentonite. Minerals identified included montmorillonite (12.5, 5.1, 4.48, 2.56, 1.49 Å), gypsum (7.6 Å), quartz (3.3 Å), calcite (3.0 Å), orthoclase feldspars (3.7 Å), and andesine (3.24 and 3.20 Å)

Rodent Study Outcomes

Consumption of Na-bentonite at either low or high dose did not cause any observable toxicity throughout the study. Importantly, the EFSA Panel on Additives and Products or Substances used in Animal Feed (EFSA-FEEDAP) recently concluded that bentonite at inclusion levels of 2% w/w in complete feed is safe for all animal species (EFSA FEEDAP Panel, 2017). In the current study, all rats remained healthy and active. Males gained more weight than the females ($p \leq 0.0001$), as expected (Fig. 4). Weight-gain over time was different in at least one of the treatment groups ($p \leq 0.0001$), but the interaction between treatment and sex was not significant ($p = 0.6780$). Rats in the control group gained more weight than rats in the 2% Na-bentonite group by time 5 (day 13) ($p = 0.0019$) and consecutively up to time 30 (day 88) ($p \leq 0.0001$). Similarly, animals in the control group gained more weight than the 0.25% Na-bentonite group by time 20 (day 58) ($p = 0.0131$) and consecutively at time 30 (day 88) ($p = 0.0002$). In the females, a significant difference was observed for FBW between the control and the Na-bentonite treatment groups (Table 3). The TFC and IBW were not significant factors for explaining the difference in FBW. Similarly, in males, IBW was not a significant factor for explaining the difference in FBW. However, in males a linear relationship was observed between TFC and FBW in both treatment groups ($p = 0.0278$). Differences in growth parameters between male and female rats were explained by Slob and Van Der Werff Ten Bosch (1975), revealing that neonatal androgens play the most important part in increasing growth in males while ovarian secretions starting in puberty suppress growth rate in females.

Hematological and Serum Analysis

Some differences between the control and at least one of the Na-bentonite treatment groups were observed for hematological, serum vitamin, and micronutrient parameters (Tables 4 and 5). Relevant findings included increased vitamin E levels observed in both females and males of Na-bentonite treatment groups suggesting a potential protective effect of the clay on the vitamin, or the ability of the clay to

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Table 2 ATR and KBr analysis of Na-bentonite

Assignment	Na-bentonite	
	ATR (cm^{-1})	KBr (cm^{-1})
Montmorillonite (OH-stretching of structural hydroxyl groups)	3636	3632
CO_2	2512	2926
	1858	2366
	–	2344
OH deformation of water	1637	1631
	–	–
	–	–
Si–O-stretching (longitudinal mode)	1108	–
Si–O-stretching	–	1038
AlAlOH deformation	930	918
AlFeOH deformation	–	881
AlMgOH deformation	–	845
Si–O stretching of quartz and silica	799	799
Si–O	–	693
	650	–
Coupled Al–O and Si–O, out of plane	–	622[a]
Al–O–Si deformation	–	524
	504	–
Si–O–Si deformation	–	467

[a] The 622 cm^{-1} band of Na-bentonite may also be referring to cristobalite, although it was not found in the SEM. Infrared bands identification was based on Madejová and Komadel (2001), Paluszkiewicz et al. (2008) and Alabarse et al. (2011)

enhance vitamin E bioavailability; however, more research is needed to delineate the specific mechanisms involved with this observation. With regards to vitamin A, male rats ingesting 2% Na-bentonite had significantly lower serum levels of this vitamin than the control group. Interactions of vitamin A and bentonites have been observed previously in rats and chickens at high dietary inclusion rates of bentonite (i.e. >3% w/w) (Briggs & Spivey, 1954; Laughland & Phillips, 1954;). However, more recent studies involving SD rats, using 2% inclusion rates of a similar bentonite, have shown no significant changes in serum vitamin A levels, when compared to the control (Afriyie-Gyawu et al., 2005; Marroquin-Cardona et al., 2011). The difference between the effects of Na vs. Ca bentonites on vitamin A may be attributed to differences in particle size and/or swelling characteristics.

Concerning serum cations, an interesting finding was the significant reduction in serum K of both males and females from Na-bentonite treatment groups when compared to control groups. Limited information on the effects of these types of clays on serum K is available though some clinical reports have linked the consumption of soils or clay to hypokalemia in pregnant and non-pregnant women (McKenna, 2006; Severance Jr. et al., 1988; Trivedi et al., 2005; Ukaonu et al., 2003). Nevertheless, these reports lack specific information regarding the mineral identification of the ingested material. Other studies using ionic K-concentrated groundwater revealed the ability of bentonite to adsorb K from the water over a period of 9 months, while findings for Na were opposite, for which an increase was observed over time (Melamed & Pitkänen, 1996). This may be related to inherent cation exchange abilities of different types of bentonites. Interestingly, a study using a Ca-bentonite has shown similar effects on serum K levels in SD rats (Marroquin-Cardona et al., 2011). Importantly, in both studies the serum K levels were within normal physiological ranges for SD rats. Moreover, no effect of Ca-bentonite clay was observed in serum K level of humans ingesting up to 3 g a day for up to 3 months (Afriyie et al., 2008b).

Serum Zn was another serum cation for which significant differences from the control were observed, although this only occurred in the males of the Na-bentonite groups. Interactions of similar bentonite clays (e.g. hydrated sodium calcium aluminosilicate; HSCAS) with Zn have been reported by Chung et al. (1990). In that study, the authors observed significantly ($p < 0.05$) lower Zn levels in the tibia of chickens fed 1% HSCAS in the diet. Tibial Zn concentration is a more reliable indicator of Zn levels in the body than serum (Wedekind & Baker, 1990); however, serum and plasma Zn have both been reported as useful indicators of short-term changes in dietary zinc for SD rats (Baltaci et al., 2005; Sunar et al., 2009). Normal clinical ranges of serum Zn for 3-month-old SD rats are not available in the literature, but the levels shown in the present study were within the normal clinical ranges reported for 6-month-old SD rats (Afriyie-Gyawu et al., 2005). Results from the present study also agree with data from a Ca-bentonite study (Marroquin-Cardona et al., 2009). The main differences between

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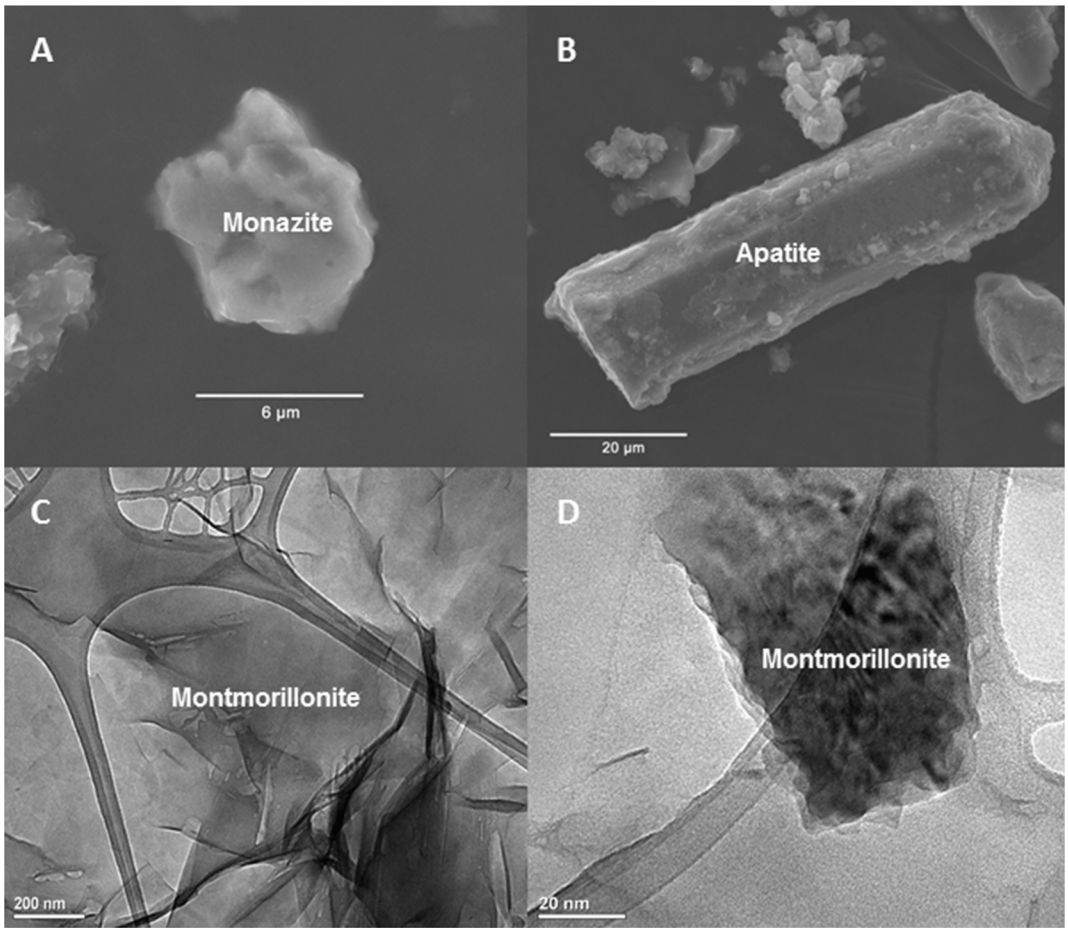

Fig. 2 **A** SEM image of monazite particle in Na-bentonite sample. **B** Hydoxylapatite particle in Na-bentonite. **C** Smectite particles in sheets and rolls with approximate length >1 µm, according to the scale bar. This morphology is commonly observed for effective aflatoxin adsorbents, according to Mulder et al. (2008). **D** Moiré patterns observed in a platelet of Na-bentonite

these types of bentonites (Ca and Na) are their cation saturation and swelling capacity upon hydration. Both bentonites have high CEC values, and these types of clays may retain cation exchange abilities in the gastrointestinal tract. These chemical properties may potentially explain the observed reduction in serum Zn in the males (see Table 4), in agreement with the suggestion that some geophagic clays may interfere with the absorption of cations (Young et al., 2011). Nonetheless, the Na-bentonite effects on Zn were observed only in males (not in females) and further work is needed to confirm this finding. The supplementation of some cations by soils has also been proposed (Wiley & Katz, 1998) and this may be related to the observed increase in serum Na (see Table 5) in both males and females of Na-bentonite groups. Serum Ca was also significantly increased in males from Na-bentonite treated groups. Similar studies using SD rats have found significant increases in serum Ca levels in females ingesting 0.5% and 2.0% of Ca-bentonite (Afriyie-Gyawu et al., 2005), and in females and males ingesting 0.25 and 2% of a refined Ca-bentonite (Marroquin-Cardona et al., 2011). In the present study, the females from the 2% Na-bentonite treatment had significantly increased levels of Ca, but this did not occur at the lower dose (0.25% Na-bentonite). Calcium sources in Na-bentonite may include calcite, gypsum, and montmorillonite as evidenced on the XRD patterns (Fig. 1), and the potential supplementation of Ca by clays has been discussed previously (Wiley & Katz, 1998; Wilson,

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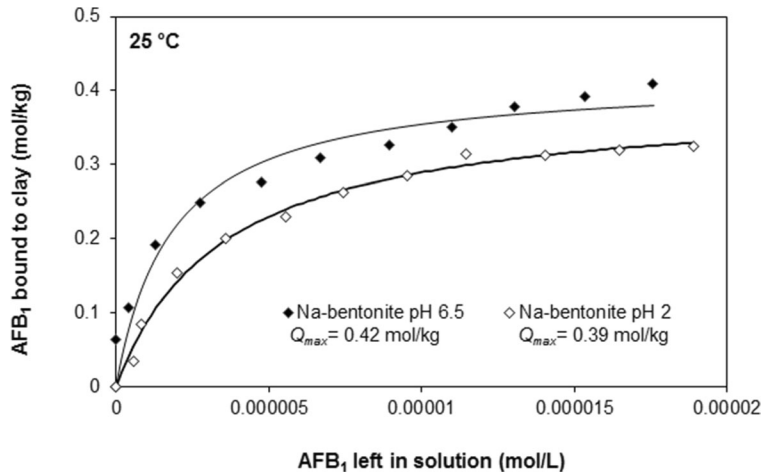

Fig. 3 Aflatoxin B_1 adsorption isotherm for Na-bentonite at pH 6.5 and 2. An L-shaped pattern was observed at both pH levels. The bend in the pattern indicated that the sorbent reached saturation as the concentration of the solute was increased. The Q_{max} (mol AFB_1/kg) values were similar at both pH levels. Q_{max} was calculated according to the formula: $Q_{max} = q/((K_dC_w/(1+K_dC_w))$, with q being the concentration of AFB_1 adsorbed (mol/kg), Q_{max} the maximum capacity (mol/kg), K_d the distribution constant, and C_w the equilibrium concentration of AFB_1 (Grant & Phillips, 1998)

2003). Other notable outcomes, in males and females of both Na-bentonite groups, included increased serum Na/K ratio (Tables 4 and 5). The interactions of clays with cations may not always lead to deficiencies, but, depending on the chemistry of the cation, in some cases supplementation may occur. Importantly, in the present study, no effects of the clay were observed for serum P as other studies have suggested. For instance, reduction of serum P in chickens was attributed to diets

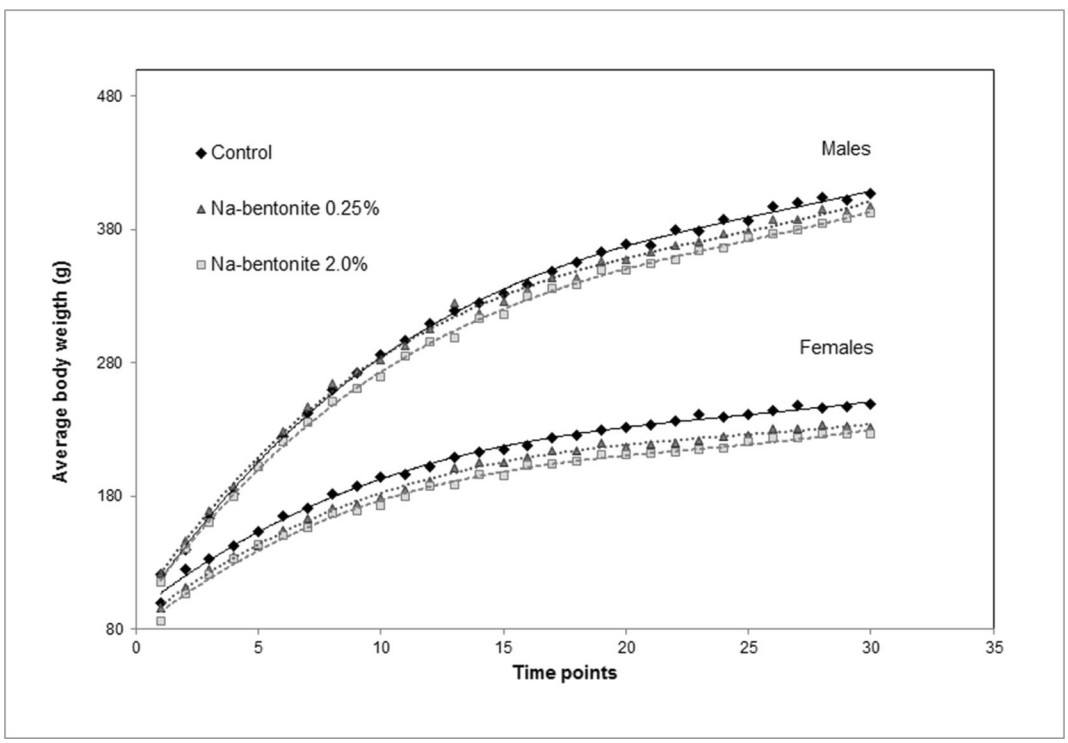

Fig. 4 Growth charts of SD rats ingesting Na-bentonite. Mean body weight (g) of males and females are plotted versus time. In total, the body weights were recorded 30 times

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Table 3 Growth parameters and organ weights of SD rats receiving the control diet and a diet with 0.25 and 2.0% Na-bentonite for 3 months

Parameter	Group		
	Control	Na-bentonite 0.25%	Na-bentonite 2.0%
Females			
FBW (g)	243.85±13.49	231.03±7.46[a]	226.60±15.63[a]
FCE (g)	13.24±2.74	14.72±1.74	15.82±1.69
IBW (g)	99.52±4.75	95.30±3.14	86.33±4.62[a]
TBWG (g)	144.33±10.12	135.73±7.35	140.27±13.70
TFC (g)	1899.17±341.57	1995.90±231.58	2205.14±169.50
Brain	1.63±0.11	1.58±0.07	1.59±0.08
Heart	0.82±0.06	0.80±0.05	0.82±0.07
L. kidney	0.80±0.07	0.75±0.04	0.73±0.05
Liver	6.82±0.40	6.30±0.74	6.51±0.88
Lung	1.20±0.10	1.17±0.08	1.15±0.13
R. kidney	0.79±0.06	0.74±0.03	0.74±0.03
Spleen	0.52±0.06	0.54±0.05	0.51±0.06
Tibia	0.75±0.13	0.75±0.08	0.69±0.03
Uterus	0.63±0.13	0.59±0.10	0.75±0.31
Males			
FBW (g)	406.45±19.99	397.39±24.44	392.73±19.19
FCE (g)	7.42±0.70	7.78±0.53	7.68±0.45
IBW (g)	120.87±3.65	121.83±6.40[b]	115.66±4.96[ab]
TBWG (g)	285.58±20.18	275.56±21.93	277.07± 21.89
TFC (g)	2110.08±104.45	2135.83±101.44	2120.53±66.08
Brain	1.65±0.09	1.76±0.13[a]	1.76±0.09[a]
Heart	1.36±0.09	1.35±0.10	1.31±0.19
L. kidney	1.31±0.11	1.24 ±0.10	1.17±0.10
Liver	12.34±0.82	11.16±0.88[a]	10.84±1.00[a]
Lung	1.64±0.21	1.53±0.11	1.61±0.14
R. kidney	1.35±0.14	1.27±0.11	1.21±0.11
Spleen	0.77±0.07	0.77±0.05	0.79±0.17
Tibia	1.04±0.04	0.95±0.08	1.03±0.05

[a] Means with asterisk are significantly different from the control at the 0.05 level

[b] Significantly different between Na-bentonite groups at the 0.05 level

FBW Final body weight, *FCE* Feed conversion efficiency, *IBW* Initial body weight, *TBWG* Total body weight gain, *TFC* Total feed consumption. Data are reported as mean values ± standard deviation

containing 0.5% inclusion of bentonite (Santurio et al., 1999). In the current research, serum P levels in rats from Na-bentonite groups were not significantly different from the control.

The rest of the parameters measured in blood and serum were either not significantly different from the control group or, if different from the control, they fell within normal clinical ranges and showed no evidence of dose dependency (Tables 4 and 5).

Organ Weights

Isolated differences were observed for some of the organ weights for males (Table 3). Specifically, liver and brain weights of males of both Na-bentonite groups were different when compared to the control. However, this was not observed in the females and no indication of a dose dependent effect was found.

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Table 4 Blood and serum parameters of female SD rats receiving dietary Na-bentonite at 0.25 and 2% for 3 months

Parameters	Control	Na-bentonite 0.25%	Na-bentonite 2.0%	Normal values	Reference
Blood					
Hb (g/dL)	14.19±0.33	13.90±0.72	13.68±0.31[a]	14.30±0.83	1
MCH (pg)	18.44±1.70	18.66±1.98	19.62±3.10	19.8±0.94	1
MCHC (g/dL)	34.43±0.72	34.20±0.71	33.95±0.79	34.40±0.50	2
MCV (fl)	55.85±1.76	57.96±1.00[a]	57.77±1.20[a]	57.90±2.10	2
PCV (%)	41.24±1.53	40.65±1.99	40.32±1.03	38.10±2.43	1
Platelets ($x10^3$)/μL	468.20±77.83	483.00±138.59	484.66±280.62	471.68±38.77	3
RBCs ($x10^6$)/μL	7.39±0.29	7.02±0.36[a]	6.98±0.21[a]	7.02±0.40	2
WBC μL	3944.00±962.96	5029.00±1314.55[b]	12595.00±4759.51[ab]	7840.00±2950.00	4
Serum					
A/G Ratio	2.41±0.11	2.22±0.18[a]	2.38±0.15	2.13±0.18	1
ALB (g/dL)	3.74±0.15	3.74±0.18	3.84±0.13	3.41±0.23	5
ALP (U/L)	58.40±14.23	57.70±19.13	71.10±19.67	117±41.7	4
ALT (U/L)	54.70±9.47	56.78±9.52	50.10±6.95	44.00±23.9	2
AMYL (U/L)	1258.40±112.41	1247.30±155.68	1531.10±447.64	1703±164.32	1
AST (U/L)	138.20±42.38	155.77±52.67	92.80±16.98[a]	93.00±30.3	2
BUN (mg/dL)	18.44±1.70	18.66±1.98	19.62±3.10	21.00±3.90	2
Ca (mg/dL)	9.34±0.32	9.62±0.24[b]	10.02±0.41[ab]	10.36±0.32	5
CHOL (mg/dL)	86.73±9.33	85.01±11.97	85.53±4.59	89.00±23.00	2
CK (U/L)	815.11±360.20	1124.11±802.88[b]	363.80±116.44[ab]	210.00±109	5
Cl (meq/L)	94.50±19.54	101.50±1.43	101.20±1.13	104.00±2.40	4
CRT (mg/dL)	0.29±0.03	0.33±0.03	0.31 ±0.02	0.55±0.10	5
Fe (μg/dL)	254.67±38.20	247.73±52.63	233.02±40.54	220±130	4
GGT (U/L)	<3.0	<3.0	<3.0	<3.0	1
GLOB (g/dL)	1.55±0.08	1.69±0.16	1.62±0.11	1.91±0.09	1
GLUC (mg/dL)	180.10±15.62	181.70±23.35	186.50±29.45	199.80±21.60	5
K (meq/L)	5.71±1.33	4.91±0.34[a]	4.57±0.53[a]	4.76±0.44	5
Na (meq/L)	129.80±25.68	140.60±0.97[a]	140.40±1.35[a]	142.00±2.00	5
Na/K Ratio	23.04±2.99	28.77±1.92[a]	31.08±3.42[a]	~29	5
P (mg/dL)	5.56±0.87	5.51±0.40	5.29±0.45	5.80±1.10	4
T-BIL (mg/dL)	0.09±0.05	0.14±0.02	0.10±0.04	0.30±0.24	2
TP (g/dL)	5.29±0.21	5.43±0.28	5.46±0.20	6.17±0.33	5
Vitamin A (μg/L)	267.31±27.78	270.12±17.75	250.16±31.42	254.10±6.00	5
Vitamin E (μg/L)	3560.00±40.00	8030.00±1220[a]	9180.00±1210[a]	NA	NA
Zn (μg/mL)	1.24±0.18	1.16±0.11	1.14±0.14	1.05±0.09	5

[a] Means with asterisk are significantly different from the control at the 0.05 level

[b] Significantly different between NaB groups at the 0.05 level

[c] Data are reported as mean values ± standard deviation

Hb hemoglobin, *MCH* mean cell hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *MCV* mean corpuscular volume, *PCV* packed cell volume, *RBC* red blood cell, *WBC* white blood cell. *ALB* albumin, *ALP* alkaline phosphatase, *ALT* alanine aminotransferase, *AMYL* amylase, *AST* aspartate aminotransferase, *BUN* blood urea nitrogen, *CHOL* cholesterol, *CK* creatine kinase, *CTR* creatinine, *GGT* gamma-glutamyl transferase, *GLOB* globulin, *GLUC* glucose, *T-BIL* total bilirubin, *TP* total protein. References: (1) Wolford et al. (1986); (2) Lillie et al. (1996); (3) Almodovar-Cuevas et al. (1985; (4) Kohn & Clifford, (2002); (5) Afriyie-Gyawu et al. 2005

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Table 5 Blood and serum parameters of male SD rats receiving dietary Na-bentonite at 0.25 and 2% for 3 months

Parameters	Control	Na-bentonite 0.25%	Na-bentonite 2.0%	Normal values	Reference
Blood					
Hb (g/dL)	14.51±0.46	14.98±0.56[b]	14.22±0.35[b]	14.70±1.22	1
MCH (pg)	18.78±0.45	18.76±0.27	18.75±0.59	19.00±1.09	1
MCHC (g/dL)	33.77±0.71	34.05±0.53	33.58±0.37	34.10±0.40	2
MCV (fl)	55.78±2.25	55.07±1.06	55.84±1.40	59.50±2.00	2
PCV (%)	43.10±1.73	44.00±1.94	42.34±0.80	39.90±3.49	1
Platelets ($x10^3$)/μL	418.60±133.39	543.10±145.15	668.10±122.03[a]	471.68±38.77	3
RBCs ($x10^6$)/μL	7.73±0.32	7.99±0.31[b]	7.58±0.19[b]	7.82±0.83	1
WBC μL	7238.00±2462.55	8235.00±1509.11	10850.00±2912.90[a]	7840.00±2950.00	4
Serum					
A/G Ratio	1.93±0.15	1.80±0.14	1.79±0.13	1.37±0.18	1
ALB (g/dL)	3.42±0.15	3.68±0.15[a]	3.64±0.11[a]	3.40±0.20	5
ALP (U/L)	79.20±9.47	69.20±15.87[b]	91.10±19.15[b]	130±43.7	4
ALT (U/L)	61.30±19.38	56.40±14.45	51.70±9.93	49.00±24.1	2
AMYL (U/L)	2316.00±211.13	2386.40±211.69	2461.68±214.64	2671.00±89.00	1
AST (U/L)	116.80±49.97	105.40±26.47	89.40±23.17	97.00±28.40	2
BUN (mg/dL)	19.41±2.63	22.03±2.46	22.05±1.92[a]	20.00±3.20	2
Ca (mg/dL)	9.12±0.39	9.67±0.19[a]	9.91±0.30[a]	10.48±0.28	5
CHOL (mg/dL)	85.37±10.16	92.38±13.49	89.22±9.12	75.00±19.80	2
CK (U/L)	432.22±227.32	368.30±86.66	399.36±248.12	222.00±109.00	5
Cl (meq/L)	98.30±2.26	100.60±0.97	100.20±1.35	103.00±1.90	4
CRT (mg/dL)	0.26±0.03	0.29±0.02[a]	0.28±0.01	0.54±0.08	5
Fe (μg/dL)	115.89±10.92	109.96±16.69	124.32±23.81	152±70	4
GGT (U/L)	<3.0	<3.0	<3.0	<3.0	1
GLOB (g/dL)	1.78±0.15	2.05±0.18[a]	2.04±0.12[a]	2.51±0.25	1
GLUC (mg/dL)	192.70±37.05	186.60±23.14	204.20±16.90	212.40±28.8	5
K (meq/L)	7.23±0.66	5.30±0.30[a]	5.16±0.13[a]	5.30±0.39	5
Na (meq/L)	135.20±2.74	140.60±0.97[a]	140.50±2.01[a]	142.00±2.00	5
Na/K Ratio	18.86±1.74	26.62±1.51[a]	27.23±0.81[a]	~26	5
P (mg/dL)	5.92±0.39	6.18±0.24	6.24±0.47	7.30±1.50	4
T-BIL (mg/dL)	0.01±0.03	0.11±0.02[a]	0.02±0.04	0.30±0.16	2
TP (g/dL)	5.20±0.25	5.73±0.27[a]	5.68±0.13[a]	5.85±0.23	5
Vitamin A (μg/L)	530.80±39.44	519.74±77.78	441.55±67.45[a]	567.40±24.80	5
Vitamin E (μg/L)	3440.00±50.00	7210.00±870[a]	8570.00±1300[a]	2106.17±1261.98	6
Zn (μg/mL)	1.50±0.09	1.33±0.09[a]	1.23±0.08[a]	1.29±0.04	5

[a] Means are significantly different from the control at the 0.05 level

[b] Significantly different between Na-bentonite groups at the 0.05 level

[c] Data are reported as mean values ± standard deviation

Hb hemoglobin, *MCH* mean cell hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *MCV* mean corpuscular volume, *PCV* packed cell volume, *RBC* red blood cell, *WBC* white blood cell, *ALB* albumin, *ALP* alkaline phosphatase, *ALT* alanine aminotransferase, *AMYL* amylase, *AST* aspartate aminotransferase, *BUN* blood urea nitrogen, *CHOL* cholesterol, *CK* creatine kinase, *CTR* creatinine, *GGT* gamma-glutamyl transferase, *GLOB* globulin, *GLUC* glucose, *T-BIL* total bilirubin, *TP* total protein. References: (1) Wolford et al., (1986); (2) Lillie et al. (1996); (3) Almodovar-Cuevas et al. (1985); (4) Kohn & Clifford, 2002; (5) Afriyie-Gyawu et al. (2005); 6) Seifi et al. (2009)

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Conclusions

Na-bentonite is a material with a large montmorillonite content and with favorable sorption patterns for AF. In general, no adverse health effects resulted from the ingestion of Na-bentonite at 0.25% and 2% inclusion in diet for up to 3 months in SD rats. The consumption of Na-bentonite was associated with increased serum Na and vitamin E, and decreased serum K in males and females, at both inclusion levels. Calcium serum levels were increased in males of both Na-bentonite groups and in females from the high dose group. Reduction of nutrients such as serum vitamin A and Zn were noted in the 2% Na-bentonite group (males-females) and in the males of both 0.25% and 2% inclusion levels, a trend previously observed for other bentonites. Besides the differences observed when comparing Na-bentonite treatments with control groups, no evidence of dose dependency was found, and all parameters fell within normal clinical ranges reported for SD rats. Nonetheless, due to the inherent cation exchange capacity of these types of clays, the current findings with Ca, Na, K, and Zn warrant further research.

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Code Availability Not applicable

Authors' Contributions Alicia G. Marroquin-Cardona: experimental work of animal study, samples collection, writing, submission.

Youjun Deng: supervision of mineral analyses, revision of data and manuscript.

Jose F. Garcia-Mazcorro: statistical analysis.

Natalie M. Johnson: experimental work in animal study, sample collection.

Nicolle J. Mitchell: experimental work in animal study, sample collection.

Lili Tang: vitamin analyses.

Jia-Sheng Wang: vitamin analyses.

Roger B. Harvey: co-advisor, revision of documents.

Timothy D. Phillips: graduate advisor and sponsor of this research.

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Data Availability Not applicable

Declarations

Ethics Approval The animal use protocol (AUP) 2008-39 was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University.

Consent to Participate Not applicable

Consent for Publication All authors give consent for publication.

Conflict of Interest The authors declare that they have no conflict of interest.

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