PAPER

In vitro models to evaluate the capacity of different sequestering agents to adsorb aflatoxins

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Abstract

Eight potential aflatoxin-sequestering agents (SAs) were tested for their ability to adsorb aflatoxin B_1 (AfB_1) and aflatoxin G_1 (AfG_1) *in vitro*. They belong to main SA classes: silicate minerals (calcium, magnesium and sodium bentonites, kaolinite, zeolite and clinoptinolite), activated carbon and yeast cell wall-derived. The AfB_1 and AfG_1 used in present work were extracted from a contaminated corn meal (82.21 mg/kg of AfB_1 and 97.20 mg/kg of AfG_1). Three single-concentration adsorption tests, consisting of a simply-water (W), a gastro-intestinal simulating monogastric model (MM) and a ruminant model (RM) were used. The methods differed for dilution media, incubation steps and pH condition in which they were conducted. In particular, one step (2h at 39°C) at pH 7 for W; two steps (4h at 39°C) at pH 2 and 7 for MM; and a pre-incubation in rumen fluid (pH 7 for 2h at 39°C) + two steps (4h at 39°C) at pH 2 and 7 for RM, characterized each method. The AfB_1:SA ratio (g/g) and dilution factor (ng of incubated AfB_1:mL of volume) were chosen (1:500,000 and 4.1, respectively) to reflect field conditions. The AfB_1 and AfG_1 recovered in controls were 92.3% and 104.9% in W and 89.5% and 101.5% in MM; while in RM were 65.2% and 81.9%; respectively. This supported the idea of intrinsic rumen fluid factors could be involved in sequestering of aflatoxins. In the present study, three SAs (activated carbon, Mg bentonite and Na bentonite) were very efficient to sequester the available AfB_1, with a sequestering activity of over 99.0% with each method. The Ca bentonite and clinoptinolite were able to bind available AfB_1 in MM and RM methods, while they appeared inefficient (available AfB_1 sequestered less than 80%) when W was used. The adsorption ability of zeolite was confirmed only with the W method. Ineffective or limited sequestering activity were obtained with kaolinite and yeast cell wall-derived products with each method. The AfB_1 and AfG_1 sequestering efficiencies observed in the present work resulted very similar showing strong and positive correlation ($P<0.001$) within methods ($r=0.79$, $r=0.96$ and $r=0.99$, respectively for W, MM and RM methods). The two simulated gastrointestinal methods (MM and RM, respectively) gave similar results and could be considered useful for *in vitro* pre-screening of potential sequestering agents. However, the major practical and analytical implications related to rumen fluid method suggested that MM method should be used.

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Introduction

Aflatoxins are hepatocarcinogenic molecules (IARC, 2002) produced mainly by *Aspergillus flavus* and *A. parasiticus* either in field, storage or transport of many crops (Scheidegger and Payne, 2003). The primary aflatoxins produced by these fungi are aflatoxin B_1 (AfB_1), aflatoxin B_2 (AfB_2), aflatoxin G_1 (AfG_1), and aflatoxin G_2 (AfG_2).

The AfB_1, the most toxic and carcinogenic aflatoxin (Roebuck and Maxuitenko, 1994), once ingested by dairy animals is rapidly absorbed in the gastro-intestinal tract, and rapidly appears in blood (Gallo *et al.*, 2008) and in milk as aflatoxin M_1 (AfM_1), the principal AfB_1 milk metabolite (Veldman *et al*., 1992; Battaccone *et al*., 2003; Masoero *et al*., 2007). The AfB_1 carry over (CO) rate into milk as AfM_1 has been determined to range from 1% to 3% in lactating dairy cows and to be principally affected by milk yield (Diaz *et al*., 2004; Van Eijkeren *et al.*, 2006; Masoero *et al.*, 2007), with a reported maximum value of about 6% (Veldman *et al*., 1992).

These findings supported the European Union (EU) decision to fix limits for AfB_1 in animal feeds at 20 μg/kg and in complete feedingstuffs for dairy animals at 5 μg/kg (European Commission, 2003). In milk, the EU set the AfM_1 maximum permitted level at 0.050 μg/kg (European Commission, 2006).

A practical approach to control aflatoxicosis in livestock is the addition of aflatoxin-sequestering agents (SA) to diet (CAST, 2003). These compounds are effective to sequester toxin molecules, such as aflatoxins, forming a complex in the gastrointestinal tract (Jouany, 2007; Masoero *et al*., 2009) through electronic elementary charges, hydrogen bonds, Van der Waals bonds and physical capture (Phillips *et al*., 1990; Diaz and Smith, 2005; Yiannikouris *et al*., 2005; Phillips *et al.,* 2008). Besides, they are added to animal diet in small amount and are impurities-, flavour- and odours-free (Diaz and Smith, 2005; Jouany, 2007).

A variety of SAs has been tested and currently traded. Some of the most commonly used SAs belong to three different groups: silicate materials or clay minerals (Phillips *et al.*, 1991; Ramos and Hernandez, 1996; Rao and Chopra, 2001; Diaz *et al.*, 2003; Jouany, 2007), yeast cell wall-based products (Karaman *et al*., 2005; Yiannikouris *et al.*, 2005) and activated carbons (Galvano *et al.*, 1996; Rao and Chopra, 2001; Diaz *et al.*, 2003; Diaz and Smith, 2005).

Several *in vitro* adsorption tests have been proposed to pre-screen SAs before their use *in vivo*. Differences in the findings of *in vitro* studies are based upon the tested aflatoxins (crystalline pure AfB_1 or extracted from natural contaminated feeds), the dilution factor (AfB_1 concentration, w/v), the AfB_1:SA ratio (w/w), the pH conditions and the media in which adsorption test was conducted (Ramos and Hermandez, 1996; Grant and Phillips, 1998; Ledoux and Rottinghaus, 1999). Moreover, these methods often oversimplify the gastrointestinal conditions, such as pH, temperature and interaction with biological fluids (Lemke *et al*., 2001; Avantaggio *et al*., 2003; Moschini *et al*., 2008).

The objective of this work was to evaluate the efficacy of different typologies of SAs (mineral clays, activated carbon and yeast cell wall-based product) to adsorb AfB_1 and AfG_1 either in aqueous or in simulated monogastric/ruminant gastrointestinal fluids.

Materials and methods

Eight potential-aflatoxin SAs were evaluated for their capability to sequester aflatoxins in vitro. The SAs were: magnesium bentonite (Grupo Tolsa, Madrid, Spain); calcium bentonite (Tecnozoo, Padova, Italy); sodium bentonite (Amcol International Corp., Arlington Heights, IL, USA); kaolinite (Fluka 03584, Sigma-Aldrich Chemie Gmbh, Switzerland); zeolite (Fluka 96096, Sigma-Aldrich Chemie Gmbh, Switzerland); clinoptinolite (Tecnozoo, Padova, Italy); yeast cell wall-based product (Mycosorb®, Alltech Italy, Bologna, Italy), and activated carbon (Acque Nymco, Milan, Italy). Only some of these products (i.e., magnesium bentonite, calcium bentonite, clinoptinolite and yeast cell wall-based product) are specifically marketed as mycotoxin SA by the producers.

Chemicals

Solvents used were of grade ACS-ISO. Acetonitrile, acetone, chloroform and methanol were HPLC grade (purity 99.5%, 99.0%, 99.0% and 99.9%; respectively) from Merck (Darmstadt, Germany). Purified water was obtained from a Milli-Q Gradient A10 water purification device (Millipore, Bedford, MA, USA). Phosphate-buffered saline (PBS) was prepared by dissolving 0.20 g KCl, 0.20 g KH_2PO_4, 1.16 g anhydrous Na_2HPO_4 and 8.00 g NaCl in 1 L of water. The pH was adjusted to 7.4 with NaOH (0.1 mol/L).

Preparation of aflatoxin test solution (solution A)

Aflatoxins (AfB_1 and AfG_1) were extracted from a contaminated corn meal inoculated with the Aspergillus flavus strain MPVP 2092 (Institute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore, Piacenza, Italy). The corn meal was incubated at 25°C and 0.99 water activity for 7 days, respecting the optimum mycotoxin production conditions (Giorni *et al.*, 2007). The final aflatoxin concentrations of the corn meal were: 82.21±0.01 mg/kg for AfB_1 and 97.20±0.01 mg/kg for AfG_1.

A stock solution (solution A) of AfB_1 and AfG_1 was prepared by extracting aflatoxins from 1 g of the contaminated corn meal using a water/methanol solution (80:20, v/v) in ratio of 1:100 (g/mL), at room temperature and in agitation (150 shake/min) for 120 min. The obtained solution A concentrations were 0.821 µg/mL for AfB_1 and 0.974 µg/mL for AfG_1, respectively.

Single concentration adsorption tests

A simply-water (W), a gastro-intestinal simulating monogastric model (MM) and a ruminant model (RM) were used to test the sequestering capacity of the different SAs. The W and MM were performed in agreement with Lemke *et al.* (2001), while the RM consisted of a pre-incubation with rumen fluid (Moschini *et al.*, 2008), followed by incubation steps applied for MM model.

The three methods differed for the dilution media, incubation steps and the pH conditions in which the adsorption tests were conducted:

W method: one step in deionized water at 39°C for 2h;
MM method: two steps at 39°C for 4h in two buffers (pH 2 and 7) to simulate gastrointestinal conditions of monogastri;
RM method: one step in rumen fluid (pH 7 for 2h at 39°C) followed by the two MM steps (pH 2 and 7 for 4h at 39°C), to simulate the ruminant digestible tract.

The pH 2 buffer used in the first step of MM method was prepared in agreement with Lemke *et al*. (2001) as follows: 1.250 g/L of pepsin enzyme, 0.500 g/L of citric acid monohydrate, 0.500 g/L of malic acid disodium salt, 0.050 mL/L of acetic acid and 0.042 mL/L of lactic acid. In the second step, the pH was increased up to 7 with sodium bicarbonate, followed by addition of pancreatine (20 mg/sample) and bile salts (70 mg/sample).

The RM method consisted of a pre-incubation in rumen fluid. The rumen fluid was collected from two fistulated cows housed at the CERZOO research and experimental centre (San Bonico, Piacenza, Italy) before the morning meal (08.00), filtered with a two layers cheesecloth, stored at 39°C under $C0_2$ and used within 1 h. The measured pH of the rumen fluid was 7.0±0.3. After this step, HCl 1M was used to reach pH 2 in test tubes and 50 mg/sample of pepsin was added to each sample. The third step of RM method was carried out by increasing pH up to 7 with sodium bicarbonate followed by the addition of pancreatine (20 mg/sample) and bile salts (70 mg/sample).

The AfB_1:SA ratio used in the current study were designed to be about 1:500,000 (w/w), in agreement with AfB_1:SA ratio used in our previous work (Moschini *et al.*, 2008). This ratio was chosen to reflect possible field conditions. In particular, dairy cows fed with an AfB_1 contaminated diet near to the EU limit of 5 µg/kg (EU, 2003) consumed, on average, 100-120 µg/head/day AfB_1 in presence of a SA dose of about 50-60 g/head/day, as suggested by the producers for on farm utilization. A similar approach could be used for swine fed with a diet containing 1% of a SA with an AfB_1 contamination of 20 µg/kg, representing the swine EU feedstuff limit (EU, 2003). The dilution factor, considered as the ratio between the amount of incubated AfB_1 (ng) and the total volume (mL) in which the absorption test was conducted, was similar for the three methods and equal to 4.1 (ng/mL), in agreement with Moschini *et al*. (2008).

Incubation procedure

Single SAs were weighted (82.0±0.1 mg) in 100-mL test tubes (Pyrex®; Barloworld Scientific Ltd., Staffordshire, UK) for each method (W, MM and RM). Then, 0.200 mL of solution A and 40 mL of the three media, precisely deionized water (W), pH 2 buffer (MM) or rumen fluid (RM), were added to each test tube and gently shacked for 5 minutes every 15 minutes. Each single-concentration adsorption test was repeated twice.

Control samples (n=3) for each method (W, MM and RM, respectively) were prepared by adding 0.200 mL of solution A and 40 mL of specific media into 100-mL test tubes without the addition of the SA.

At the end of incubations for all samples, the supernatant was removed after centrifugation (3500 x g for 15 min at 4°C) and then the precipitate washed twice with 10 mL of water. The recovered supernatant (about 30 mL) as a whole was analyzed by HPLC. In particular for RM samples, to measure AfB_1 and AfG_1 sequestered by rumen fluid, the precipitate of the controls were treated in agreement with Moschini *et al.* (2008) and analyzed for aflatoxin contents: the supernatant was removed and the precipitate was re-suspended in 10 mL chloroform before centrifugation. This step was repeated twice, and the recovered solution was analyzed by HPLC to measure the AfB_1 and AfG_1 contained in the rumen pellet of controls.

The sequestered aflatoxins (AfB_1 and AfG_1) by the SAs in each sample were calculated as percentage ratio between the aflatoxin quantity of supernatant and its amount in the control sample.

Animals

The ruminal fluid cow donors were fed on a diet formulated in agreement with dairy cow requirements (NRC, 2001) and based on grass hay (700 g/kg), corn silage (200 g/kg) and concentrate (100 g/kg) on a dry matter basis. The research protocols and animal care were performed in accordance with the EC council

Table 1. Elementary composition and total ash content (mean ± SD) of the tested adsorbents.

Adsorbents	Elements (%)									Total ash
	C	O	Al	Si	Na	Mg	K	Ca	Fe	(%)
Activated carbon	89.2±0.82	11.1±0.67	nd	nd	nd	nd	nd	0.2±0.03	nd	2.7±0.2
Magnesium bentonite	3.5±0.67	51.7±0.56	1.7±0.12	21.9±0.46	1.3±0.02	14.8±0.21	0.4±0.02	0.5±0.04	0.8±0.11	94.3±0.8
Sodium bentonite	3.2±0.62	52.7±0.23	11.6±0.12	29.5±0.43	3.2±0.02	1.5±0.06	0.2±0.01	0.6±0.03	2.3±0.08	90.9±1.2
Calcium bentonite	9.6±0.23	51.6±0.32	6.1±0.11	16.5±0.48	0.9±0.9	1.3±0.06	1.5±0.12	8.3±0.16	3.7±0.19	93.3±0.6
Clinoptinolite	2.2±0.20	51.5±0.13	6.7±0.32	30.1±0.22	1.4±0.06	2.1±0.12	0.9±0.14	1.6±0.02	1.8±0.04	99.8±0.2
Kaolinite	0.9±0.24	56.0±0.87	17.2±0.15	22.0±0.33	nd	0.3±0.01	1.3±0.11	0.1±0.04	0.4±0.06	90.1±1.1
Yeast cell wall-based	63.4±1.41	33.9±0.15	nd	nd	0.1±0.01	0.2±0.01	0.9±0.11	1.3±0.76	nd	8.1±0.6
Zeolite	2.6±0.17	57.0±0.28	13.8±0.55	14.7±0.89	11.7±0.28	0.2±0.01	0.3±0.02	0.1±0.03	0.2±0.02	99.8±0.2

nd: not detectable (under the LOD).

directive guidelines regarding the use of animals for experimental and other scientific purposes (EEC, 1986).

Aflatoxin and SAs analysis and apparatus

Aflatoxins (AfB_1 and AfG_1) were analyzed in agreement with Moschini *et al.* (2008). Briefly, 10 grams of dried contaminated corn meal were extracted with 100 mL of an acetone:water solution (85:15, v/v), shacked at 150 rpm for 45 min (Universal table Shaker 709) and filtered with Schleicher&Schuell 595.5 filter paper (Dassel, Germany). Elutions of 5 mL with 45 mL of bi-distilled water were performed and passed through an immuno-affinity column (Aflatoxin Easi-extract, Rhône diagnostics technologies, Glasgow, UK). The supernatant solution was passed through an immunoaffinity column previously washed with 20 mL of PBS. The column was than washed with 5 mL water and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, re-dissolved in 1 mL of an acetonitrile:water (25:75, v/v) solution and filtered (Millipore Corporation, Bedford, MA, USA, HV 0.45 µM) prior to HPLC analysis for the AfB_1 and AfG_1 contents.

Analysis was performed using an HPLC instrument consisting of a LC-200 pump (Perkin Elmer, Norwalk, CT, USA) an AS-2055 sampling system, a FP-1520 fluorescence detector (Jasco Corporation, Tokyo, Japan), and a UV derivatizer (UVE TM derivatizer, LC tech, Dorfen, Germany); the instrument was controlled by Borwin 1.5 software (Jasco). A Superspher RP-18 column (4 µm particle size, 125x4 mm i.d., Merck) was used at room temperature with a mobile phase of water: methanol:acetonitrile (64:23:13, v/v/v) at 1 mL/min. The AfB_1 and AfG_1 were detected after post-column photochemical derivatization to AfB_{2a} and AfG_{2a}, respectively. The detector was set at 365 nm excitation and 440 nm emission wavelengths.

The elemental components of the SAs were determined by a semi-quantitative X-ray fluorescence analysis using the scanning electron microscope Phillips XL 30 E-SEM (Phillips electron Optics B.V., Eindhoven, Netherlands) equipped with an energy-dispersive X-ray detector model Genesis (Edax Inc., Mc Kee Drive, Mahwah NY, USA) operating in low vacuum. The SA samples were assayed in duplicate according to the AOAC (1990) to determine total ash content (procedure 942.05).

Statistical analysis

The *in vitro* data were analyzed using a complete randomized design and the general linear model of the SAS® (SAS Institute Inc., Cary, NC; version 9.1.3). A factorial arrangement was used and fixed effects of model included SA (n=8), method (n=3) and associated first order interaction. When data suggested the first order interaction, the SLICE option of LSMEANS statement was used to compare SAs within each tested method. The correlation between AfB_1 and AfG_1 sequestering efficiency was calculated with the Spearman rank correlation coefficient using PROC CORR of SAS (SAS Institute Inc., 2001) within each method. Significance was declared at $P<0.05$.

Results and discussion

The most used SAs belong to the silicate mineral, the activated carbon and the yeast cell-wall product (Diaz and Smith, 2005; Jouany, 2007) classes. The widest SA class is the silicate minerals, which could be grouped in phyllosilicate and tectosilicate sub-classes. The SAs belonging to phyllosilicate sub-class are montmorillonite/smectite, such as bentonite and kaolinite clays, while the tectosillicate sub-class includes zeolite and clinoptinolite clays (Diaz and Smith, 2005).

In the present work, the tested clays had an ash content higher than 90% (Table 1). In particular for bentonite clays, the dominant cation characterized the type of bentonite, as confirmed by elementary chemical composition of magnesium bentonite (Mg 14.8%), calcium bentonite (Ca 14.8%) and sodium bentonite (Na 3.4%). Zeolite and kaolinite clays appeared different with respect to other clays having a similar Al and Si content (14.1% and 14.6% for zeolite and 18.6% and 21.0% for kaolinite, respectively). The high carbon content of activated carbon (89.6%) and yeast cell wall (61.4%) suggested an organic origin, even if the available analytical data can not exclude a possible clay presence, particularly for the yeast cell wall product.

Single concentration adsorption tests

Before their use in animal diets, *in vitro* pre-screening tests of potential SAs are necessary to evaluate the sequestering efficiency of these materials in controlled experimental conditions. If a SA sequesters less than 80% of the available *in vitro* aflatoxins, it could be considered as ineffective and not tested *in vivo* (Ledoux and Rottinghaus, 1999; Diaz and Smith, 2005).

Several lab methods have been proposed, but they differed for experimental conditions (Phillips *et al.*, 1988; Galvano *et al.*, 1996; Ramos and Hermandez, 1996; Grant and

Table 2. Aflatoxin B_1 (AfB_1) and aflatoxin G_1 (AfG_1) recovered in supernatants, in rumen pellet and as total in water, simulating gastro-intestinal monogastric model and ruminant total tract model methods in control samples.

	In vitro models				
	Water (W)	Gastro-intestinal (MM)	Ruminant total tract (RM)		
			Supernatant	Rumen pellet	Total
AfB_1	92.3 (3.25)	89.5 (1.70)	36.6 (0.18)	28.6 (0.46)	65.2 (0.40)
AfG_1	104.9 (4.06)	101.5 (4.89)	55.6 (0.37)	26.3 (0.18)	81.9 (0.54)

Standard error is in brackets.

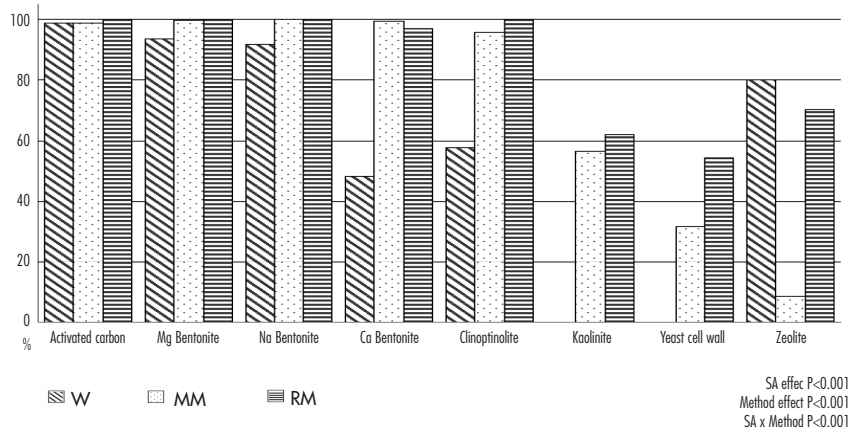

Figure 1. Data represent the sequestered AfB_1 from each adsorbent (SA) in the three methods: water (W), gastro-intestinal (MM) and ruminant model (RM), respectively (P of the model <0.001; SEM= 5.771).

Phillips, 1998; Ledoux and Rottinghaus, 1999; Lemke *et al*., 2001; Diaz *et al*., 2003; Spotti *et al.*, 2005; Vekiru *et al*., 2007; Moschini *et al*., 2008; Thieu and Petterson, 2008). These differences among methods could cause contrasting results for the same SA, when tested with different adsorption tests: Moschini *et al.* (2008) reported the effect of AfB_1:SA ratio (i.e., 1:5000; 1:50,000 and 1:500,000) on the sequestering performance of SAs. Also Vekiru *et al*. (2007) showed that adsorption abilities of a commercial HSCAS and a bentonite were influenced by the constituents and the pH of the incubation media (i.e. acetate buffer *vs* artificial or real gastro intestinal media). Similar discrepancies among methods were obtained by Lemke *et al.* (2001) testing a clinoptinolite in two single-concentration adsorption studies conducted in water media or in a gastro-intestinal model media. These authors emphasized the importance of using methods simulating the animals' physiological conditions. More recently, Spotti *et al.* (2005) and Moschini *et al*. (2008) tested the SA efficiency using an *in vitro* method conducted in rumen fluid media, demonstrating a partial sequestering activity of rumen fluid against AfB_1.

In the present work, the average AfB_1 and AfG_1 recovered in control samples in absence of any type of sequestering agents were 92.3% and 104.9% in W method (Table 2), in accordance to our previous results (Moschini *et al*., 2008). Similar results were obtained in MM method (89.5% and 101.5%, respectively for AfB_1 and AfG_1), in agreement with Lemke *et al.* (2001). The acid condition (pH 2) in which the first step of MM method was conducted did not influence the amount of AfB_1 recovered, showing the absence of the reaction of AfB_1 to AfB_{2a} (Vekiru *et al.*, 2007). This could be related to the limited incubation time. The AfB_1 presence observed in control samples of W and MM methods determinate an AfB_1:SA ratio of about 1:500,000, as designed before experiments.

In RM method, the aflatoxin determine in the supernatant of the control after the centrifugation of the rumen fluid media were equal to 36.6% for the AfB_1 and 55.6% for AfG_1. The residual AfB_1 extracted with chloroform from the precipitate was 28.6% and similar to AfG_1 rumen pellet content, in agreement with previous published data (Spotti *et al.*, 2005; Moschini *et al.*, 2008). The total AfB_1 and AfG_1 recovered in control samples of RM method were 65.2% and 81.9%, respectively. Therefore, the AfB_1:SA ratio for RM method was different with respect to other methods, being about 1:750,000. This supports the idea that some rumen fluid components such as chlorophyllin, bacteria and yeast cell wall could be effective in sequestering aflatoxins (Breinholt *et al*., 1999; Peltonen *et al*., 2000; Diaz and Smith, 2005; Gratz *et al*., 2005; Oatley *et al.*, 2005). In particular, Arimoto-Kobayashi *et al*. (1997) suggested that chlorophyllin structure could form a complex between porphyrin-like structure of chlorophyllin and the polycyclic structures of carcinogenic molecules, like AfB_1. Also specific cell wall components in yeast (Dawson *et al.*, 2001) and bacteria, i.e. lactobacilli, interacted with mycotoxins forming a stable complex (Gratz *et al*., 2005; Oatley *et al*., 2005; Niderkom *et al*., 2009).

Upadhaya *et al*. (2009) recently reported a loss of AfB_1 ranging from 8 to 20% when *in vitro* incubated at different times (0, 3, 6, 9 and 12 h) with rumen fluids collected by goats and steers. These authors supposed that AfB_1 was degraded by rumen microflora. Niderkorn *et al*. (2007), testing more than 200 bacterial strains to verify their ability to bind and/or biotransform mycotoxins (i.e., deoxyvalenol, zearalenon and fumonisin B_1 and B_2), reported that most strains were capable to bind *Fusarium* toxins. In particular, these authors indicated *Streptococcus* spp. and *Enterococcus* spp. as the most effective genera, while only few strains (i.e., 8 *Lactobacilli* spp. and 3 *Leiconostoc* spp.) could be involved in the biotrasformation of zearalenone in α-zearalenol. However, these bacteria did not biotransform other studied mycotoxins as deoxynivalenol and fumonisins B_1 and B_2.

SA sequestering activity

Our results showed that three potential SAs (i.e., activated carbon, Mg bentonite and Na bentonite) exhibited strong AfB_1-sequestering capacity, with values higher than 99.0% (Figure 1), in all experimental condition and with no differences among methods (Table 3).

Table 3. Analysis of variance (ANOVA) of sequestering agents, method and first order interaction effects on the AfB_1 and AfG_1 sequestering efficiency data.

Source	Degrees of freedom	AfB_1 (P value)	AfG_1 (P value)
Sequestering agents (SA)	7	<0.001	<0.001
Method (M)	2	<0.001	<0.001
SA x M interaction	14	<0.001	<0.001
Mean square error	24	63.98	56.60
SA x M interaction sliced by SA			
Activated carbon	2	0.996	0.996
Mg bentonite	2	0.515	0.703
Na bentonite	2	0.683	0.657
Ca bentonite	2	<0.001	<0.001
Clinoptinolite	2	0.004	0.004
Kaolinite	2	<0.001	<0.001
Yeast cell wall-derived	2	<0.001	<0.001
Zeolite	2	<0.001	<0.001

The high affinity of the activated carbon for AfB_1 molecula confirmed the results obtained by other authors (Galvano *et al*., 1996; Lemke *et al*., 2001; Rao and Chopra, 2001; Vekiru *et al*., 2007). The mechanism involved in the sequestering of aflatoxin could be the physical capture by the pore of activated carbon structure, even if it could depend by other factors, such as surface area, structure and dose of mycotoxins (Galvano *et al.*, 2001). However, the use of activated carbons as chemoprotective against aflatoxins produced variable results *in vivo*. Galvano *et al.* (1996) reported that only one of the two tested activated carbons at 2.0% inclusion level in the animal diet resulted effective to reduce AfM_1 excretion in lactating dairy cows, while Diaz *et al*. (2004) did not observe a significant reduction of the AfM_1 milk concentration when an activated carbon was added to the diet at 0.25% inclusion level.

Two bentonites (Mg and Na bentonites) were highly efficient to adsorb the available AfB_1 *in vitro*. These results were in agreement with the ones by different authors (Dvorak, 1989; Ramos and Hermandez, 1996; Rao and Chopra, 2001; Diaz *et al*., 2004; Moschini *et al.*, 2008), suggesting bentonite's ability to adsorb AfB_1 in several media, such as water, buffer solution, saline solution, swine stomach and bovine rumen fluids. More recently, Veriku *et al*. (2007), testing 61 different bentonite materials, concluded that AfB_1 was generally strongly bound by these clays, both in a pH 5 acetate buffer or in a pH 7 phosphate buffer. These results are consistent with data reported by Diaz *et al.* (2003), comparing different SAs (i.e., Na and Ca bentonite, esterified glucomannan and activated carbons) at pH 3, 7 and 10. Moreover, Moschini *et al.* (2008) reported that the AfB_1-bentonite complex was very stable in the gastro-intestinal tract of lactating dairy cows, with an estimated release of the sequestered AfB_1 lower than 5%.

Masoero *et al.* (2009) testing the Mg bentonite *in vivo* on lactating dairy cows, measured a significant reduction in the AfM_1 milk concentration (44%) and in the AfB_1 carry over into milk (47%), when the SA was added at a level of 100 g/cow/day to an AfB_1-contaminated diet causing an ingestion of 174 μg of AfB_1/cow/diet.

In the present study, more than 97% of the available AfB_1 was adsorbed by the Ca bentonite using MM and RM methods, while an inefficient sequestering activity was observed using the W method (i.e., 48%). Similar differences among methods were observed with clinoptinolite clay, that could be considered effective to bind AfB_1 if tested with MM and RM methods (96% and 100% sequestering activities, respectively), while it appeared inefficient in W media. In agreement with our observation, Lemke *et al*. (2001) reported that under MM conditions, a clinoptinolite showed a sequestering activity not observed in other methods. These authors concluded that the MM model was the most physiologically relevant method to pre-screen potential SAs.

Ineffective or limited sequestering activity was observed for kaolinite and yeast cell wall products in all methods. In particular, these products were not able to sequester AfB_1 when tested in W media, while they showed poor sequestering activities both in MM and in RM (57% and 62% for kaolinite and 32% and 54% for yeast cell wall, respectively in MM and RM method) (Figure 1).

The results obtained for yeast cell wall-derived products confirmed our previous data, in which a low *in vitro* efficiency was measured in all experimental conditions (Moschini *et al.*, 2008). In particular, the pH and the solvent type appeared to be critical in the reduction of the ability of β-d-glucans, a major component of the inner layer of the yeast cell wall, to complex mycotoxins (Yiannikouris *et al*., 2005). A previous report by Dawson *et al*. (2001) suggested that a pH of 4.0 was optimal for yeast cell wall product activity.

Differences among methods showed that the two gastro-intestinal model tests (MM and RM methods) gave higher (P<0.05) sequestering affinity than W method when Ca bentonite, clinoptinolite, kaolinite and yeast cell wall products were tested. This was not true for zeolite, which resulted efficient at binding AfB_1 in W, sequestering about the 80% of the available AfB_1, while it appeared inefficient using the MM method. Also Thieu *et al*. (2008), found that a zeolite product had a different capacity to adsorb AfB_1 using a single concentration gastro intestinal method at pH 3 or pH 7 (80% and 20% sequestering activities, respectively).

Contrasting *in vivo* data were obtained adding zeolite to animal diet (Shariatmadari, 2008). Modirsanei *et al*. (2004) reported that zeolinite at 0.50% and 0.75% inclusion in the broiler chick diet did not reduce any adverse effects due to AfB_1 ingestion (1.0 mg/kg of diet). On the contrary, Miazzo *et al*. (2005) suggested that zeolinite is able to counteract same AfB_1-depended toxic effects in growing broiler chicks ingesting 2.5 mg of AfB_1/kg of diet. Similarly, Abdel-Wahhab *et al*. (2002) reported improved haematological and bio-

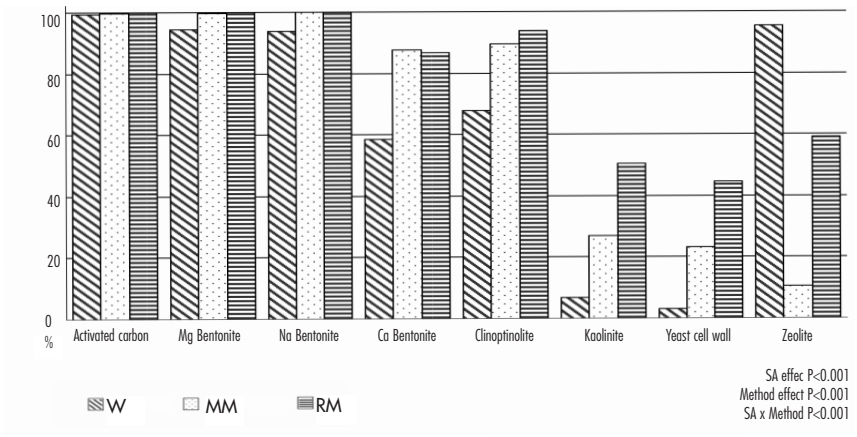

Figure 2. Data represent the sequestered AfG_1 from each adsorbent (SA) in the three methods: water (W), gastro-intestinal (MM) and ruminant model (RM), respectively (P of the model <0.001; SEM= 5.320).

chemical parameters in rats fed with a contaminated AfB_1 (2.5 mg/kg) diet containing sorbent compound derived from a natural zeolite.

The AfB_1 and AfG_1 sequestering efficiencies observed in this experiment with the three methods resulted very similar (Figure 2). This was confirmed by statistical analysis of the data: strong and positive correlations ($P<0.001$) were measured between AfB_1 and AfG_1 sequestering activity in MM and RM methods ($r=0.96$ and $r=0.99$, respectively). Less strong correlation was found for the W method ($r=0.79$; $P<0.001$).

Despite some small differences between the chemical structure of the aflatoxin molecules (AfB_1 and AfG_1 belong to cyclopentenone and lactone series, respectively), these data seem to confirm suggestions of Phillips *et al*. (2008) concerning the mechanism of aflatoxin absorption by clays: the process should be not site-specific because it involves the β-dicarbonyl system of AfB_1 and AfG_1 molecules, thought the chelation of metal ions at the surface and within the interlayer of silicate clays. The carbons of the β-dicarbonyl system have a partial positive charge and they could be attracted through an electron donor acceptor mechanism by the negatively charges of platelets of silicates. Phillips *et al*. (2006) reported a good correlation between the magnitude of partial positive charges on carbons C_{11} and C_1 of the β-dicarbonyl system and the strength of AF-SA complex.

Conclusions

Based on the results of the current paper, obtained with the in *vitro* pre-screening tests, three SAs products (activated carbon, Mg bentonite and Na bentonite) could be considered efficient at sequestering the available AfB_1, resulting as promising agents for *in vivo* trials. The Ca bentonite and clinoptinolite products were able to adsorb available AfB_1 in MM and RM model methods, while they appeared inefficient when the adsorbent test was performed in water media. The adsorption ability of zeolite was confirmed only by the W method.

The simulated gastrointestinal methods presented in the current paper (MM and RM, respectively) gave similar results and could be considered useful for i*n vitro* pre-screening of potential sequestering agents. However, the major analytical complexity of the rumen fluid method supports the idea that the 2-steps MM method proposed by Lemke *et al.* (2001) should be used.

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