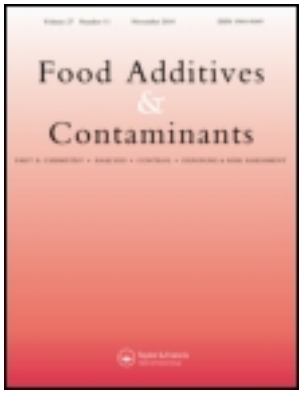

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Yeast cell based feed additives: studies on aflatoxin B_1 and zearalenone

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Yeast cell based feed additives: studies on aflatoxin B_1 and zearalenone

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Thirty commercially available yeast cell wall products and two reference bentonites were tested for their ability to bind aflatoxin B_1 (AFB1) and zearalenone (ZON) in buffer solutions at pH 3 and pH 6.5 as well as in real gastric juice. For most products, the binding efficacy of AFB1 correlated with the ash content, which was between 2.6 and 89%, and constituted the inorganic non-volatile components, such as mineral clays, of the samples. Samples with smectite as the main ash component showed the highest binding efficacy; yet, a correlation with the content of mannanooligosaccharides (MOS) and β-glucans from yeast cell walls was not observed. Products containing >30% ash showed AFB1 adsorption values >90% at least in one of the investigated media whereas most products with <10% ash did not exceed adsorption rates of 20%. In the case of ZON, adsorption efficiency ranged between 10 and 60%. It tended to be lowest for products with MOS and β-glucan contents <10% and greatest for products with MOS and β-glucan contents >50%. However, there was no general correlation between the adsorption of ZON and the concentration of MOS and β-glucans. Different products of one brand sold in different countries were observed to bind AFB1 to different degrees, which was explained by the difference in ash contents and mineral composition. In the case of ZON, differences in adsorption between products of the same brand were less pronounced.

Keywords: animal feed; aflatoxins; mycotoxins; mycotoxins, zearalenone; chromatography, HPLC, LC/MS

Introduction

Mycotoxins are an inherent problem in feed production and animal nutrition. In warm and damp countries, aflatoxins are among the main representatives. Aflatoxin B_1 (AFB1) is a potent carcinogen and can be metabolised *in vivo* to AFM1 which, in turn, can be found in milk and other animal products (Prandini et al. 2009). Zearalenone (ZON) is a frequently occurring mycoestrogen produced by several *Fusarium* species. Due to its structural similarity to estrogens, such as 17-β-estradiol, ZON can cause reproductive problems in mammals (Bennett et al. 2003).

Primary strategies to reduce the risk of mycotoxin contamination include good agricultural practices in the field (crop rotation, soil cultivation, weed and insect control, careful use of fungicides) and upon harvest, as well as transportation and storage under dry and cool conditions (Jouany 2007). However, complete avoidance of mycotoxins is not possible. There are several approaches to cope with the problem: lower the mycotoxin concentration in contaminated crops by physical or chemical treatment (Jouany 2007), reduce the animals' mycotoxin uptake by addition of feed additives working on the basis of biological decontamination through microbes or enzymes (Fuchs et al. 2002; Molnar et al. 2004), or the adsorption of mycotoxins to binding agents in the animals' digestive system. Binders should be non-toxic, have a high adsorption capacity (so that their inclusion level into feed may be low) and bind mycotoxins selectively and irreversibly at different pH values and in complex systems, such as the digestive tract. Several different types of binders have been tested: bentonites, for example, NovaSil plus (NSP, formerly called hydrated sodium calcium aluminosilicate, HSCAS, but structurally a calcium montmorillonite clay); zeolites; organoclays; charcoal; diatomaceous earth; and yeast cell wall-derived products (YCP). Unlike charcoal, NSP and other bentonites have been found to selectively bind aflatoxins through chemisorption (Phillips 1995; Grant et al. 1998; Vekiru et al. 2007). However, the adsorptive performance (adsorption capacity, selectivity, etc.) of the binders can be very different even if they belong to the same mineralogical group. Limitations of clays are that they accumulate in

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manure, may be contaminated with toxic metals and dioxins which requires rigorous testing before use and that they bind other mycotoxins than aflatoxins only to a limited degree (Yiannikouris et al. 2004a).

Glucans can be found in a wide variety of cereals, plant algae, bacteria, fungi and yeast sources (such as *Saccharomyces cerevisiae*). Yeast glucans are major cell wall components often present as the inner wall layer and associated with other cell wall components such as chitin (together they represent 50–60% of the wall dry weight). Strongly glycosylated mannoproteins form the outer cell wall layer. Glucan-associated activity is affected by the glucan-characterising parameters of primary structure, solubility, degree of branching, molecular weight, as well as polymer charge and/or solution conformation (e.g. triple or single helix) (Zekovic et al. 2005). Mannanoligosaccharides (MOS) are also constituents of yeast cell walls. They are widely used in animal nutrition to enhance growth performance and gastrointestinal health. However, they have not been reported to bind mycotoxins.

Some manufacturers of yeast cell wall derived products claim that preparations containing yeast cell wall glucans, besides being nutritional aids and growth promoters, bind a variety of mycotoxins *in vitro* and *in vivo*, especially ZON, without changing the nutritional value (regarding mineral and vitamin profile) of the feed. The mechanism of binding by β-D-glucans has been investigated and has been discussed in several publications (Yiannikouris et al. 2004a,b,c, 2006). It was shown that it is important to differentiate between the adsorption affinity of whole yeast cell wall and processed cell wall. A further factor influencing binding ability is the presence of chitin which stiffens the cell wall structure and restricts access of, for example, ZON to the binding sites of β-D-glucans (Yiannikouris et al. 2004c). Therefore, the preparation of cell wall material prior to its application as a mycotoxin binder is of major importance and greatly influences the adsorption ability of the final product. Hence, to describe the precise properties of β-D-glucan preparations, origin, molecular structure and purity are important as they are known to influence the activity of β-D-glucan preparations. It would be very useful and provide transparency for the end-user if cell wall derived products were accompanied by a certificate of analysis reporting parameters such as moisture, glucan, MOS and ash content as well as other parameters such as protein and fat content.

Low percentages of clays (1–6%) are commonly added to feed as technological anti-caking feed additives. Greater percentages indicate targeted addition of clays for mycotoxin removal by binding. Possible ways to examine clay addition in products based on yeast cell wall ingredients are powder X-ray diffraction analysis or determination of the ash content of the product, which indicates the non-volatile inorganic matter therein.

The aim of this work was to test a great number of yeast cell wall based products for their ability to bind AFB1 and ZON, and to correlate the respective adsorption rates with the glucomannan and ash content of the samples. In addition, variations in binding efficiency within products of the same brand, but marketed in different countries and stemming from different lots, were assessed.

Materials and methods

Reagents and solutions

Solid standards of AFB1 and ZON were provided by Romer Labs Diagnostic GmbH (Tulln, Austria). Acetonitrile (ACN, HPLC-gradient grade) was purchased from VWR (Vienna, Austria) and trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (Vienna, Austria). Reagents for preparation of buffer solutions were purchased from Merck (Darmstadt, Germany) and Riedel-deHaën (Seelze, Germany). Milli-Q water, prepared using an ultra-pure water system (Millipore, Molsheim, France), was used in all experiments.

Stock solutions of AFB1 (85 mg/l) and ZON (100 mg/l) were prepared in ACN. The preparation of buffer solutions (citrate buffer pH 3.0 and phosphate buffer pH 6.5) has been described earlier (Vekiru et al. 2007). Real gastric juice (from swine) was cleaned from suspended particles by centrifugation and filtration.

Tested products

Thirty products containing different percentages of yeast cell wall constituents, mineral components and other ingredients were purchased worldwide. In addition, two mineral binders (products 1 and 2) were used as reference materials and for quality control purposes during our experimental work. Activated carbon and cholestyramine (Sigma-Aldrich and Fluka, Vienna, Austria) were chosen as positive control for binding of ZON. Product details, including manufacturers' information and our experimentally determined data (ash content and percentage of MOS and β-glucans) are listed in Table 1, headed by the two reference bentonites and then sorted by decreasing ash content. In the case of four products (Mycosorb, Microbond, Integral, MTB-100), different batches – in part sold in different countries – were analysed to investigate product variations in ash, MOS and β-glucan content as well as in binding efficacy of AFB1 and ZON.

Adsorption tests at different pH values and in real gastric juice

Adsorption tests were carried out at pH 3 (citrate buffer), pH 6.5 (phosphate buffer) and in real gastric

Table 1. Product data.

Product No.	Product name	Provider; marketed in	Intended use	Manufacturer's information	Mineral composition	Ash	β-Glucan	MOS	β-Glucan +MOS
1	Bentonite GB7	Directly from a mine in Greece; not available for the end user	Anti-caking and adsorption of aflatoxins	Untreated bentonite	Smectite**, calcite 3%	87.5	0.0	0.0	0.0
2	NovaSil plus	Product of Trouw Nutrition Int., USA, manufactured by Engelhard, USA; USA	Anti-caking and adsorption of aflatoxins	HSCAS	Smectite**, quartz*, calcite 2%	85.4	0.0	0.0	0.0
3	Select Biocycle	Product of Select Sires, USA, manufactured by Agrarian Marketing Corporation, USA; Canada	Positive effects on gut microflora and rumen microorganism	Distillers dried grains with solubles, $CaCO_3$, *Sacc. cer.* active dry yeast, potassium iodide, dried egg, lactic acid, calcium lactate, malic acid, acetic acid, tartaric acid, fumaric acid and sodium benzoate	Quartz*, mica*, calcite 1%	89.0	5.0	1.4	6.5
4	Captex FUSA	Dox-al; Bulgaria	Against fusarium toxins	4.2–4.6% moisture, 608 mg/kg clinoptilolite, 116 mg/kg Resindox™, 207 mg/kg esterified glucomannans (*Sacc. cer.*) and 185 mg/kg $CaCO_3$	Zeolite*, feldspars*, calcite 1%, dolomite 60%	89.0	5.2	3.4	8.6
5	Finale	Marsyt, USA; USA	MT removal by adsorption	Montmorillonite clay, diatomaceous earth, *Sacc. cer.*, magnesium silicate, calcium propionate	Quartz**, mica*, smectite*, calcite 3%	88.9	2.8	1.4	4.2
6	MT.X Plus®	Olmix, France; UK	MT removal by adsorption	Amadéite, montmorillonite, diatomaceous earth, yeast cell walls	Quartz*, mica*, calcite 13%, dolomite 2%	75.0	5.9	4.3	10.2
7	Mastersorb Gold®	Grasp, Brasil; Brasil	MT (fumonisins, ZON, T2, DON, AFB1) removal by adsorption, protection against pathogens	Mineral, phycolytic and phytogenic components, details unknown	quartz*, smectite*, kaolinite*, mica*, calcite 2%	65.2	10.0	5.9	15.8
8	Tonilys	Nordos, France[1]; France	MT removal (AFB1, ZON, DON) by adsorption, stimulation of liver function, immune stimulation due to MOS and vitamin E, antioxidative effect	Mineral clay (sodium bentonite), dried yeast extract, antioxidants (BHT)	Smectite**, quartz*, Gibbsite*, calcite 3%	59.1	8.5	6.1	14.6

(*continued*)

Table 1. Continued.

Product No.	Product name	Provider; marketed in	Intended use	Manufacturer's information	Mineral composition	Ash	β-Glucan	MOS	β-Glucan +MOS
9	Mycosorb_Japan_1	Alltech, USA; Japan	MT removal by adsorption	*Sacc. cer.* extract >60%, HSCAS, $CaCO_3$	Smectite**, quartz*, feldspars*, calcite 3%, dolomite 2%	29.7	19.7	13.0	32.7
10	Mycosorb_Japan_2	Alltech, USA; Japan	See product No. 9	See product No. 9	Smectite**, quartz*, calcite 3%, dolomite 1%	28.1	25.9	12.3	38.1
11	Integral_Canada_1	Alltech, USA; Canada	MT removal by adsorption	Yeast by-product	Calcite 17%, dolomite 7%	28.1	22.5	11.4	33.9
12	Mycosorb_Japan_3	Alltech, USA; Japan	See product No. 9	See product No. 9	Smectite**, quartz*, calcite 2%, dolomite 1%	27.1	27.9	11.2	39.0
13	Microbond_USA	Cenzone, USA; USA	Immunostimulation, protection against pathogens, improvement of digestion and nutrient adsorption, diminishing of adverse effects of MTs	β-Glucans, MOS, digestive enzymes	Smectite**, quartz*, sodium sulfate*, aluminium sulfate hydrate*, calcite ~0.5%	26.7	22.8	9.6	32.4
14	Microbond_Southafrica	Cenzone, USA; South Africa	See product No. 13	See product No. 13	Smectite**, quartz*, sodium sulfate*, aluminium sulfate hydrate*, calcite ~0.5%	26.2	21.1	9.0	30.1
15	Mycosorb_Japan_4	Alltech, USA; Japan	See product No. 9	See product No. 9	Smectite**, quartz*, calcite 2%, dolomite ~0.5%	26.0	26.9	11.9	38.8
16	Microbond_Japan	Cenzone, USA; Japan	See product No. 13	See product No. 13	Smectite**, quartz*, sodium sulfate*, aluminium sulfate hydrate*, calcite ~0.5%	24.9	26.4	11.6	38.0
17	Integral_Canada_2	Alltech, USA; Canada	See product No. 11	See product No. 11	Calcite 15%, dolomite 7%	23.8	24.3	11.1	35.4
18	Mycosorb_Australia	Alltech, USA; Australia	See product No. 9	See product No. 9	Calcite 14%, dolomite 6%	23.6	22.5	11.8	34.3
19	Nutricell® Polysorb	Biorigin, Brasil; Brasil	MT (AFB1, ZON, T2-toxin) removal by adsorption (produced from YCW by hydrolysis, extraction and further treatment)	Max. 8% moisture, 17–25% ash, >45% carbohydrates (β-glucans + MOS) 20 ± 5 proteins, 5-12% fat	Smectite**, quartz*, feldspars*, cristobalite*	22.4	22.3	25.3	47.6
20	Mycosorb_China_1	Alltech, USA; China	See product No. 9	See product No. 9	Calcite 13%, dolomite 6%	20.2	30.5	19.0	49.5
21	MTB-100_Vietnam	Alltech, USA; Vietnam	US Patent No 6045834, MT removal by adsorption	Molasses distillers dried solubles, maize condensad distillers solubles, HSCAS, $CaCO_3$ min. crude protein 22%, max. crude fat 0.5%, max. crude fiber 11%, max. moisture 15%	Calcite 12%, dolomite 5%	19.2	27.9	14.9	42.8
22	DETOXA PLUS 168	Alinat, Argentine; China	Biological degradation of ZON, FB1, OTA, T2 by *Sacc. telluris* strains and YCW for adsorption of aflatoxins	Min. crude protein 32%, max. fiber 1%, max. moisture 8%, max. ash 18%, min. β-glucans 14%	Quartz*, zeolite*, K-feldspar*	16.4	25.0	13.8	38.7

23	Safmannan	Saf Agri (Lesaffre), Mexico; Brasil	Improvement of GI and immune system (obtained by autolysis of *Sacc. cer.* yeast)	Moisture 2–3%, proteins 14–17%, β-glucans 24–26%, MOS 22–24%, fat 20–22%, ash 3–5%	Calcite ~0.5%	9.8	25.0	21.3	46.3
24	Mycosorb_China_2	Alltech, USA; China	See product No. 9	See product No. 9	Quartz*, zeolite*, calcite 2%	8.1	27.1	17.0	44.0
25	Mycosorb_Cyprus	Alltech, USA; Cyprus	See product No. 9	See product No. 9	Not determined	7.8	25.8	14.9	40.7
26	Mycosorb_Irland	Alltech, USA; Ireland	See product No. 9	See product No. 9	Quartz*, zeolite*, calcite 1%	6.8	26.1	18.9	45.0
27	Mycosorb_Brasil	Alltech, USA; Brasil	see product No. 9	See product No. 9	Not determined	6.7	28.6	20.0	48.6
28	ActiveMOS	Biorigin, Brasil; Brasil	Protection against pathogens, positive effects on gut microflora (prebiotic rich in MOS, produced from cell wall of *Sacc. Cer.*)	Max. protein 30%, max. moisture 8%, MOS 25 ± 3%, β-glucans 30 ± 3%, ash 6%	Not determined	6[2]	37.6	19.9	57.5
29	Betamune	Biorigin, Brasil; Brasil	Modulation of cellular and humoral immune response, positive effects on animal growth; action based on cell wall of *Sacc. cer.*	Min. β-glucan 70%, max. moisture 10%, ash 5.7%	Not determined	5.7[2]	67.0	0.8	67.8
30	MTB-100_Denmark	Alltech, USA; Denmark	See product No. 21	See product No. 21	Calcite 4%	5.6	36.9	18.6	55.5
31	Nutricell MOS 55	Biorigin, Brasil; Brasil	Protection against pathogens, benefits for the GI system	Details unknown	Not determined	4.4	70.0	2.0	72.0
32	Biolex® MB40	Leiber GmbH, Germany; Brasil	Removal of MTs and pathogenic bacteria by adsorption, stimulation of immune system and prebiotic properties (contains extracted *Sacc. cer.*)	Crude protein 25%, crude fat 7.5%, β-glucan 35–30%, MOS 20–25%, 4% ash	Undefined mineral traces	3.8	26.1	27.5	53.5

Notes: *Sacc. cer.*: *Saccharomyces cerevisiae*; MT: mycotoxin; YCW: yeast cell wall; BHT: butylated hydroxytoluene.
[1]Daughter company of Trouw Nutrition.
[2]Manufacturer's information.
**Main component (>25% of mineral content).
*Minor component (between ~1% and 25% of mineral content).
Percentage of carbonates refers to the total composition of the product.

juice from swine (pH 5): 10 mg of adsorbent were weighed accurately (±0.3 mg) into 15-ml polypropylene test tubes (Sarstedt, Nuembrecht, Germany), 5 ml of citrate buffer, phosphate buffer or gastric juice, each containing 0.2 mg/l of AFB1 or 0.5 mg/l of ZON, were added corresponding to 0.2% (w/v) of binder and the tubes were vortexed and incubated at 37°C for 1 h under shaking in a vertical position in a rack on a microtiter plate shaker (1000/min). For each medium, 5 ml of a reference solution containing toxin but no binder were treated like the samples. Immediately after incubation, the vials were centrifuged (3700 rpm, 15 min) and aliquots of the buffer solutions were transferred into autosampler vials. In the case of gastric juice, 0.5 ml of supernatant was mixed with 0.5 ml of ACN, centrifuged to precipitate proteins and an aliquot of the clear solution was used for HPLC analysis. As AFB1 can transform into AFB2a in acidic solutions (Vekiru et al. 2007), chromatograms were checked for AFB2a, which was found to elute earlier in previous experiments. However, formation of AFB2a was not detected in any of the investigated media. Each adsorption test was carried out in triplicate for each medium, product and reference solution. Each replicate was analysed by HPLC once. The two reference bentonites were worked-up and analysed in every experimental run for quality control purposes.

The percentage of adsorption was calculated by dividing the difference of the concentrations in the reference solution without binder (maximum concentration) and in the test solution after adsorption by the concentration in the reference solution and multiplying by 100.

Chemisorption tests

Adsorption tests were carried out with selected products separately for AFB1 (0.2 mg/l) and ZON (0.5 mg/l) at a binder concentration of 0.5% (w/v) and at pH 6.5 (phosphate buffer), as described above. After shaking for 1 h at 37°C and centrifugation, the supernatant was removed for HPLC analysis and the pellet was re-suspended by vortexing in 1 ml of buffer. The re-suspended pellet was washed twice with 1 ml of the buffer used for the preceding adsorption experiment and subsequently extracted three times with 1 ml each of methanol by shaking at 37°C (600/min) for 0.5 h. Supernatants of each washing and extraction cycle were collected and analysed separately.

The chemisorption index (Cα) was calculated by dividing the difference of toxin adsorbed during the adsorption assay and of toxin desorbed in the course of the desorption steps by the total amount of toxin used for the adsorption assay. The percentage of desorption was determined by dividing the amount of toxin desorbed by the amount of toxin originally adsorbed and multiplication by 100.

Adsorption rate at increasing amounts of AFB1

A constant quantity (0.2% w/v) of selected products (which showed high AFB1 binding efficiency in the previous test) was mixed with increasing amounts of AFB1 at 37°C and the amount of toxin bound was evaluated. Adsorption tests were performed in gastric juice as described above using 0.2, 2.0, 4.0 and 8.0 mg/l AFB1 with the exception that the samples were incubated under shaking for 24 h (instead of 1 h).

HPLC analysis

The HPLC system used consisted of an Agilent G1311A quaternary pump, a G1322A vacuum degasser, a G1313A autosampler, a G1316A column compartment and a G1315A UV-diode array detector. AFB1 was analysed on a Zorbax SB Aq column (150 × 4.6 mm, 5 µm particle size) equipped with a pre-column at 35°C at a flow-rate of 0.7 ml/min using 50% aqueous ACN adjusted to pH 2.9 with TFA as mobile phase. The injection volume was 100 µl and the total run time was 7 min. UV-absorbance was monitored at 365 nm. Calibration was performed in the range 10–4500 µg/l

For HPLC analysis of ZON, 25 µl of sample or standard solutions were injected and separated isocratically on an Eclipse XDB-C8 column (150 × 4.6 mm, 5 µm) equipped with a pre-column at 25°C and at a flow-rate of 0.8 ml/min. The mobile phase consisted of 54% aqueous methanol containing 1% of acetic acid (v/v) and the UV absorbance was recorded at 275 nm. The total runtime was 8 min. Calibration was carried out in the range 10–1000 µg/l.

Ash content

The ash content was determined based on the AOAC Official Method 942.05. In short, 5 g of sample were dried overnight at 105°C and subsequently subjected to a temperature gradient with 550°C as maximum and 105°C as final temperature prior to cooling in a dessicator.

Mineral composition

The mineral composition of the products was determined by powder X-ray diffraction (Moore et al. 1997). In addition, the content of carbonates was determined by Scheibler method (ÖNORM L 1084) and simultaneous thermal analysis (STA, Smykatz-Kloss 1974).

Content of MOS and β-glucans

The total content of MOS and glucans was determined as described at http://www.eurasyp.org/public.technique.home.screen with minor modifications. The method involves pre-solubilisation of the yeast cell wall (YCW) with concentrated sulphuric acid, subsequent acid hydrolysis, neutralisation and determination of the released glucose and mannose (which are formed in proportional quantities from the glucans and mannans) by ion chromatography.

Results and discussion

Products

The ash, MOS and β-glucan content and the mineral composition determined in this work are given in Table 1. Regarding the ash and MOS plus β-glucan content of the tested products, three groups are distinguished: pure yeast or yeast cell wall products without inorganic binders added, having an ash content below 10% and generally highest percentages of MOS and glucans (41–72%); mixtures of yeast or yeast cell wall products (30–50%) and inorganic binding agents, containing between 10 and 30% ash; and products with ash contents >30% composed mainly of inorganic binder with smaller percentages of yeast (0–16%).

Ten of the investigated products contained between 3.8 and 9.8% ash. Low percentages of ash are common in yeast cell-based products, because some mineral components are necessarily added during manufacturing for anti-caking purposes. Five of the products containing <10% ash were intended for welfare of animals by offering benefits to the GI system and by stimulating the immune system, whereas the four Mycosorb products claimed to have mycotoxin-binding properties. Mycotoxin-binding is also the main aim of the majority of products containing 10–30% ash and 30–50% MOS and glucans. Three further products of this group were intended for well-being of animals by protecting against pathogens and by reducing the adverse effects of mycotoxins. One further product claimed to detoxify various mycotoxins by biological degradation. Finally, seven of the eight products (five of the six yeast cell wall based products) containing more than 30% ash and less than 16% of MOS and glucans were aimed at mycotoxin removal by binding and only one of these products was intended mainly to help maintain a healthy GI system.

Adsorption tests at different pH values and in real gastric juice at pH 5

Homogenous products with small particle size can easily be tested at small scale (5 ml) for their binding efficacy by indirect pipetting technique, which includes preparation of a binder slurry and addition of slurry-aliquots to test-tubes containing a certain concentration of the toxin. Using this technique, binder concentrations as low as 0.002% can be added reproducibly, so that differentiation of high capacity binders (which all bind 100% of AFB1 at 0.2% of binder concentration) becomes possible. Yeast cell wall preparations, however, are not homogenous and product particles are rather coarse, so that indirect pipetting technique is not applicable. To minimise the volume of toxin-spiked solution required for the test, we used 0.2% of the product (10 mg/5 ml) for performing the adsorption tests. This resulted in 100% binding of AFB1 by the reference bentonites, but allowed differentiation of the other products.

AFB1

The results indicated a trend that products with high ash content showed high binding efficiency of AFB1 (Figure 1A). For instance, all eight products containing >30% ash showed adsorption values >90% at least at pH 6.5. Likewise, eight of the 10 products containing <10% ash did not exceed adsorption values of 25% under any of the tested conditions (pH 3, pH 6.5, gastric juice). Of the 14 products containing between 10 and 30% ash, five reached adsorption efficiencies of 100%, three further products showed at least 50% adsorption in buffer solutions, five had adsorption rates between 30 and 50% and one was a very poor AFB1 binder, binding <15%. Hence, not only the amount, but also the type of mineral constituting the ash is a key factor in adsorption of AFB1. Therefore, the mineral composition of the individual products was determined by powder X-ray diffraction. Smectite, reported as the active ingredient in additives used as aflatoxin binders (Kannewischer et al. 2006; Marroquin-Cardona et al. 2009), was the main ingredient in 11 and a minor component in two further products containing >20% ash. With the exception of the three products of the Microbond line (13, 14, 16), all of these products showed >90% AFB1 adsorption in buffer and >70% adsorption in gastric juice. Products 3 and 4, in which smectite was not detected, showed <50% adsorption in gastric juice. One further indication that the binding capacity of yeast cell wall derived products depends strongly on the amount and type of mineral component added is the poor binding efficacy for AFB1 of several products (e.g. products 28–32) exhibiting high MOS and β-glucan content. This observation is in accordance with the finding by Baptista et al. (2004) that manno-oligosaccharides and thermolysed yeast are not able to inactivate aflatoxins *in vivo*.

Of the three media tested, highest adsorption values were obtained in phosphate buffer at pH 6.5, in most cases followed by citrate buffer at pH 3.

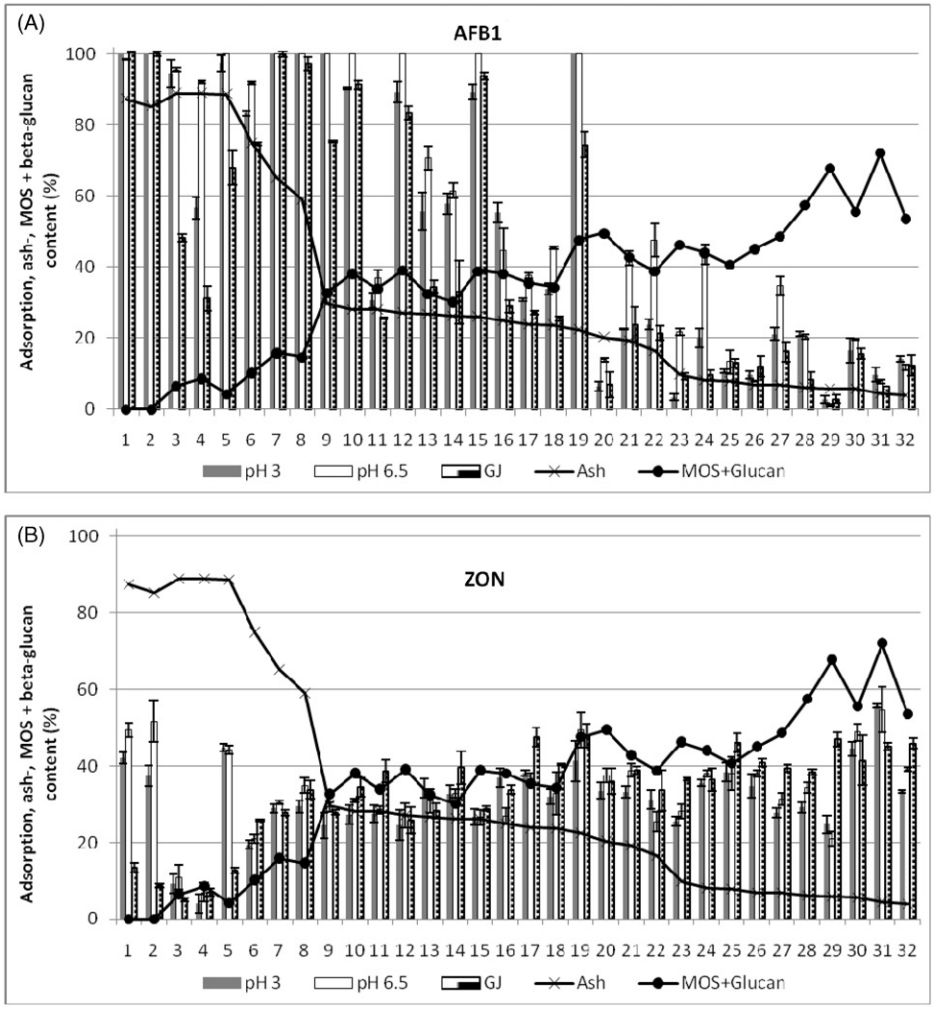

Figure 1. Adsorption efficiency, ash, beta-glucan and MOS content of 32 products for (A) AFB1 and (B) ZON at pH 3, 6.5 and in gastric juice.

Adsorption rates in gastric juice were lowest, possibly due to adsorption of gastric juice components to the binder, reducing the binder's capacity for AFB1 (see later for further explanations).

ZON

In the case of ZON, most products showed adsorption rates between 20 and 40% (Figure 1B). Two products (No. 3 and 4) were very poor binders, binding less than 15% under all of the tested conditions (pH 3, pH 6.5, gastric juice). Only 10 products showed adsorption capabilities greater than 40% in at least one of the investigated media. Interestingly, the two reference bentonites, NSP and GB7, and one further product with high ash content (No. 5, 88.9% ash) were among the best adsorbents for ZON at pH 3 and 6.5, whereas two further products containing about 90% ash bound less than 10%. This result indicates that the type and composition of mineral binder greatly affects the product's efficiency for adsorption of ZON in buffer solutions, whereas the ash content itself does not seem to be a factor.

Contrary to the results in buffer solutions, adsorption in gastric juice was low (<15%) for all products with ash contents of about 90% and MOS and β-glucan contents below 10%. This suggests that these types of product are hardly qualified for an *in vivo* application. The other seven products showing adsorption capacities greater than 40% in at least one of the investigated media also contained considerable amounts of MOS or β-glucans, which seemed to improve ZON adsorption rates especially in real gastric juice. Moreover, several products rich in β-glucans showed greater adsorption in gastric juice than in buffer solutions.

In our study, all products containing >10% MOS and β-glucans showed ≥20% adsorption in all tested media. However, although the products with greatest MOS and β-glucan content were among those with

greatest adsorption, adsorption rates did not generally increase with greater MOS and β-glucan content. The type of polysaccharide (MOS or β-glucan) was not a determinant factor, either.

Activated carbon and cholestyramine, tested as positive control samples for ZON adsorption, showed adsorption rates of 100 and 74.5% at pH 3, of 100 and 87.6% at pH 6.5, and of 100 and 78.2% in gastric juice.

Influence of the adsorption medium

Although the adsorption rate of AFB1 was different to that of ZON for each individual product, the ratio of adsorption of ZON to adsorption of AFB1 was similar at pH 3 and at pH 6 for most products. However, the ratio of adsorption of ZON to adsorption of AFB1 was greater in gastric juice than in buffer solutions for 14 products of all ash and MOS plus β-glucan contents. On the other hand, the ZON to AFB1 adsorption ratio was significantly smaller in gastric juice than in buffer solutions for the three products with high ash content which showed great adsorption of ZON in buffer solutions, but not in gastric juice (products 1, 2 and 5). One explanation might be that gastric juice, although subjected to filtration and centrifugation prior to use, still contains components which interfere with adsorption. For instance, adsorption of ZON to gastric juice particles occurred up to 31% in the absence of binder. Hence, adsorption rates in gastric juice were corrected by the adsorption of AFB1 and ZON, respectively, in "blank" solutions of the respective toxin prepared without binder in gastric juice. Still, adsorption rates in gastric juice were increased compared to adsorption rates in buffer solutions for 14 products (see above).

However, gastric juice components can also adsorb to the binder and thereby reduce its capacity for binding the target mycotoxins as observed for products 1, 2 and 5 in the case of ZON. In the case of AFB1, the capacity of the reference bentonites NSP and GB7 is great enough so that this effect was not observed.

Translation of this finding into *in vivo* behaviour is tricky. Adsorption to gastric juice particles constitutes only a temporary mycotoxin binding as gastric juice particles are digested later in the intestinal system. Hence, the released mycotoxins should then be able to bind to mineral or MOS and β-glucan components or to other indigestible diet constituents in the intestinal tract before absorption. However, due to the complexity of the involved matrices differentiation of the different adsorption processes in the GI tract is hardly possible.

Natural variation in composition and binding efficacy between different products of one brand

Frequently, products of the same brand and manufacturer, but at least in part produced in different countries are manufactured with mineral components (e.g. anti-caking agents) from local mines to avoid raw material transportation. Hence, products of the same brand but sold in different countries can differ in the type, content and composition of clay which may result in different mycotoxin adsorption efficacy. In this work, the natural variation of composition and binding efficacy of products of the same brand but of different lots and/or sold in different countries was investigated by analysing 10 products of Mycosorb sold in six different countries; three products of Microbond, each sold in a different country; two products of Integral sold in the same country; and two products of MTB-100, each sold in two different countries.

AFB1

The greatest variations were observed within the Mycosorb products (Figure 2). The four products sold in Japan (JP 1-4, products 9, 10, 12, 15) contained $27.7 \pm 1.6\%$ ash with smectite as the main component, whereas the average ash content of four other products sold in four different countries (CN 2, CY, IE, BR, No. 24, 25, 26, 27) was $7.3 \pm 0.7\%$. Two further products (AU, CN 1, No. 18, 20) were in between, containing $21.9 \pm 2.4\%$ ash, with calcite as the main inorganic component. Likewise, the adsorption of AFB1 was significantly higher in the Japanese products where it amounted to $86.0 \pm 5.2\%$ in gastric juice, $92.3 \pm 8.3\%$ at pH 3 and $100 \pm 0.0\%$ at pH 6.5. In the other six investigated products (of low and medium ash content, no smectite) the adsorption of AFB1 was below 30% at pH 6.5 and even below 20% at pH 3 and in gastric juice. Similarly, the two products of MTB-100 sold in Vietnam and Denmark (products 21, 30) differed widely in their ash contents (5.6 and 19.2%) and in their adsorption efficacy for AFB1 (differences by a factor of 1.4–2.2 in the individual media, see Figure 1A), even though both contained calcite as the main component. In contrast to Mycosorb and MTB-100, products of Microbond sold in the USA, South Africa and Japan (products 13, 14, 16) had similar ash contents ($25.9 \pm 0.9\%$), similar mineral composition and showed similar adsorption under different conditions ($56.3 \pm 1.4\%$ at pH 3.0, $58.9 \pm 13.2\%$ at pH 6.5, $32.1 \pm 2.9\%$ in gastric juice). The same is true for the two Integral products sold in Canada (products 11, 17, relative standard deviations (RSDs) of ash content 11.7%, RSDs of adsorption under different conditions $<4.5\%$).

ZON

The MOS and β-glucan contents in the 10 different products of Mycosorb varied between 32.7 and 48.6% with an average value of $41.1 \pm 5.4\%$ (Figure 2B). The

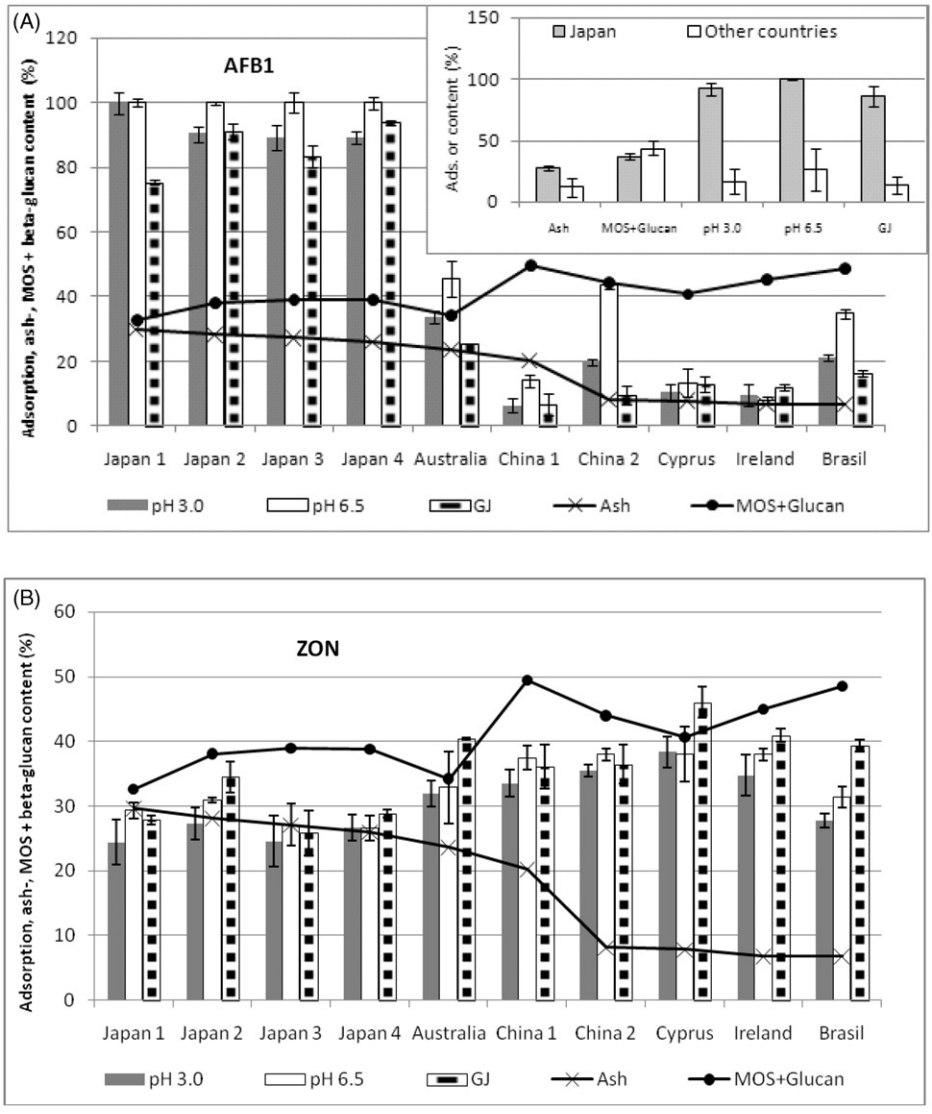

Figure 2. Variability in 10 different batches of Mycosorb regarding ash content, MOS and β-glucan content and adsorption in different media for (A) AFB1 and (B) ZON. Error bars denote the analytical standard deviation.

differences in adsorption of ZON between the different products were also much lower compared to the differences in adsorption of AFB1. Adsorption rates for ZON versus AFB1 were, respectively, between 24.1 and 38.4% at pH 3, 26.6 and 38.0% at pH 6.5 and between 25.9 and 46.0% in gastric juice. The two MTB products (21, 30) differed both in MOS and β-glucan content (by a factor of 1.3) and in the adsorption of ZON in different media (by a factor of 1.07, 1.25 and 1.34 in gastric juice, at pH 6.5 and at pH 3, respectively). The two Integral products from Canada (11, 17) had similar MOS and β-glucan contents, but different adsorption values (between factor 1.23 in gastric juice and factor 1.39 at pH 3). On the other hand, the three Microbond products (13, 14, 16) from different countries had a relative standard deviation (RSD) of MOS and β-glucan contents of 12.2% and RSDs of adsorption in different media between 5.9 (pH 3) and 16.6% (gastric juice).

Chemisorption tests

In Figure 3, the chemisorption indices (Cα) of selected products are plotted together with the percentages of adsorption and desorption determined in the chemisorption experiments. In the case of AFB1, there was a strong correlation between the ash content and Cα. This is in part explained by the definition of Cα, which reflects both the initial capacity of adsorption and the strength of the interactions between mycotoxin and binder. Hence, products with low adsorption under the test conditions cannot reach a Cα of 1, even if no desorption takes place. In our experiments, Cα was

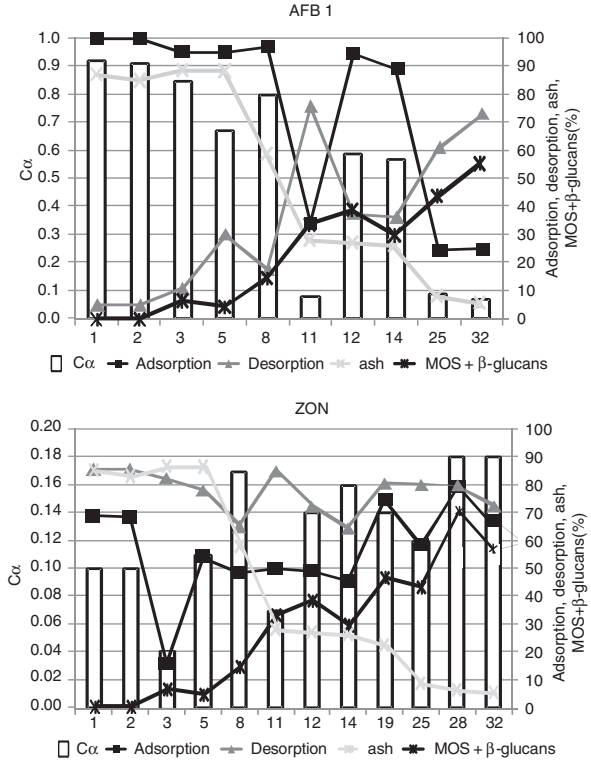

Figure 3. Cα and percentages of adsorption and desorption of selected products for AFB1 and ZON.

greatest for the mineral binders and lowest for products with low ash and high MOS and β-glucan content. In addition, product 11, despite having an ash content of 28%, showed adsorption of only 33% and thus a desorption of 75%, which is in contrast to other products of medium ash content. In general, products with low adsorption had greatest desorption values and vice versa.

In the case of ZON, the increased ratio of product used for this test (0.5% w/v) resulted in significantly greater adsorption rates than the standard test using 0.2% w/v (see above). Yet, none of the tested products exceeded a chemisorption index of 0.2. This is explained by high desorption of all products (65–85% of the adsorbed amount) rather than by low adsorption values as adsorption under test conditions with 0.5% binder was between 45 and 80% for all products but one (product 3). Although products with greatest MOS and β-glucan content had greatest chemisorption indices, there was no direct correlation between Cα and the MOS plus β-glucan content. The ash content was not a factor either. The low chemisorption indices and the high desorption values of all products indicate that ZON is adsorbed by physisorption, i.e. weak interactions with the adsorbent material and that no strong chemical bonds are formed.

Adsorption rate at increasing amount of AFB1

In this test, the adsorption rate at increasing amount of the toxin was evaluated to compare the performance of different products which yielded similar results in the screening adsorption tests. As binder saturation during the screening test was not obtained for any of the products in the case of ZON, this test was only performed for AFB1.

On the one hand, establishment of adsorption isotherms for calculation of affinity and maximum binding capacity requires work in the range of binder saturation. On the other hand, different products should be compared under the same experimental conditions. Hence, the same percentage of binder as used in adsorption tests was chosen (0.2% w/v), which resulted in an almost vertical line in the isotherm plot for the reference bentonite (No. 1) up to an initial concentration of 4 mg/l AFB1 (before addition of binder) and in significantly flatter lines for the yeast cell based products No. 7 and 10 (Figure 4). Therefore, although calculation of affinity and maximum binding capacity was not possible from the obtained data, different products with similar binding efficiency in adsorption tests could well be differentiated by means of their adsorption rates at increasing amount of AFB1.

Points to consider when comparing experimental data with other published articles

Table 2 summarises results of published *in vitro* studies for the commercially available products investigated in this study. Unfortunately, for most of the products it was not possible to obtain independent experimental results (provided by someone other than the manufacturer of the product) regarding *in vitro* binding action. It was even more difficult to find results which refer to the product as it is sold on the market and not to formulations used for research work, which do not exactly represent the content of the commercially available product. Formulations used for research work (e.g. model compounds) are usually used to investigate the individual differences of various product ingredients in their mode of action and to determine factors that influence their efficacy (e.g. models of β-glucans as used by Yiannikouris et al. (2004d) or purified β-glucan fractions of yeast cell walls isolated from different strains of *S. cerevisiae* (Yiannikouris et al. 2004c).

Further inconveniences are different experimental conditions used by the individual research groups (percentage of binder, concentration of the toxin, type and pH of the medium, incubation parameters), which renders comparison of the presented data difficult. One also has to distinguish between adsorption isotherms allowing the calculation of the maximum binding

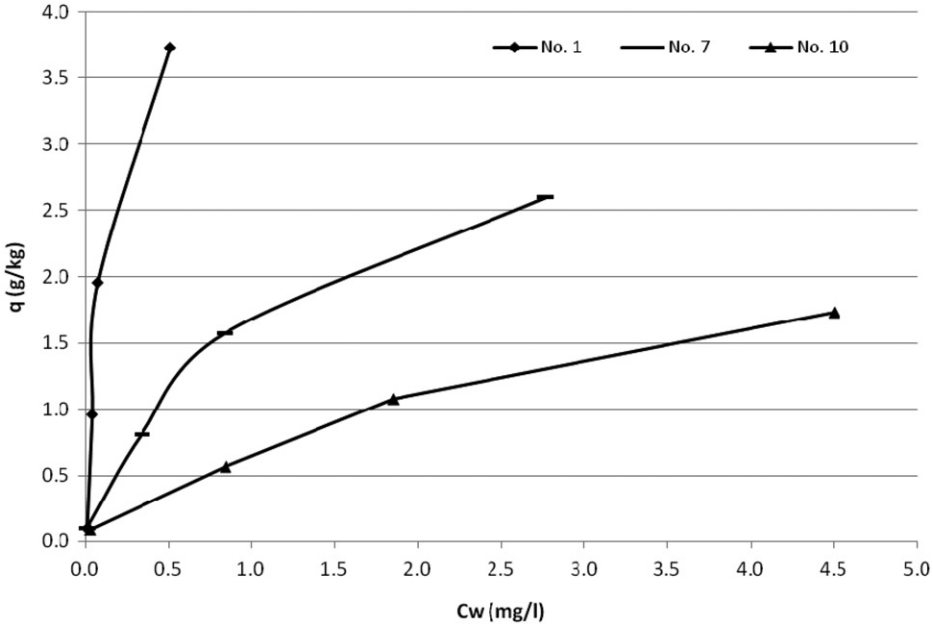

Figure 4. Adsorption rate at increasing concentrations of AFB1 (products No. 1, 7 and 10). Cw: concentration of AFB1 left in the test solution supernatant after adsorption (mg/l); q: amount of AFB1 (g) adsorbed to 1 kg of product.

capacity and single adsorption studies giving just the percentage of adsorption and the amount of mycotoxin bound/kg of binder under the chosen conditions.

For instance, the amount of AFB1 bound/kg of the reference binders GB7 and NSP (products 1 and 2) was 0.32 mmol in the present work (use of 10 mg of product, 5 ml of solution containing 0.2 mg/l AFB1). The same value was obtained for several other products investigated (No. 5, 7, 8, 9, 10, 12, 15, 19) in at least one of the tested media. The difference in the adsorption capacity of the products became only visible when tests at increasing amount of AFB1 were performed (see above and Figure 4). Comparison of our data obtained in single adsorption studies for the reference binders with literature values for Mycosorb and MTB-100 tested in single adsorption studies (Table 2) points out the impact of the experimental conditions on the amount of AFB1 bound/kg of binder. Use of 10 mg of binder (0.2%) and 5 ml of 0.2 mg/l AFB1 solution leads to 0.32 mmol AFB1 bound/kg of binder for 100% adsorption whereas use of 0.1% of binder in a solution containing 0.5 mg/l AFB1 gives 1 mmol AFB1 bound/kg of binder for an adsorption value of just 62%. Hence, comparison of values obtained in single adsorption studies does not necessarily give a ranking of the best binders.

Determination of maximum adsorption capacities, which are a more useful tool for comparisons, demonstrates the superiority of mineral binders compared to yeast cell products in the adsorption of AFB1 (maximum adsorption capacities between 9 and 18 mmol AFB1/kg binder for YCP and between 260 and 420 mmol/kg binder for mineral binders; Table 2). This efficiency of YCP may be too low for effective binding under more complex matrix conditions (as in the intestinal tract of animals).

In the case of ZON, the best performing Mycosorb products in our investigations were able to bind 0.31 mmol ZON/kg yeast cell based product. This is in the lower range of the already reported data for single adsorption studies (see Table 2B). Again, serious comparison with literature values was hampered by different experimental conditions. Isothermal analysis has only been reported for ZON and organoclays, not for ZON and Mycosorb. The maximum adsorption capacity of organoclays is up to 390 mmol ZON/kg binder (Lemke et al. 1998), a value which cannot be reached by YCP considering the low adsorption efficiencies at 0.2% of binder and 0.5 mg/l ZON in solution (see Figure 1B).

Conclusions

This is the first study comparing the AFB1 and ZON adsorption capabilities of a great number of commercially available yeast cell products and correlating these with the ash, MOS and β-glucan content of the products. Commercially available yeast cell based products, even of the same brand, were shown to differ in type and content of MOS and β-glucan, as well as in ash content and mineral composition. Differences in the content and type of mineral clay components account for different binding capabilities of AFB1. The adsorption rate at increasing amount of

Table 2. Overview of articles investigating adsorption of (A) AFB1 and (B) ZON (single adsorption) onto YCP included in our study.

Product name	Percentage of binder used (%)	Solvent	pH	Toxin conc [mg/l]	Type of study	mmol AFB1 bound/kg binder	Reference
(A) AFB1 adsorption							
Bentonite GB7	0.002% (w/v)	Buffer	7.0	0.4–8	Adsorption isotherm	Max 420	(Vekiru et al. 2007)
NovaSil plus	0.02 mg/ml 0.002% (w/v)	Buffer	2.0 & 6.5	0.4–8	Adsorption isotherm	Max 396	(Marroquin-Cardona et al. 2009)
NovaSil plus	0.002% (w/v)	Buffer	7.0	0.4–8	Adsorption isotherm	Max 390	(Vekiru et al. 2007)
HSCAS	0.002% (w/v)	Water	Unadjusted	0.4–8	Adsorption isotherm	Max 260	(Lemke et al. 2001)
HSCAS (NovaSil)	0.001% (w/v)	Water	Unadjusted	0.4–8	Adsorption isotherm	Max 336	(Grant et al. 1998)
Mycosorb	0.02 mg/ml 0.002% (w/v)	Water	2.0 & 6.5	0.4–8	Adsorption isotherm	Max 9 & 15	(Marroquin-Cardona et al. 2009)
Mycosorb	1 mg/ml 0.1% (w/v)	Aqueous solution	Not given	2–10	Adsorption isotherm	Max 17.6	(Dawson et al. 2001)
Mycosorb	0.1% (w/v)	Unknown	Not given	0.5	Single adsorption (62% adsorption)	1	(Newman 2000)
MTB-100	1% (w/v)	Water/MeOH (9:1)	3, 7, 10 and unadjusted	5.0	Single adsorption (97% adsorption)	1.55	(Diaz et al. 2002)

Product name	Percentage of binder used (%)	Solvent	pH	Toxin conc [mg/l]	% adsorption	mmol ZON bound/kg binder	Reference
(B) ZON adsorption							
Mycosorb	1 mg/ml 0.1% (w/v)	Buffer	3.0 & 8.0	2	38 & 42	2.4 & 2.6	(Avantaggiato et al. 2005)
Mycosorb	1 mg/ml 0.1% (w/v)	Buffer	3.0 & 8.0	20	23 & 18	1.4 & 1.1	(Avantaggiato et al. 2005)
Mycosorb Extra	10.2 mg/ml 1.02% (w/v)	Buffer	Changed during incubation from 3–7	0.334	88.7	0.09	(Doell et al. 2004)
Mycosorb	1 mg/g 0.1% (w/w)[1]	Not given	Unknown	0.25–2	22–40	<2.5	(Newman 2000)
Mycosorb	1% (w/v)	Buffer	4.5	40	68	8.5	(Völkl et al. 1999)

[1]As stated in the original article.

the toxin (determined to differentiate between products with similar AFB1 binding efficiency in adsorption tests) revealed big differences between "pure" mineral binders and yeast cell based products with mineral components added.

Whereas AFB1 adsorption did not correlate with the MOS and β-glucan content of the investigated products, the ZON adsorption rate tended to increase with increasing MOS and β-glucan content. However, the adsorption capability was very low for the applied high inclusion rate of 0.2% w/v of the products and based on weak interactions. *In vivo* trials using mycotoxin biomarkers could be used to clarify whether the beneficial effect of yeast cell products against mycotoxicosis is based on effective binding of the toxin or on the general beneficial biological action of MOS and β-glucans to animals. In our study, ZON adsorption was independent of the type of yeast cell based product; claimed toxin binders (see Table 1) had similar or even lower binding capacities than yeast cell based additives designed for improving gastrointestinal health. Of the tested pure clay mineral products (>90% ash), some showed surprisingly good adsorption of ZON in buffer solutions (ca. 40%), but none of them adsorbed more than 15% of ZON in gastric juice.

The impact of the product composition on the adsorption properties and the influence of experimental conditions on the amount of mycotoxin bound/kg of binder stresses how important it is that manufacturers present detailed product information (moisture, β-glucan and MOS as well as ash content, and also other parameters such as protein and fat content) and that authors pass on this information and add experimental details (percentage of binder used, concentration of toxin, medium and pH of the binding study) in their articles for better comparability of literature results.

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