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13 Effect of Rumen Fluid on in Vitro Aflatoxin Binding Capacity of Different Sequestering Agents and in Vivo Release of the Sequestered Toxin

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13.1 Abstract

Aluminosilicates (Atox® and NovasilTM Plus) and a yeast cell wall derivate (Mycosorb®) were used as sequestering agents (SAs) to verify their capacity on binding aflatoxin B1 (AFB1) *in vitro*. SAs were individually mixed at three different ratio with AFB1 (1:5000, 1:50000 and 1:500000, w/w) in water (CTR), rumen fluid of lactating cow with low rumen pH (LRS) or rumen fluid of dry cow with high rumen pH (HRS), then used in a 3x3x3 factorial arrangement of a completely randomized design. Complexes between AFB1 and SAs (AF:SA) were obtained by mixing one litre of AFB1 contaminate solutions (0.845, 0.790, 0.832 and 0.911 μg/mL) with 50 g Atox®, 150 g Mycosorb®, 50 g NovasilTM Plus and 4000 mL rumen fluid, respectively. The unbound AFB1 was eliminated from the AF:SA complexes after centrifugation and washing of precipitates. The strength of complexes were investigated *in vivo* by measuring the aflatoxin M1 (AFM1) in milk and the AFB1 recovery rate (RR) in milk as AFM1 in dairy cows fed-drench, before the morning meal, 300 mL/cow of the prepared AF:SA complex suspension for a total of 0.447, 0.360, 0.460 and 0.367 μg/mL AFB1, respectively for Atox®, Mycosorb®, NovasilTM Plus and contaminated rumen fluid (R-SA). In in vitro condition and at the 1:500000 AF:SA ratio Atox® and NovasilTM Plus sequestered over 0.87 and 0.98 of the AFB1 in CTR and rumen solutions (LRS and HRS), respectively. The efficacy decreased when the amount of clays was reduced, with higher values ($P<0.001$) for Atox® compared to NovasilTM Plus (0.50 vs. 0.28 in CTR; 0.58 vs. 0.16 in LRS and 0.44 vs. 0.27 in HRS). Mycosorb® had a lower sequestering efficacy ($P<0.001$) in all the tested experimental conditions, with 0.34 as the maximum value obtained in the CTR solution. When the prepared AF:SA complexes were given to cows, differences ($P<0.05$) were

observed for the total AFM1 excreted with amounts (ng) of 199, 870, 2394 and 1056, respectively for Atox®, Mycosorb®, NovasilTM Plus and R-SA. Between used aluminosilicates, Atox® had the lowest RR value compared to NovasilTM Plus (0.002 vs. 0.017; $P<0.05$), however with no difference compared to Mycosorb® and R-SA. Higher amount ($P<0.05$) of AFB1 was released from the NovasilTM Plus AF:SA complex compared to the Atox® complex. R-SA showed a sequestering activity and a low release of the sequestered AFB1 from the AF:SA complex.

Keywords: *Aflatoxin B1; Aflatoxin M1; Dairy cows; Milk; Sequestering agents*

Abbreviations: ADFom, acid detergent fiber exclusive of residual ash; AFB1, aflatoxin B1; AFM1, aflatoxin M1; AF, Aflatoxin; CO, carry-over; CP, crude protein; CTR, water solution; DM, dry matter; F:C, forage to concentrate ratio; HRS, high rumen pH; HSCAS, hydrated sodium calcium aluminosilicates; LRS, low rumen pH; aNDFom, neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash; NSC, nonstructural carbohydrates; PBS, phosphate-buffered saline; PeNDF, physical effective neutral detergent fibre; RR, recovery rate; R-SA, contaminated rumen; SA, sequestering agent; S.D., standard deviation; S.E.M., standard error of the mean; TMR, total mixed ratio; VFA, volatile fatty acid;

13.2 Introduction

Aflatoxins (AFs) are secondary metabolites produced primarily by Aspergillus flavus and A. parasiticus and are common contaminants of corn (Zea mais), cotton and peanuts and their by products, either in field or storage conditions. Aspergillus spp. have been described as ubiquitous (Scheidegger and Payne, 2003) and were isolated from all latitudes, however, higher frequencies were described in desert climates and at latitudes ranging from 16 to 35°, in tropical, subtropical and warm temperate climates (Klich et al., 1992).

The aflatoxins, as a group (aflatoxin B1, B2, G1, G2 and M1), are classified as group 1 carcinogens (IARC, 2002). Because of its lipophilic proprieties and low molecular weight, once ingested, aflatoxin B1 (AFB1) was rapidly adsorbed through the rumen wall and intestine (Moschini et al., 2007) by a non-described passive mechanism (Yiannikouris and Jouany, 2002) and quickly appeared, also as the metabolite aflatoxin M1 (AFM1), in blood just after 15 minutes (Moschini et al., 2007) and in milk at the first milking (Diaz et al., 2004) after intake of aflatoxin contaminated feeds. Efforts have been put in trying to understand major metabolites pathways (figure 13-1) of production and excretion following AFB1 intake.

The AFM1 excretion in milk occurred for several days after a single AFB1 dose (Battacone et al., 2003) with a carry-over (CO) rate even higher then 0.03 in dairy cows (Diaz et al., 2004). The excretion was affected mainly by milk yield and also by a different AFB1 liver metabolism in early and late lactation (Veldman et al., 1992; Van Eijkeren et al., 2006). The maximum allowed concentration of AFB1 in dairy animal feeds and concentrates is 5 μg/kg in the European Union (EC, 2003a). The concentration of AFM1 in milk is limited to 0.05 μg/kg in the European Union (EC, 2006) and 0.5 μg/kg in the US (Berg, 2003).

Within the food chain, the best way to avoid the risk of mycotoxicosis is the reduction of the mycotoxin production controlling harvesting conditions, grain maturity etc. (Huwig et al., 2001). Biological, chemical and physical strategies have been also developed to preserve feeds from post-harvest mycotoxins contamination.

The utilization of sequestering agents (SAs) capable of reducing the free toxin available for gastro-intestinal absorption is widely used for counteracting the biological negative effects of aflatoxin in diets. Huwig et al. (2001) summarized the binding capacity and affinity of several binders for AFs. Among most studied binders were smectite clay like hydrated sodium calcium aluminosilicates (HSCAS) (Phillips et al., 1990; Harvey et al., 1991), montmorillonites (Ramos and Hernandez, 1996), bentonite (Shell et al., 2000), zeolite (Piva, 1995); also activated carbons (Diaz et al., 2003) and yeast cell walls esterified glucomannans (Aravind et al., 2003; Karaman et al., 2005) had been used. The sequestering capacity of smectite clay are through electric elementary charges between the beta-carbonil portion of the aflatoxin molecule to sites containing aluminion ions (Phillips et al., 1990) or through hydrogen and van der Waals bonds between the single helix of β-D-glucans in yeast cell walls and the lactone groups of AFB1 (Yiannikouris et al., 2005; Jouany, 2007).

Several *in vitro* methods have been proposed to screen different SAs, however results were not always comparable to the *in vivo* responses having the pH, AFB1:sequestering agent (AF:SA) ratio, temperature (Ramos and Hermandez, 1996; Grant and Phillips, 1998; Lemke et al., 2001) and biological fluid (Ledoux and Rottinghaus, 1999) as important affecting factors. Differences of published *in vitro* works were: kind of AFs (AFB1 from standard or extracted with a methanol solution from natural aflatoxin contaminated feeds), dilution factor (AF:Volume ratio, μg/mL), AF:SA ratio, pH conditions. Also, authors did not consider the possible competition between AFs and other biological molecules for binding sites of SAs in the gastro-intestinal tract.

Even though works have been published about effects of SAs using the monogastrics and polygastrics models (Lemke et al., 2001; Spotti et al., 2005), there are few information about the adsorption mechanism and fate of the AF:SA complex in ruminants.

The objectives of the work were to compare the sequestering capacity, in water or in rumen fluid, of two aluminosilicates and a derived yeast cell walls esterified glucomannan, used at different AF:SA ratios. Also, the strength of the AF:SA complex was investigated *in vivo* by measuring the appearance of AFM1 into milk.

13.3 Materials and methods

13.3.1 AFB1 contaminated corn meal

The AFB1 contaminated corn meal was obtained after inoculation with Aspergillus flavus type strain MPVP 2092 (Istituto di Entomologia e Patologia Vegetale, Università Cattolica del Sacro Cuore, Piacenza, Italy). The AFB1 was produced at 25°C and 0.99 water activity (Giorni et al., 2007). The final AFB1 contamination of the corn meal was 82.21±0.01 mg/kg.

13.3.2 In vitro experimental design

Three commercial binders (Atox®, Grupo Tolsa, Madrid, Spain; NovasilTM Plus, Trouw Nutrition International, Verona, Italy; Mycosorb®, Alltech Italy, Bologna, Italy) were used to evaluate the *in vitro* AFB1 sequestering capacity. The Atox® and NovasilTM Plus products are sold (EC, 2003b) in the sub-classification "Binders, anti-caking agents and coagulants" whereas the Mycosorb® is sold as raw material for animal use. Binders, from now on identified as SA, were individually mixed at three different ratio with AFB1 (1:5000, 1:50000 and 1:500000, w/w) in water (CTR), rumen fluid of lactating cow with low rumen pH (LRS) or rumen fluid of dry cow with high rumen pH (HRS), then used in a 3x3x3 factorial arrangement of a completely randomized design. The rumen liquors were taken from two rumen fistulated cows three hours after the morning meal, filtered with a three layers cheese cloth, then stored at 39°C in anaerobic conditions.

Preparation of AFB1 solution

The AFB1 was extracted from a natural contaminated corn meal (82.21±0.01 mg/kg) using a water/methanol solution (20:80 v/v) in ratio of 1:100 (g:mL), at room temperature and light agitation (150 shake/min) for 120 minutes. The obtained concentration was 0.821 µg/mL of AFB1 (solution A).

Ten mL of the solution A were added to 2000 mL of water (CTR) or rumen fluids (LRS or HRS) and stirred for ten minutes to obtain the basal contaminated solutions (solutions B: calculated

AFB1 concentration of 4.1 µg/L). Solutions B were centrifuged (3500 g for 15 minutes), then supernatants were sampled for AFB1 (CTR, LRS and HRS) and aflatoxicol (LRS and HRS) analysis. AFB1 in precipitates were extracted into 10 mL chloroform and evaporated to dryness under nitrogen before being recovered into 1 mL acetonitrile:water solution (25:75 v/v) for HPLC analysis.

Then, twenty-seven 50 mL sub samples/solution were poured into a 150 mL glass beakers with a pre-weighted SA, three replicates for each SA and level (1:5000, 1:50000 and 1:500000, w/w). Samples were incubated at 39°C for one hour under gentle shake of five minutes every 15 minutes, then three 10 mL sub-samples were obtained for each beaker, cooled down at 4°C to stop any possible fermentation activity and centrifuged at 3500 g for 15 minutes.

The supernatant was recovered and the precipitate was suspended into 10 mL water before centrifugation at 3500 g for 15 minutes. The step was cycled three times and the recovered supernatant as whole was analyzed by HPLC for AFB1 content.

The AFB1 sequestered by SA in each sub-sample was obtained by difference to one hundred of the percentage of total AFB1 recovered in the supernatant over the AFB1 found in the solution B (water or rumen fluids).

13.3.3 In vivo trial

Preparation of the AF:SA complex

Solutions A were prepared for each AF:SA complex as previously described in the *in vitro* trial. The obtained AFB1 concentrations were 0.845, 0.790, 0.832 and 0.911 µg/mL respectively for Atox®, Mycosorb®, NovasilTM Plus and rumen fluid.

Atox® (50 g), NovasilTM Plus (50 g), and Mycosorb® (150 g) were mixed with 4000 mL water at room temperature, stirred for 60 min to complete hydration and suspension, then added 1000 mL of solution A. Amounts of SAs used for the AF:SA complexes preparation were based on the different sequestering efficacy observed in the *in vitro* experiment (table 13-4). This was to obtain a similar amount of bound AFB1 among the AF:SA complexes, then used in the *in vivo* trial.

The contaminated rumen (R-SA) was obtained by adding 1000 mL solution A to 4000 mL rumen fluid (LRS).

Solutions were gentle stirred for two hours, then each solution was divided into twelve fractions and centrifuged at 4000 g for 15 minutes. The supernatant was separated, measured and stored at 5°C until HPLC analysis for AFB1 content. The precipitate of each centrifugation tube was washed with 200 mL of distilled water, centrifuged and the supernatant recovered and analyzed

for AFB1 content. The washing was cycled until the supernatant AFB1 concentration was lower than 0.05 µg/L. For the twelve fractions, the number of washings needed were: five (12 L) for Atox®, seven (16.8 L) for NovasilTM Plus, 15 (36 L) for Mycosorb® and 16 (38.4 L) for the R-SA. Then, residues were pooled by solution and re-suspended into 1800 mL water (37°C) before animal drench (300 mL/cow).

13.3.4 Animals

The trial was carried out at the CERZOO research and experimental center (San Bonico, Piacenza, Italy). The research protocol and animal care was in accordance with the EC council directive guidelines for animals used for experimental and other scientific purposes (EEC, 1986).

The rumen liquor at low pH for the *in vitro* experiment was collected from a fistulated cow fed the same total mixed ratio (TMR) used in the *in vivo* experiment (LRS). A dry fistulated cow fed a TMR based on grass hay (700 g/kg), corn silage (200 g/kg) and concentrate (100 g/kg) on a dry matter (DM) basis was used as the high rumen pH liquor donor (HRS).

Twenty-four Holstein lactating cows were used in the *in vivo* experiment. Cows were housed in a free stall, had free access to water and were fed a TMR (table 13-1) formulated according to the nutrient requirements of dairy cattle (NRC, 2001) for an average cow weight of 600 kg, 140 days in milk and 33 kg milk yield (38.0 g/kg fat and 33.5 g/kg protein). The bulk of the diet on a DM basis was: corn silage (312 g/kg), alfalfa hay (167 g/kg), grass hay (41 g/kg) and energy-protein supplement (480 g/kg).

The diet was fed once a day (0800 hours) ad libitum (5% expected refusal). Cows were milked twice a day (0230 and 1330 hours) and the individual milk yield was recorded at each milking (Afimilk system, Afikim, Israel).

Animals (6 cows/treatment) were fed-drench 300 mL of the AF:SA complex suspension (0.447, 0.360, 0.460 and 0.367 µg AFB1/mL respectively for Atox®, Mycosorb®, NovasilTM Plus and R-SA) before the morning meal.

The recovery rate (RR) of AFB1 excreted in milk as AFM1 was calculated as the ratio total AFM1 in milk (sum of the AFM1 excreted in four consecutive milkings) over the total AFB1 ingested by the cow.The total AFM1 ingested was cleaned by the base diet contamination contribution. The total AFM1 excreted in milk was also cleaned of the contribution from the contamination of the base diet, estimated by the AFM1 content in milk measured in two consecutive milkings before the cow oral drench, and assumed as constant value for the four collected milkings after oral drench.

13.3.5 Milk and Feed samples

TMR samples were collected the day before treatment, dried at 55°C in a ventilated oven until constant weight, then ground with one mm sieve (Thomas-Wiley Laboratory Mill, Arthur H. Thomas Co., Philadelphia, PA) and stored for analysis.

Samples were assayed in duplicates according to AOAC (1990) for DM (procedure 930.15), crude protein (CP) (procedure 975.06), ash (procedure 942.05), crude lipids (procedure 954.02) and acid detergent fibre exclusive of residual ash (ADFom) (procedure 973.18). The neutral detergent fibre (aNDFom) assayed with a heat stable amylase and expressed exclusive of residual ash, according to Mertens (2002), without sodium sulfite and using the Ankom equipment (Ankom220, USA) for extraction and filtering.

Individual milk samples were collected at each milking and for six consecutive milkings (two before and four after treatment) for AFM1 content determination. Cumulative AFM1 excretion were individually calculated based on daily milk AFM1 concentration and milk yield for four milkings.

13.3.6 Aflatoxin Analysis

AFB1 and aflatoxicol assays

Ten grams of dried feed were extracted with 100 mL of a methanol:water solution (80:20 v/v), shacked at 150 rpm for 45 minutes (Universal table Shaker 709) and filtered with Schleicher&Schuell 595 ½ filter paper (Dassel, Germany). Then, 5 mL were eluted with 45 mL of bi-distilled water and passed through an immunoaffinity column (Aflatoxin Easi-extract, Rhône diagnostics technologies, Glasgow, UK).

One mL of the supernatant solution was added to 9 mL of phosphate-buffered saline (PBS) solution (pH 7.4) and passed throw an immunoaffinity column previously washed with 20 mL of a PBS solution.

The column was than washed with 5 mL water and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in 1 mL acetonitrile:water (25:75 v/v) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 μm) before HPLC analysis for AFB1 (feed and supernatant) and aflatoxicol (supernatant) contents.

AFM1 assay in milk samples

The extraction was by immunoaffinity technique according to Mortimer et al. (1987). Fifty mL of defatted milk (centrifuged at 7000 rpm for 10 minutes at 4°C) were filtered with Schleicher&Schuell 595 ½ filter paper (Dassel, Germany). Then, 20 mL were passed through an immunoaffinity column (Aflatoxin Easi-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a PBS solution (pH 7.4). Then columns were washed with 5 mL water, and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in 1 mL acetonitrile:water (25:75 v/v) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 μm) before HPLC analysis.

Chromatography

The HPLC analysis was performed by a Perkin Elmer LC (Perkin Elmer, Norwalk, CT, USA) equipped with a LC-200 pump and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). The system and data acquisition were controlled by Jasco Borwin Chromatography PC software.

The AFB1 and aflatoxicol were separated with a reverse-phase C18 Superspher column (4 μm particle size, 125 x 4mm i.d.; Merck, Darmstadt, Germany) at room temperature (18°C) and isocratic conditions, with a mobile phase of water and acetonitrile:methanol (17:29 v/v) solution (64:36 v/v). The flow rate was 1mL/min. Then, the AFB1 and aflatoxicol were detected by fluorescence, after postcolumn derivatization (Jasco 2080 Plus HPLC pump) with pyridinium hydrobromide perbromide (PBPB) at 0.1 mL/min flow. The fluorescence detector was set at 365 nm excitation and 440 nm emission wavelengths for AFB1 and 360 nm excitation and 418 nm emission wavelengths for aflatoxicol. The standard stock solution was checked for AFB1 and aflatoxicol concentrations according to AOAC method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

The AFM1 was separated with a reverse-phase C18 LiChospher 100 column (Merck, Darmstadt, Germany, 5 μm particle size, 125 x 4 mm I.D.) at room temperature (18°C), with a water and acetonitrile (75:25 v/v) mobile phase and flow rate set at 1 mL/min. The fluorescence detector was set at 365 nm excitation and 440 nm emission wavelengths. The standard stock solution was checked for AFM1 concentration according to the AOAC method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

13.3.7 Statistical Analyses

The *in vitro* data were analyzed as completely randomized design using the general linear model procedure of SAS® (Statistical Analysis System Institute, 2001). A factorial arrangement was used and fixed effects in the model included SA, solution and AFs:SA ratio.

The AFB1 intake, AFM1 in milk concentration and RR were analyzed as completely randomized design using the general linear model procedure of SAS®. Fixed effects were the different preparation of AF:SA complexes and the experimental unit was the single treated animal.

Both *in vitro* and *in vivo* means were considered different for $P<0.05$.

13.4 Results

13.4.1 In vitro experiment

Analytical parameters of rumen fluids showed the predictable effects due to the forage:concentrate ratio, energy and nonstructural carbohydrates on pH (6.1 and 6.7), N-NH3 (4.23 and 8.20 mg/100mL), total volatile fatty acids (138.57 and 124.56 mmol/L) and fatty acids molar concentration respectively for LRS and HRS (table 13-2).

Recoveries of AFB1

The average AFB1 recovered from the CTR was 0.93. The average AFB1 recovered in the supernatant after centrifugation of rumen fluids solutions were 0.53 and 0.53 and the average residual AFB1 extracted with chloroform from precipitate were 0.23 and 0.25, respectively for the LRS and HRS solutions. Thus, the total average AFB1 recovery (supernatant + precipitate) were 0.76 for LRS and 0.78 for HRS (table 13-3).

None of supernatants and precipitates analyzed for aflatoxicol content had concentration values over the detectable limit (0.03 μg/L).

Binding efficacy of SA

The *in vitro* AFB1 sequestering activities of used SA in different solutions and at different AF:SA ratios are reported in table 13-4. Data analyzed as a factorial arrangement showed the effect ($P<0.01$) of SA, solution, AF:SA, and of the first (SA x AF:SA; SA x solution, solution x AF:SA) and second (SA x solution x AF:SA) order interactions.

The higher sequestering capacity was observed for Atox® and NovasilTM Plus at the 1:500000 AF:SA ratio. Over 0.87 and 0.98 of AFB1 was sequestered by the SA, respectively in water and rumen solutions. The efficacy decreased when the amount of the SA was reduced with higher values for the Atox® compared to NovasilTM Plus, independently of the solution being used (0.50 vs. 0.28 in CTR; 0.58 vs. 0.16 in LRS and 0.44 vs. 0.27 in HRS). Comparing Atox® and NovasilTM Plus at the same level of inclusion no differences between the two SAs were observed at the higher dosage (1:500000), whereas the sequestering efficiency was higher ($P<0.001$) for Atox® compared to NovasilTM Plus for the lower and intermediate level of inclusion (1:5000 and 1:50000, respectively) independently of the used media solution (water or rumen fluids).

The Mycosorb® showed a lower ($P<0.001$) AFB1 sequestering capacity compared to the two aluminosilicate SAs in all experimental conditions (table 13-4), with the maximum efficacy (0.34) in CTR solution at the higher dosage (1:500000). When Mycosorb® was incubated at the same dose in the HRS media, its sequestering capacity decreased to the 0.02 at the 1:500000 dose.

13.4.2 In vivo experiment

Animals used in the trial had a daily milk yield of 27.5 ± 2.1 kg and a DM intake of 23.3 ± 0.8 Kg (mean ± S.D.).

The TMR had a base AFB1 content of 0.14 ± 0.01 µg/kg contributing to a bulk milk AFM1 content of 15.25 ± 9.10 ng/kg before treatment (mean ± S.D.).

During the AF:SA complex preparation for the *in vivo* trial the fraction of AFB1 not bound plus the AFB1 washed were 0.05, 0.18, 0.004 and 0.28, respectively for Atox®, Mycosorb®, NovasilTM Plus and R-SA. Data indicated a non uniform strength of the AF:SA complex between tested SAs.

Mycosorb® sequestered 0.82 of the AFB1. The high AF:SA ratio used in this trial for the preparation of the AF:SA complex could be the key to get good sequestering performances if compared to results of the *in vitro* trial in which completely different dilution and AF to SA ratio were used.

The highest AFM1 milk concentration was detected for each SA as soon as the first milking after oral drenching (8.38 ± 7.65 µg/kg for Atox®, 26.78 ± 15.20 for Mycosorb®, 85.25 ± 82.56 for Novasil™ Plus and 35.65 ± 30.90 for R-SA) (figure 13-2). Differences ($P<0.05$) were also observed for total AFM1 excreted in four milkings after drenching and RR. The amount of AFM1 excreted (ng) were 199, 870, 2394 and 1056 and RR were 0.002, 0.008, 0.017 and 0.01, respectively for Atox®, Mycosorb®, NovasilTM Plus and R-SA (table 13-5).

13.5 Discussion

Dietary supplementation with SA is the most practical and the most widely studied technique to mitigate the consequences of aflatoxin exposure (Diaz and Smith, 2005).

The HSCAS, a smectite clay (Philliphs et al., 1990), was reported to form AF:SA complex which reduced the absorption of the aflatoxin across the intestinal epithelium (Dawson et al., 2001; Abdel-Wahhab et al., 2002) and prevented negative effects due to mycotoxins intake in broilers (Pimpukdee et al., 2004), in pigs (Schell et al., 2000) and in goats exposed to 100 and 200 µg/kg of AFB1 (Smith et al., 1994).

When used in dairy cows (AFB1 content ranging from 55 to 200 µg/kg in TMR) the HSCAS and yeast derivate cell walls reduced from 24 to 65% the AFM1 in milk (Harvey et al., 1991; Nelson, 1993; Diaz et al., 2004). As far as we know there are no published works reporting on NovasilTM Plus and Atox® in dairy cows.

Mycosorb®, a yeast cell wall derived glucomannan, was reported to adsorb a large range of mycotoxins (Dawson et al., 2001) and it improved the performance in broilers (Aravind et al., 2003; Karaman et al., 2005) exposed to AFB1 ranging from 168 to 2000 µg/kg and in pigs (Casteel et al., 2003) fed 500 µg AFB1/kg of diet.

The AFB1 concentration used in our *in vitro* experiment was 4.1 µg/L, a level lower than what used in previous works (Galvano et al., 1996; Lemke et al., 2001; Diaz et al., 2003; Spotti et al., 2005; Ledoux and Rottinghaus, 1999) (table 13-6) and close to the concentration that could be found in the rumen of a cow with a daily AFB1 intake between 200 and 250 µg and considering a rumen liquid phase of 50-60 L. The level used was based on our preliminary observation of rumen AFB1 concentration on a dry cow at one (4.07 µg/kg) and three (5.91 µg/kg) hours after feeding a bolus containing 200 µg AFB1.

The amount of the SA used ranged between the on farm suggested dose as being effective (1:500000) and the suggested level for *in vitro* study (1:5000) as from Ledoux and Rottinghaus (1999). Compared to previous works (table 13-6), our *in vitro* experimental conditions were different particularly for the dilution factor (AF:Volume ratio, µg/mL), source of AFB1 (standard solutions or extracted from a natural contaminated feed), AF:SA ratios and type of the media solution (water, digestive enzyme solutions, intestinal or ruminal fluids).

The two aluminosilicates obtained in our trial a sequestering capacity higher than 0.80, a threshold value suggested by Ledoux and Rottinghaus (1999) to classify a SAs as efficient *in vitro*.

Similar results were reported by Phillips et al. (1988) using HSCAS and in which the chemisorptions of AFs to HSCAS involved the formation of a complex by the β-keto-lactone or bilactone system of aflatoxin with uncoordinated metal ions in HSCAS (Phillips et al., 1990).

Authors reported the maximum AFB1 binding capacity of one mg of HSCAS ranged from 200 to 332 nanomoles (63 to 105 µg), without effects of temperature and pH experimental conditions.

In vitro studies indicated that cell walls of several probiotic bacteria (i.e. lactobacilli) living in the intestine can bind AFB1 (Peltonen et al., 2000; Oatley et al. 2000; Gratz et al., 2005). This binding reduced the free AFB1 in the gastro-intestinal tract, delaying but not preventing the intestinal AFB1 absorption (Gratz et al., 2005).

The chlorophyllin, a water-soluble derivative of chlorophyll, was also effective in reducing the toxicity associated to AFs (Dashwood et al., 1998; Atroshi et al., 2002). Indeed, a chlorophyllin-AFs complex was actually the main mechanism responsible for chemoprotection (Breinholt et al., 1999).

Thus, rumen liquor intrinsic factors could justify the lower AFB1 recovery we observed in the supernatant after centrifugation of rumen fluids solutions and in the residual AFB1 extracted with chloroform from precipitate. Similar results on rumen solution were previously reported by Spotti et al. (2005). However, our results on aflatoxicol content after one hour incubation in rumen fluids should not exclude the possible presence of other AFB1 metabolites.

The yeast cell wall derivate (Mycosorb®) had a very low *in vitro* efficiency in all tested conditions (table 13-4). The pH and the kind of the solvent can reduce the ability of beta-D-glucans, a major component of the inner layer of the yeast cell wall, to complex mycotoxins, indicating the involvement of non-covalent binds (adsorption) rather than a real binding type (Yiannikouris et al., 2005). Indeed, a previous work by Dawson et al. (2001) suggested a pH of four as optimal for Mycosorb® activity along with 0.5 M phosphate, conditions that can be found in the gastro-intestinal tract of monogastric animals but not in the rumen. This specific SA itself had a slight acidifying property as observed during the AF:SA complex preparation (pH after binder mixing: 4.6). Thus, the higher pH values observed in the LRS and HRS solutions along with the considerable buffer capacity of the rumen liquor did probably counteract the acidifying property of Mycosorb® resulting in a negative environment for its specific sequestering activity, which lowered or zeroed (HRS solution) the binding efficiency. It has been reported that up to 90% adsorption of aflatoxins to yeast cells is dose-dependent in *in vitro* condition (Devegowda et al., 1994).

The efficacy of Mycosorb® was reported to be higher compared to the clay-based binders when used at low mycotoxin concentration, whereas the binding capacity was greater at high mycotoxin concentration (Dawson et al., 2001). Authors reported also an higher Mycosorb® efficiency with aflatoxin concentration ranging from 2 to 10 µg/mL at 37°C.

Concentrations used in our trial were lower than the above mentioned values and the binding efficiency of Mycosorb® was considerably low (table 13-4), not as high as the sequestering efficiency observed for Atox® and NovasilTM Plus, also when implemented at higher dosages.

Results of the *in vitro* experiment were partially contradicted by the *in vivo* data. In facts, NovasilTM Plus was less effective ($P<0.05$) in keeping bound the AFB1 in the gastro-intestinal tract compared to Atox®. Even though working in controlled environment, the *in vitro* operating conditions is still an attempt to resemble the real gastro-intestinal tract conditions.

The calculated RR value of bound AFB1 to AFM1 excreted in milk were 0.002 for Atox®, 0.008 for Mycosorb®, 0.017 for NovasilTM Plus and 0.01 for Rumen-SA. Animals were given 134.0, 108.0, 138.1 and 109.9 µg of bound AFB1, respectively for Atox®, Mycosorb®, NovasilTM Plus and Rumen-SA.

When assuming a CO rate of 0.03, an expected value in high yielding dairy cows (Veldman et al., 1992), based on the amount of AFM1 recovered in milk in our experiment we can estimate the fraction of AFB1 released from the complex drenched to animals. Calculated values were 0.05, 0.27, 0.58 and 0.30, respectively for Atox®, Mycosorb®, NovasilTM Plus and R-SA. Data indicated differences in terms of bound strength within the AF:SA complexes used.

Therefore, data suggested an evidence of a labile AF:SA sequestering capacity which can lead to a release of some of the bound AFB1 in the gastro-intestinal tract, which was not supported by a previous work in different species (Phillips, 1990). However, Watts et al. (2003) did not find an effective prevention of HSCAS in chicks, suggesting a lack of efficacy of HSCAS against other mycotoxins.

Thus, more specific knowledge about the chemical and physical characteristics of Atox® and Novasil™Plus are needed to get additional answers about observed differences between *in vivo* and *in vitro* efficacies.

Up to now, no works have been published on this topic in cows and, as emphasize by Diaz and Smith (2005), a two step *in vitro* procedure must be used to correctly evaluate the efficacy of a SA: the initial sequestration (weak binding) and the desorption (strong binding). Chemical solvent extraction, like methanol or chloroform, have been used by several authors (Ramos-Girona and Gimenez, 1997; Kannewischer et al., 2006) to evaluate the strong binding.

The EU limit of AFB1 in dairy animal feeds refers to maximum allowed concentration (20 µg/kg for animal feeds and 5 µg/kg for concentrates) of the mycotoxin in marketed feeds, the same is true for the milk limit (0.05 µg/kg). However, there is no limit in terms of maximum intake of AFB1 by the animal if not the ultimate concentration of the produced milk. Thus, even feeding on regularly marketed feeds, in particular condition an high yielding dairy cow could easily intake over

40 µg AFB1/day, a value previously reported (Veldman et al., 1992) as the maximum amount for complying with the milk EU limit. Even though AFB1 CO modelling (Van Eijkeren et al., 2006) reports the effectiveness of the EU limits in preventing excessive levels of AFM1 in milk, high intake of AFB1 could justify the implement of effective SAs as a tool for complying with the AFM1 milk EU limit.

13.6 Conclusions

The *in vitro* efficiency of used SAs was related to the AF:SA ratio. There was a synergy between rumen fluid and SA on the reduction of AFB1 absorption in the gastro intestinal tract.

The discrepancy between the *in vitro* and the *in vivo* results outlines the need of further research on AF:SA complex behavior in the gastro intestinal tract.

Results suggest an higher level of inclusion for Novasil™ Plus or Mycosorb® to obtain a binding and a release efficiency similar to the Atox® product.

Table 13-1. Forage to concentrate ratio (F:C), composition (g/kg dry matter) and chemical composition (g/kg dry matter) of experimental diets fed to lactating (LRS) and dry cows (HRS)

Item	Lactating cows (LRS)	Dry cows (HRS)
F:C	40:60	80:20
Corn silage	312	200
Alfalfa hay, dehydrate	167	-
Grass hay	41	700
Cotton seed, whole with lint	85	-
Corn meal	183	30
Barley meal	66	30
Protein supplement[1]	103	20
Calcium soap[2]	9	-
Soybean meal	34	20
Chemical composition		
Crude protein	162	125
Crude lipids	48.6	25.2
ADFom[3]	204	320
aNDFom[4]	340	440
Calculated		
PeNDF[5]	266.8	405
NSC[6]	411.8	225

[1]Contains per kg of premix: Soybean meal 600 g, Sunflower meal 300 g, mineral and vitamin supplement 100 g.; 120000 IU of Vitamin A; 9000 IU of Vitamin D3; 90 mg of Vitamin E; 3.6 mg of Co; 19.2 mg of I; 1.44 mg of Se; 600 mg of Mn; 62.4 mg of Cu; 2240 mg of Zn; 1.92 mg of Mo; 360 mg of Fe.

[2]Megalac;

[3]ADFom: acid detergent fibre expressed exclusive of residual ash.

[4]aNDFom: assayed with a heat stable amylase and expressed exclusive of residual ash, according to Mertens (2002), without sodium sulfite.

[5]PeNDF: Physical effective neutral detergent fibre (Mertens, 1997) calculated according to the contribution of the single feed present into the diet (concentrates were considered with PeNDF=0; whole cotton seeds PeNDF=70)

[6]NSC (nonstructural carbohydrates)= 100-(NDF+Ash+Crude protein+Crude lipids)

Table 13-2. Rumen pH, ammonia nitrogen ($N\text{-}NH_3$), volatile fatty acids (VFA), acetic/propionic and acetic+butyric/propionic molar ratios of rumen fluids from lactating (LRS) and dry (HRS) cows

Item	LRS	HRS
Rumen pH	6.10	6.70
$N\text{-}NH_3$ (mg/100 ml)	4.23	8.20
Acetic acid (mmol/L)	99.68	93.12
Propionic acid (mmol/L)	23.43	21.69
Butyric acid (mmol/L)	12.31	7.93
Total VFA (mmol/L)	138.57	124.56
Acetic/Propionic	4.25	4.29
(Acetic+Butyric)/Propionic	4.68	4.66

Table 13-3. Recoveries (mean ± S.D.) of aflatoxin B1 (AFB1) in supernatant, in rumen pellet and as total in water (CTR) and rumen fluids from lactating (LRS) and dry (HRS) cows

Solution	Recovery		
	Supernatant	Rumen pellet	Total
CTR	0.93 ± 0.05	-	0.93 ± 0.05
LRS	0.53 ± 0.03	0.23 ± 0.01	0.76 ± 0.02
HRS	0.53 ± 0.06	0.25 ± 0.02	0.78 ± 0.03

Table 13-4. *In vitro* sequestering efficiency of Atox®, Novasil™ plus and Mycosorb® used at several ratio with aflatoxin B1 (AF:SA) in water (CTR) and rumen fluids from lactating (LRS) or dry (HRS) cows

Solution	AF:SA	Sequestering Agent			S.E.M.[1]	Main effect (P)		First order interaction (P)				Second order interaction (P)
		Atox ®	Novasil™ plus	Mycosorb®		SA[2]	Sol[3]	AF:SA	SA x Sol	SA x AF:SA	Sol x AF:SA	SA x Sol x AF:SA
CTR	1:5000	0.50	0.28	0.09	0.02	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.006	< 0.001
	1:50000	0.66	0.53	0.34								
	1:500000	0.88	0.80	0.36								
LRS	1:5000	0.58	0.16	0.15								
	1:50000	0.86	0.70	0.17								
	1:500000	1.00	0.98	0.21								
HRS	1:5000	0.44	0.27	0.00								
	1:50000	0.90	0.78	0.00								
	1:500000	0.99	0.99	0.02								

[1]S.E.M.: standard error of the mean.

[2]SA: sequestering agent.

[3]Sol: solution.

Table 13-5. Milk yield, aflatoxin B1 (AFB1) ingested, cumulative aflatoxin M1 (AFM1) excreted in four milkings and recovery rate (RR) for Atox®, Mycosorb®, Novasil™ Plus and contaminated rumen fluid (R-SA) (n=6)

Item	Atox®	Mycosorb®	Novasil™ Plus	R-SA	S.E.M.[1]
Milk yield[2] (kg/day)	27.9	27.0	27.8	27.2	0.216
AFB1 ingested (µg/cow)	134.0	108.0	138.1	109.9	-
AFM1 excreted in 4 milkings (ng/cow)	199[a]	870[a]	2394[b]	1056[ab]	455
RR	0.002[a]	0.008[ab]	0.017[b]	0.010[ab]	0.348

[1]S.E.M.: standard error of the mean.

[2]P of the model not significant.

[a, b] Means within a row with different letters differ significantly (P<0.05).

Table 13-6. *In vitro* aflatoxin:sequestering agent (SA) ratio g/g (AF:SA), volume (mL) and dilution factor (µg/mL) reported by different authors and in our xperimental condition (present work)

Authors		AF:SA ratio (g/g)	Volume (ml)	Dilution (ug/mL)
Present work		1:5000 1:50000 1:500000	50	0.004
Spotti et al. (2005)		1: 250000	1	0.1
Ledoux and Rottinghaus (2005)		1: 5000	10	2
Lemke et al. (2001)	single-concentration absorption study	1:25	5	8
	Chemisorption index	1:1250	5	8
	GI model studies	1:312.5	40	10
	Modified Isotherm studies	1:2551	5	7.84
Diaz et al. (2002)		1:2000	1	5
Galvano et al. (1996)	test 1	1:10000	1	10
	test 2,3 and 4	from 1:40 to 1:500	5	from 0.1 to 1.25

Figure 13-1. Biotrasformation pathways in liver of absorbed aflatoxin B1 (AFB1): detoxification (continuous line) and toxic or carcinogenic (dashed line) processes (adapted from Yiannikouris and Jouany, 2002)

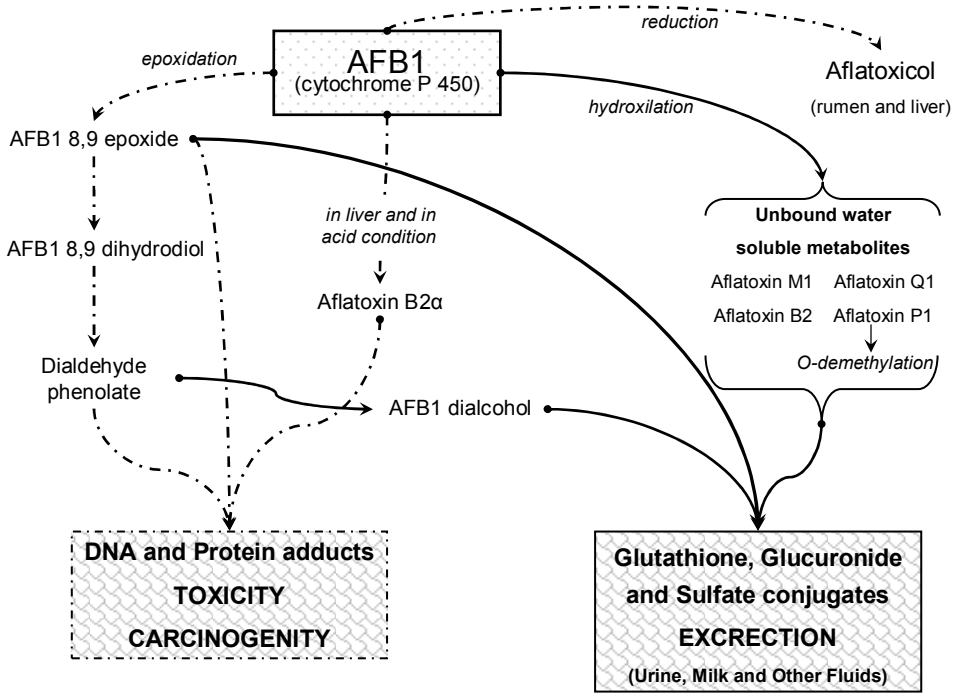

Figure 13-2. Milk AFM1 concentration (ng/kg) in four milkings after oral drench.