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To cite this version:

Severine Cavret, Nathalie Laurent, Videmann Bernadette, Mazallon Michelle, Sylvaine Lecoeur. Determination of DON adsorbents and characterisation of their efficacy using complementary in vitro tests.. Food Additives and Contaminants, 2009, 27 (01), pp.43-53. 10.1080/02652030903013252 . hal-00573859

HAL Id: hal-00573859

https://hal.science/hal-00573859v1

Submitted on 5 Mar 2011

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Journal:	*Food Additives and Contaminants*
Manuscript ID:	TFAC-2008-130.R2
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	24-Mar-2009
Complete List of Authors:	cavret, severine; isaralyon nathalie, laurent; ISARA Lyon bernadette, videmann; INRA, Animal Health michelle, mazallon; INRA, Animal health Lecoeur, Sylvaine; INRA, Animal Health
Methods/Techniques:	Survey
Additives/Contaminants:	Mycotoxins - trichothecenes
Food Types:	Animal feed

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Assessment of deoxynivalenol (DON) adsorbents and characterisation of their efficacy using complementary *in vitro* tests.

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Abstract

Deoxynivalenol (DON) is a prevalent and resistant mycotoxin found in cereals and their related products. Adsorbents appear to provide an opportunity to decrease DON absorption in animals, but it is very difficult to evaluate their real efficacy due to their specificity. It is often senseless to extrapolate results obtained with one mycotoxin to another one and even to extrapolate results obtained *in vitro* in buffer to *in vivo* situation. We have carried out experiments to characterize the properties of potential DON adsorbents. Tests in buffer pH 7 first allowed us to focus on six adsorbents: activated charcoal, cholestyramin, *saccharomyces cerevisiae* mannans, algae β-glycan, fungus β-glycan and leguminous plant. The use of equilibrium sorption models suggested non-saturated phenomenon and involved variable mechanisms according to the specific material. Then, tests with Caco-2 cell model showed a high reduction of DON cytotoxicity on proliferative intestinal cells and of DON absorption by differentiated intestinal cells, when adsorbent was added (except for cholestyramin). Otherwise, values were not always in accordance with those obtained in buffer. Our work allowed us to point out five potential DON adsorbents and to propose a complementary *in vitro* test allowing the enhancement of the determination of adsorbent properties.

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Keywords: mycotoxins; deoxynivalenol; adsorption; intestinal transfer

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Introduction

Fusariotoxins are secondary metabolites from certain species of the *Fusarium* genus, present in soil which probably produces the most important quantity of toxins in temperate Northern hemisphere (North America, Europe or Asia) (Placinta 1999). Among fusariotoxins, deoxynivalenol (DON) is the most frequently detected (Placinta et al. 1999; Bennett and Klich 2003; Mac Donald et al. 2004) and is commonly found in cereals and their related products, and cereal components constituting an important part of animal feed (Miraglia 1996; Bennett and Klich 2003; Sudakin 2003). Studies measuring plasmatic concentration of DON after oral administration demonstrated that the toxin is effectively absorbed by the organism (Prelusky et al. 1986; Prelusky et al. 1988; Azcona-Olivera et al. 1995; Meky et al. 2003). In monogastric animals, and particularly in pigs, this toxin often causes a decrease of alimentary uptake, digestive perturbations, lesions, and sometimes even the animal's death (Larsen et al. 2004; Eriksen and Pettersson 2004; Sklan et al. 2003). Economic loss can have an important impact on breeding and the question of a potential transfer of the toxin or some residue to animal products has not yet been solved.

In spite of preventative efforts, crop contamination is often inevitable (Birzele 2000; Edwards 2004; Schrödter 2004). Furthermore, DON appears very resistant and it is currently really difficult to decontaminate a crop (Aziz et al. 1997; McKenzie et al. 1997; Yumbe-Guevara et al. 2003). One of the alternative solutions is the use of adsorbents. The chemical affinity of some molecules for mycotoxins could allow a reduction of their bioavailability,

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decreasing intestinal absorption, blood concentration and distribution to target organs. Adsorbents are characterised by their affinity, their adsorption capacity and their action mechanisms (Huwig et al. 2001). Substrate properties (polarity, solubility…) intervene too and it is very difficult to evaluate the efficacy of any one adsorbent on any one substrate. Many studies have already dealt with the characterisation of mycotoxin adsorbents, but adsorbent properties appear to be closely dependent on the molecule to be bound. As published data principally focuses on aflatoxins, few results are available for DON or fusariotoxins. All activated or not activated charcoals prove to bind most mycotoxins, and notably trichothecenes although their adsorption capacity may change (Galvano et al. 1998; Avantaggiato et al. 2004). Among aluminosilicate minerals, only montmorillonite, bentonite and sepiolite seem capable to partially adsorb a fusariotoxin, namely zearalenone (ZEA) (respectively 192 µg/g, 112 µg/g and 74 µg/g) (Ramos et al. 1996). Avantaggiato et al. (2004) demonstrated a small but detectable adsorption of fusariotoxins using resins (poly-(vinylpolypyrrolidone) (PVPP) and cholestyramin). It is also more and more common to envisage microorganisms and their cellular wall as mycotoxin adsorbents too, many studies focusing on cell wall components like mannans or β-glycans, capable of binding some mycotoxins *in vitro* (Yiannikouris and Jouany, 2002; Huwig et al., 2001). Other materials are sometimes cited as potential mycotoxin adsorbents and may be of interest: chlorophyllin, Bismuth salts, leguminous plants (Versantvoort et al. 2004; Mata et al. 2004; Scott 1998; Strangoom and Smith 1984).

Moreover, many studies concern *in vitro* binding between adsorbents and mycotoxins but *in vitro* efficacy is not always a reliable indicator of their actual efficacy *in vivo* (Ledoux et al. 2003). In parallel, *in vivo* tests remain quite rare and hardly convincing as many variation factors can influence the animal's response to mycotoxin presence in feed (age, weight, sex, breeding conditions). It is then very difficult to designate effective adsorbents, on the basis of published results. Models simulating conditions in gastrointestinal tract have been

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related (Avantaggiato et al. 2003; Sabater-Vilar et al. 2007) but it would be of great interest to test adsorbent properties in the presence of intestinal cells in order to focus on what happens at the intestinal level. Indeed, the greater part of DON appears to be absorbed at the level of the first intestinal segments (Eriksen et al. 2003; Danicke et al. 2004). The use of a model simulating pig gastrointestinal tract confirmed that DON absorption principally occurred at the small intestinal level, and specified jejunum preponderance (Avantaggiato et al. 2004).

Our purpose was first to determine those adsorbents that effectively bind DON with adsorption/desorption tests. After a screening of 18 potential adsorbents, 6 were selected and their properties for DON characterised. Then, in order to focus on effects at the intestinal level, intestinal cells were used to better describe consequences of the presence of adsorbents in response to 2 questions. On the one hand, our experiments aimed at evaluating an eventual protector role of adsorbents against DON cytotoxicity on proliferative intestinal cells. On the other hand, we studied the adsorbent impact on DON transfer through intestinal epithelium in order to specify an eventual effect on DON bioavailability at intestinal level. These experiments were carried out on the enterocyte model Caco-2. Cultures of Caco-2 cell monolayers, isolated from a human colon carcinoma, are commonly used as a model system to study intestinal uptake and transport processes. Differentiated post-confluent Caco-2 cells exhibit well-developed microvilli and, grown on semi-permeable supports, form tight monolayers with a polarized distribution of brush border enzymes (Artursson et al. 2001).

Material and methods

Chemicals. charcoal, bentonite, sepiolite, *Saccharomyces cerevisiae* and short and long bladed leguminous plant were kindly supplied by NEAR (Nutrition Experimentation Advisers Research) company (Orvault, France). Barley, bacteria, algae, fungus and lichen β-glycans came from Carbomer (San Diego, USA). DON (CAS Number: 51481-10-8) and all

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other chemicals were purchased from Sigma Aldrich (St Quentin Fallavier, France). DON was diluted in ethanol (0.3 M).

3.1. *Adsorption/desorption tests in aqueous buffer*

Screening.

18 adsorbents were tested, corresponding to the principal adsorbent categories (table 1). Adsorption studies were carried out in triplicate, at pH 7 (0.05M phosphate buffer) and pH 3 (0.1M HCl), for 2 DON concentrations (1 and 2 µM), either with or without a constant amount of adsorbent (1 mg/ ml). Incubation was performed in 5 ml centrifugation tubes in a metabolic shaker at 37°C for 90 min. The adsorbent-DON suspension was then centrifuged for 10 min at 10000 rpm. The concentration of unadsorbed DON in supernatant was measured by GC analysis described later. The amount of DON adsorbed by each testing material was calculated according to the difference between the initial and final concentrations of DON in the testing solution.

Adsorption and desorption experiments.

Screening allowed us to determine 6 potential DON adsorbents. Previously described experiments were repeated with buffer solution (pH 7 and 3) containing increasing concentrations of DON (1, 2, 6, 8 10 and 12 µM). Residues of samples obtained at pH 3 were re-suspended in phosphate buffer pH 7, homogenised, centrifuged and supernatants were analysed to highlight an eventual desorption phenomenon.

Use of equilibrium sorption models.

Sorption data was tested against the linear, Freundlich and Langmuir adsorption isotherm models.

Linear isotherm equation was:

$$C_a = a + b\,C_e$$

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where: C_a=DON amount adsorbed at equilibrium (µg/ml); C_e=DON concentration in buffer at equilibrium (µg / ml); a, b=constants determined from the slope and intercept calculated using linear regression (Microsoft Excel function, Excel:PC 2003).

Plotting log C_a against log C_e, we used the linear equation below testing the Freundlich model:

$$\log C_a = \log Kf + (1/n) \log C_e$$

where: C_a=DON amount adsorbed at equilibrium (µg / ml); C_e=DON concentration in buffer at equilibrium (µg / ml); Kf, n= Freundlich constants determined from the slope and intercept calculated using linear regression (Microsoft Excel function, Excel:PC 2003).

The linear form of the Langmuir model as shown below was used:

$$1/C_a = 1/(Kl \times b \times C_e) + (1/b)$$

where: C_a=DON amount adsorbed at equilibrium (µg / ml); C_e=DON concentration in buffer at equilibrium (µg / ml); Kl, b= Langmuir constants determined from the slope and intercept calculated using linear regression (Microsoft Excel function, Excel:PC 2003).

2. *Efficacy tests on Caco-2 cells*

Cell culture.

The Caco-2 cell line was obtained from American Tissue Culture Collection (ATCC N°HTB37) and was used between passage 24 and 34. The cells were maintained in Dulbecco's Modified Eagle's Medium containing 10 % heat-inactivated foetal bovine serum, 1 % non-essential amino acids, 5 % penicillin (100 U/ml) and streptomycin (100 mg/ml) in a humidified atmosphere of 95 % air and 5 % CO_2 at 37°C. Cells were split every 7 days at a density of 10^6 cells/flask. For transport studies, cells were harvested with trypsin-EDTA and seeded on polycarbonate membrane inserts (0.2 µm pore diameter, 25 mm diameter; NUNC, Roskilde, Denmark) at a density of 2 x 10^5 cells/insert. Culture medium was changed 3 times

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a week and cells were used for experiments at day 21 post-seeding, when morphological differentiation was reached (Pinto et al. 1983). Cell culture media and reagents came from Invitrogen (Cergy Pontoise, France). Foetal bovine serum came from Perbio Science (Brebières, France). Culture flasks, and plates came from Falcon (VWR international, Strasbourg, France).

Cytotoxicity tests.

MTT assays were provided by Sigma Aldrich (St Quentin Fallavier, France) ("In vitro toxicology assay kit, MTT based"). Cells were seeded on 96-well plates at a density of 4 x 10^4 cells/well. MTT tests were carried out on proliferative cells at day 3 after seeding: cultures were incubated 24h with DON (0, 1, 10 and 50 µM) either with or without adsorbent material (1 mg/ml), and treated according to the manufacturer's recommendations. Acidified formazan crystals formation was measured at 570 nm, with a reference wavelength of 690 nm. The amount of dye produced was proportional to the number of metabolically active live cells. 6 repetitions were performed.

Transport studies.

The Caco-2 cell monolayers were rinsed twice with phosphate buffered saline and pre-incubated for 30 min at 37°C in Hank's balanced salt solution (HBSS). Apical (AP) and basolateral (BL) chamber volumes were maintained respectively at 1.5 and 2 ml. Transepithelial transport was assayed at day 21 after seeding by adding DON (10 µM) to the apical compartment containing or not adsorbent material (1 mg/ml) and measured for 8 hours. DON was diluted in ethanol so that final volume of ethanol added to the donor compartment did not exceed 0.5 %. At each sampling time, 200 µl of transport medium were withdrawn from the receiver basolateral compartment and replaced by fresh buffer. Four repetitions were carried out.

The same experiments were repeated pre-incubating DON and adsorbent 1 h before exposing cells or incorporating adsorbent material in apical transport medium1 h after DON

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exposure. For every transport study, monolayer integrity was checked by measuring the transepithelial electrical resistance (TEER), using a Millicell ERS (Millipore, Molsheim, France). TEER measurement was performed at the end of both the pre-incubation period and the transport experiments. Monolayers displaying TEER values inferior to 300 $\Omega.cm^2$ or in which TEER values fell more than 15 % were removed.

DON analysis

For the analysis of BL medium in transport experiments, each sample was heated 10 min at 90°C and then centrifuged at 1600 g at room temperature. Every analysed sample was evaporated to dryness under a stream of nitrogen at 60°C (Videmann et al., 2007). The dry residue was derivatized in 25 µl Trisil-TBT with 75 µl hexane, shaken and heated at 60°C for 30 min. It was then extracted with twice 1 ml hexane and phosphate buffer, pH 7 (22% NaH_2PO_4, H_2O 0.2M, pH 4.8, 32% Na_2HPO_4, H_2O 0.2M pH 9.4). Hexane phases were combined and evaporated to dryness under a stream of nitrogen at room temperature. 200 µl of 1,1-bis(p-chlorophenyl)-2,2-dichloroethylene (DDE 20 ng/ml in hexane) was added to the residue as an internal standard. Samples were analyzed by GC-ECD (Hewlett Packard 5890 set II) using a capillary column Rtx-5 (length: 60m, internal diameter: 0.25mm, film thickness: 0.25µm). GC conditions: carrier gas linear velocity, 1.0 ml/min; injector temperature, 250°C; splitless, 0.75 min; detector temperature (^{63}Ni-ECD), 300°C; column oven temperature, hold at 50°C (1 min), programmed 50-250°C at 30°C/min, 270-290°C at 15°C/min, hold at 290°C (12 min), 290-300°C at 30°C/min and hold at 300°C (3 min). The detection limit and the quantification limit were 0.01 µM and 0.03 µM respectively.

4. Statistical analysis.

Results of adsorption tests in aqueous buffer were analysed by comparison of means.

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Cytotoxicity data are expressed as a mean of 6 experiments ± S.D. Transport data are expressed as a mean flux of 3 independent experiments ± S.D. Statistical analysis was performed using one-way analysis of variance (ANOVA), and a p-value of < 0.05 was considered significant.

Results

Screening (Figure 1).

Six adsorbents significantly decreased DON concentration in phosphate buffer, regardless of the level of DON concentration (0.3 and 0.6 mg DON/g adsorbent): activated charcoal (respectively 99.9±0.1% and 98.4±0.4%), cholestyramin (97.8 ± 1.2 % and 68.6 ± 15 %), saccharomyces cerevisiae mannans (84.6±1% and 88.1±4.7%), algae β-glycan (81.1±2.3% and 78.8±2%), fungus β-glycan (80.0±1.9% and 85.6±0.7%) and chlorophyllin (85.1±3.1% and 89.6±0.4 %). Leguminous plant samples presented such heterogeneity that obtained values could show dramatic variations. Short bladed material showed better results than long bladed material (47.3±7.3% and 52.8±7% against 32.3±6.5% and 39.8±8.4%).

DON adsorption at pH 7 and pH 3 (Tables 2 and 3).

Whatever the pH and concentration, activated charcoal led to a decrease of more than 95% of DON concentration in solutions. Cholestyramin reduced DON levels in buffer by about 60% at pH 7. The amount of DON adsorbed by *Saccharomyces cerevisiae* mannans decreased when the DON concentration increased at pH 7. This phenomenon was not observed at pH 3. DON levels in buffers decreased by between 70 and 90% in the presence of algae or fungus β-glycans. DON adsorption by short-bladed leguminous plants remained constant at about 50% at pH 7 but this material hardly decreased DON concentration at pH 3.

Desorption tests never demonstrated a DON desorption phenomenon when residues obtained at pH 3 were re-suspended at pH 7 (data not shown). Linear regression of the linear,

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Freundlich and Langmuir adsorption isotherms for DON at pH 7 and pH 3 lead to their constant determination (Tables 4 and 5). Isotherm adsorption models are usually used to describe pesticide, radionuclide, ZEA adsorption (Avom et al. 2001; Hussein et al. 2004, Ramos et al. 1996). Here, they allowed for a better characterisation of the binding reactions involved. They provided a relation between DON concentration in buffer and the amount of DON absorbed per mg of adsorbent for a given temperature (37°C). The linear model is available for highly diluted medium and stationary experimental conditions. It is based on the hypothesis of unlimited adsorption capacity and constant affinity. The Freundlich model does not need any specific knowledge about adsorbent material and supposes an unlimited number of adsorption sites. Kf constant obtained is proportional to adsorption capacity of the adsorbent and n corresponds to its affinity (Avom et al., 2001; Horsfall and Spiff, 2004). It is believed that adsorption is significant as soon as 1/n is lower than 1. The Langmuir model supposes only one surface site type, an instantaneous and reversible binding, then a dynamic equilibrium, a limited number of fixation sites and an adsorption that reaches a steady state. The lower b is, the higher the adsorbent affinity (Hussein et al., 2004). Kl corresponds to the energy needed for binding.

Adsorbent effects on DON cytotoxicity on proliferative Caco-2 cells.

On the one hand, results obtained with an adsorbent alone showed no significant cytotoxic effects on proliferative intestinal cells, except with cholestyramin where cell viability decreased by 31 ± 3.5% after a 24 h exposure (Figure 2). On the other hand, after DON exposure, cell viability slightly decreased by 15±1% for a DON concentration of 1µM and was very significantly diminished for higher concentrations (respectively 35±1 and 44±2% for 10 and 50µM) (Figure 3). With the exception of cholestyramin, every adsorbent material succeeded in maintaining at least a higher cell viability than the control, for every

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DON concentration. Each result obtained was not significantly different to control samples without DON.

Adsorbent effects on DON transfer in Caco-2 cells.

Even in wells exposed to cholestyramin, TEER values reached 400 ± 10 Ω.cm^2 at the beginning of the transfer experiment and 450 ± 13 Ω.cm^2 after. These measurements indicated no diminution of epithelium integrity during transfer experiments, assessing confluent and viable cells. Cholestyramin no longer prevented DON transfer after as little as 1 h. The results showed that the presence of another adsorbent in apical medium leads to a decrease of DON transfer in basal medium (Figure 4). The pre-incubation of activated charcoal, cholestyramin or mannans with DON did not influence DON transfer in intestinal cells (Figure 5). However, incubating leguminous plant, algae or fungus β-glycans with DON 1 h before cell exposure led to a higher inhibition of DON transepithelial transfer.

After 1 h exposure, DON transfer (without adsorbent) was about 5% of the total amount transferred in 6 h. Addition of an adsorbent after 1 h exposure limited DON transfer but it needed 3 h to reach levels similar to those obtained when DON and adsorbent were added together. The presence of cholestyramin in apical medium 1 h after DON contamination had no consequence on the toxin transfer in epithelium.

Discussion

We confirmed with this study that it is difficult to extrapolate adsorption results obtained with one mycotoxin to another one, notably because of adsorbent specificity (Phillips 1999). Our work, combining different *in vitro* tests, has permitted us to increase our understanding of DON adsorption and to corroborate or refute different hypotheses presented in the literature. Screening concentrations were chosen according to presumed norms in Europe for DON in cereals (1.25 to 1.75 mg/kg) and the fact that adsorbent rate in animal

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feed can vary from 1 to 5 kg/t. We obtained a rate between DON and adsorbent of about 0.25 to 1.7 mg/g, corresponding to about 0.75 to 5.1 µM DON with our dilutions. Screening allowed us to identify 6 potential DON adsorbents, characteristics of which remained to be specified. At studied doses, DON concentration did not show significant influence on adsorption.

Our results supported the hypothesis that DON adsorption by activated charcoal would be of about 90% for DON concentrations lower than 2 mg/g and where the pH was between 3 and 8 (Avantaggiato et al. 2004; Galvano et al. 1998; Galvano et al. 2001). Variations of adsorbed DON were maybe principally due to the charcoal used. However, Avantaggio et al. (2004) described a 55% DON diminution for a concentration of 10 mg/g, suggesting that DON linkage to charcoal might be a saturable mechanism for very high toxin concentrations. Activated charcoal combined a high adsorption capacity and an important efficacy whatever the pH was, according to the Freundlich adsorption isotherm. Charcoal did not seem to significantly bind DON when not activated.

We demonstrated, as did Avantaggiato et al. (2004) in a previous study, the significant effect of cholestyramin on DON concentration in buffer at pH 7. Using adsorption isotherms, the resin showed average efficacy and capacity. However, the previous work showed an adsorption of 10 ± 1 % and 4 ± 7 % for concentrations of 2 and 10 mg/g, at pH 8, whereas our results gave a rate of about 60 % at pH 7 for concentrations corresponding to 0.3 to 3.6 mg/g. DON adsorption by cholestyramin could then be a saturable phenomenon. It seemed that pH had a great influence on cholestyramin efficacy too as the amount of DON adsorbed remained dramatically low at pH 3. It is believed that mannans can adsorb many mycotoxins, notably fusariotoxins, and to a better extent than the other categories of adsorbents (Devegowda and Castaldo, 2000, cited by Yiannikouris and Jouany, 2002). Our study confirmed that *Saccharomyces cerevisiae* mannans could bind DON and specified that

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DON adsorption rate decreased as DON concentration increased. Adsorption isotherms suggested they have an average efficacy and capacity at both pH.

Many recent studies are based on cell wall components, and β-glycans in particular. We tested different β-glycans and it appeared that bound type (1-3 or 1-6) or ramification might not be sufficient criteria to characterise their adsorption properties. Among the glycans tested, algae and fungus ones showed a better and quite efficient adsorption of DON. pH appeared to have an impact on these 2 β-glycans properties as they presented a low efficacy but a high adsorption capacity at pH 7, and the inverse at pH 3.

Chlorophyllin showed a highly significant effect on DON but its prohibited use in animal feed makes it of no interest. However, as it is composed of chlorophyll and Cu, it could be suggested that chlorophyll has an adsorption action on DON. In parallel, we expected long-bladed leguminous plants to be more efficient than short-bladed ones as Strangoom and Smith (1984) had observed that the fibrous fraction of alfalfa could neutralise negative ZEA effects on growth by rats, but we observed that short-bladed material showed a better adsorption rate than long-bladed material. Adsorption mechanisms in leguminous plant might involve chlorophyllin more than fibre. These results corroborated the hypothesis concerning the potential of chlorophyllin in DON adsorption.

The use of the three equilibrium sorption models permitted a better understanding of the mechanisms involved in DON adsorption in relation to the considered material and gave results in line with DON adsorption rate in buffer. Whatever the material and the pH, the linear model appeared the least adapted. Moreover, it was sometimes difficult to discern which of the Freundlich or the Langmuir model was the most suitable, maybe because used concentrations did not allow us to reach the final steady-state if fixation sites were limited. This suggested that phenomenon of adsorption was not saturated for any material at the given concentration. Constants of the Freundlich model demonstrated that mechanisms involved in DON adsorption were different according to the considered material.

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In contrast, pH showed itself to be a determinant factor, the rate of DON adsorbed decreasing with pH. Some material like cholestyramin or leguminous plant no longer bound DON at pH 3. Considering pH, tests in pH 3 buffer reflected stomach condition and tests in pH 7 buffer, those, approximatively, of the small intestine. Adsorbents may then be more effective at the intestinal level, the principal absorption site.

These results could not be extrapolated to an estimation of *in vivo* adsorption but it is possible to foresee a real decrease of DON absorption. To better comprehend the consequences of adding an adsorbent to animal feed on DON effects, it appeared determinant to specify what happens at the intestinal epithelium level. Caco-2 cell model first allowed assessing consequence of adsorbent use on proliferative intestinal cell viability. It indicated that only cholestyramin decreased cell viability and then might provoke lesions at the level of intestinal integrity.

DON showed a growing cytotoxicity on proliferative intestinal cells as its concentrations increased. Except for cholestyramin, the presence of adsorbent material permitted to highly decrease, indeed to cancel, DON cytotoxicity on proliferative intestinal cells. Then, we focused on adsorbent effects on intestinal transepithelial transfer of DON. DON transfer decrease remained quite stable except for cholestyramin and mannans which showed a more and more increasing transfer, suggesting adsorption mechanisms depending on a thermodynamic equilibrium function of DON concentration in medium. Comparing to experiments in pH 7 buffer at same toxin concentration, activated charcoal and algae β-glycan showed an inhibition of DON transfer in Caco-2 cells similar to their DON adsorption in buffer. Otherwise, cholestyramin and in a lesser extent fungus β-glycan presented a decrease of transferred DON less important than adsorption rates in buffer. In parallel, leguminous plant appeared more effective to diminish DON transfer than its adsorption tests could predict. These results were in concordance with the impossibility to increase the scope of buffer tests to *in vivo* reality. Intestinal cell absorption decreased apical DON concentration

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and made then varying apical medium conditions, phenomenon that could have repercussions on transferred DON amount. It is also possible that competition mechanisms were involved between absorption and adsorption sites.

Adsorbent effects could also depend on the way they are administered. It was then of great interest to test, on the one hand how pre-incubation of DON and adsorbent (as in animal feed) or, in the other hand, the addition of adsorbent after DON exposure (evaluating their efficacy subsequent to a contamination) could influence the toxin transfer in Caco-2 cells. These experiments could also allow us to better interpret the mechanisms involved. Results demonstrated that pre-incubation allowed a more efficient adsorption for leguminous plant, algae and fungus β-glycans. DON adsorption seemed thus to need an interval of time for these adsorbents. Pre-incubation globally led to a rapid and better diminution of DON absorption *in vitro*. This hypothesis should be tested *in vivo* to assess if the addition of adsorbents to feed could enhance diminution of DON bioavailability. In mice, it was demonstrated that smectite (2g/kg) did not succeed in preventing T-2 toxin (1mg/kg) affecting intestinal transit, but a 24h pre-incubation period inhibited all the toxin effects (Fioramont et al., 1987). Otherwise, the addition of an adsorbent after DON exposure could limit DON absorption, but only after at least a 3h delay. We could extrapolate that in case of ingestion of contaminated feed, adsorbent administration could reduce DON absorption at the intestinal level. The acceptable delay could not be determined here, but as it seems obvious that adsorbent efficacy weakens with time, as then the delay has to be as short as possible.

Conclusion

Due to the specificity of adsorbents, it is more often difficult to extrapolate adsorption results obtained with one mycotoxin to another one (Phillips, 1999). Here, interesting DON adsorbents were not always those already referenced for other mycotoxins, even for fusariotoxins. It was then of great interest to carry out specific tests with DON. Furthermore,

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it appeared difficult to simply evaluate the efficacy of an adsorbent using only one mycotoxin because neither *in vitro* nor *in vivo* tests described in literature prove satisfactory. We proposed here experiments that allowed for a better evaluation of the efficacy of an adsorbent, even if results remain to be tested *in vivo*.

It appeared difficult to classify DON adsorbents because of their heterogeneity. But it seems conceivable that a mix of different adsorbents would allow an additivity of their efficacy, or even a synergy due to their specific characteristics. Our results can help to elaborate not the adsorbent but a mix of adsorbents usable in animal feed. It would be all the more interesting to explore this as multi-contamination is often observed in cereals and adsorbent material has to prove efficient at dealing with several mycotoxins.

Acknowledgments

We thank NEAR Company for its participation in this work.

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	Adsorbent material	provider
Charcoals	Charcoal	NEAR
	Activated charcoal (3500 m²/g)	Sigma Aldrich
Aluminosilicate minerals	Bentonite	NEAR
	Montmorillonite	Sigma Aldrich
	Sepiolite	NEAR
Resins	Cholestyramin	Sigma Aldrich
	Poly(vinylpolypyrrolidone) (PVPP)	Sigma Aldrich
Microorganisms	Beer yeast	NEAR
	Mannans (*Saccharomyces cerevisiae*)	Sigma Aldrich
β-glycans	Barley β-glycan (1,3-1,4-β-D-glycan)	Carbomer
	Bacteria β-glycan (1,3-β-D-glycan)	Carbomer
	Algae β-glycan (1,3-β-D-glycan)	Carbomer
	Fungus β-glycan (1,3-β-D-glycan ramified in 1,6-β-D-glycan-ramification degree: 2/5)	Carbomer
	Lichen β-glycan (1,6-β-D-glycan)	Carbomer
Others	Bismuth subcarbonate	Sigma Aldrich
	Chlorophyllin	Sigma Aldrich
	Leguminous plant short bladed	NEAR
	Leguminous plant long bladed	NEAR

Table 1. Materials tested in aqueous buffer tests.

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	DON concentration (μM)					
	1	2	6	8	10	12
activated charcoal	99.9±0.1	98.9±0.4	98.8±0.6	99.2±0.0	95.1±2.3	96.5±0.1
cholestyramin	64.9±5.0	68.6±5.2	56.0±0.6	66.5±2.0	69.8±2.4	68.0±5.8
mannans	84.6±1.0	88.1±4.7	69.8±0.6	67.1±2.9	61.7±3.7	62.8±3.5
algae β-glycan	81.1±2.0	78.8±2.0	84.4±3.1	81.3±3.6	67.2±0.3	74.6±1.7
fungus β-glycan	80.0±1.9	85.6±0.7	92.8±0.9	92.2±0.6	92.8±4.6	92.6±0.8
LPSB	47.3±7.3	52.8±7.0	54.7±1.5	50.3±1.3	55.6±1.3	48.8± 0.4

Table 2. Percentages of DON bound to different adsorbents (1 mg/ml) in pH 7 buffer. (n = 3)

Note: LPSB=leguminous plant short bladed.

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	DON concentration (μM)					
	1	2	6	8	10	12
activated charcoal	99.2±0.2	98.5±0.3	98.6±0.2	97.6±1.0	98.4±0.2	98.4±0.4
cholestyramin	24.0±5.8	19.9±3.3	12.0±12.0	7.5±2.8	10.5±3.4	4.9±0.9
mannans	89.0±2.7	86.1±2.4	91.8±0.5	93.1±3.6	89.2±1.8	91.1±1.5
algae β-glycan	78.1±2.5	85.3±2.6	87.0±2.9	90.8±0.5	88.3±2.2	90.6±2.0
fungus β-glycan	98.4±0.5	91.5±0.6	79.9±3.0	80.3±0.9	79.8±1.2	78.8±1.5
LPSB	24.4±0.4	7.7±1.9	4.0±5.8	2.4±0.5	2.3±6.4	2.6±0.9

Table 3. Percentages of DON bound to different adsorbents (1 mg/ml) in pH 3 buffer.(n = 3)

Note: LPSB=leguminous plant short bladed.

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	linear	Freundlich			Langmuir		
	R^2	Kf	1/n	R^2	Kl	b	R^2
activated charcoal	0.45	7.37	0.39	0.77	1023.57	1.40	0.61
cholestyramin	0.68	1.71	1.00	0.88	0.97	2.15	0.75
mannans	0.84	1.80	0.53	0.89	4.69	1.90	0.72
algae β-glycan	0.71	2.85	0.78	0.87	0.73	6.50	0.91
fungus β-glycan	0.81	39.08	1.71	0.86	-3.90	-0.93	0.84
LPSB	0.91	1.17	1.07	0.91	0.05	22.68	0.69

Table 4. Linear, Freundlich and Langmuir constants obtained from linear regression of DON adsorption isotherms at pH 7. (n = 18)

Note: LPSB=leguminous plant short bladed.

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	linear	Freundlich			Langmuir		
	R^2	Kf	1/n	R^2	Kl	b	R^2
activated charcoal	0.56	20.21	0.68	0.85	58.77	2.62	0.86
cholestyramin	*0.20*	*0.13*	*0.35*	*0.34*	*2.37*	*0.20*	*0.33*
mannans	0.77	8.26	0.92	0.85	5.37	2.24	0.70
algae β-glycan	0.63	10.62	1.24	0.80	-1.72	-2.39	0.91
fungus β-glycan	0.93	2.74	0.48	0.95	-3.90	-0.93	0.77
LPSB	*0.07*	*0.07*	*0.17*	*0.07*	*45.39*	*0.07*	*0.00*

Table 5. Linear, Freundlich and Langmuir constants obtained from linear regression of DON adsorption isotherms at pH 3. (n = 18)

Note: LPSB=leguminous plant short bladed.

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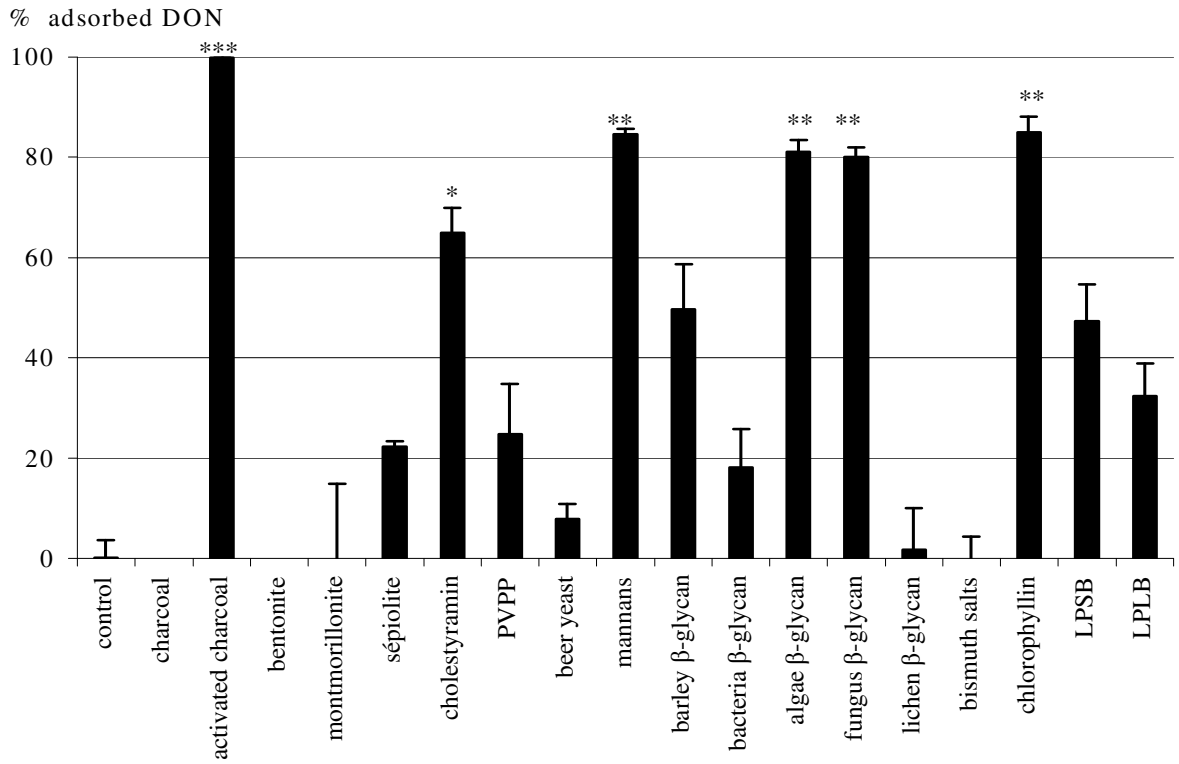
a.



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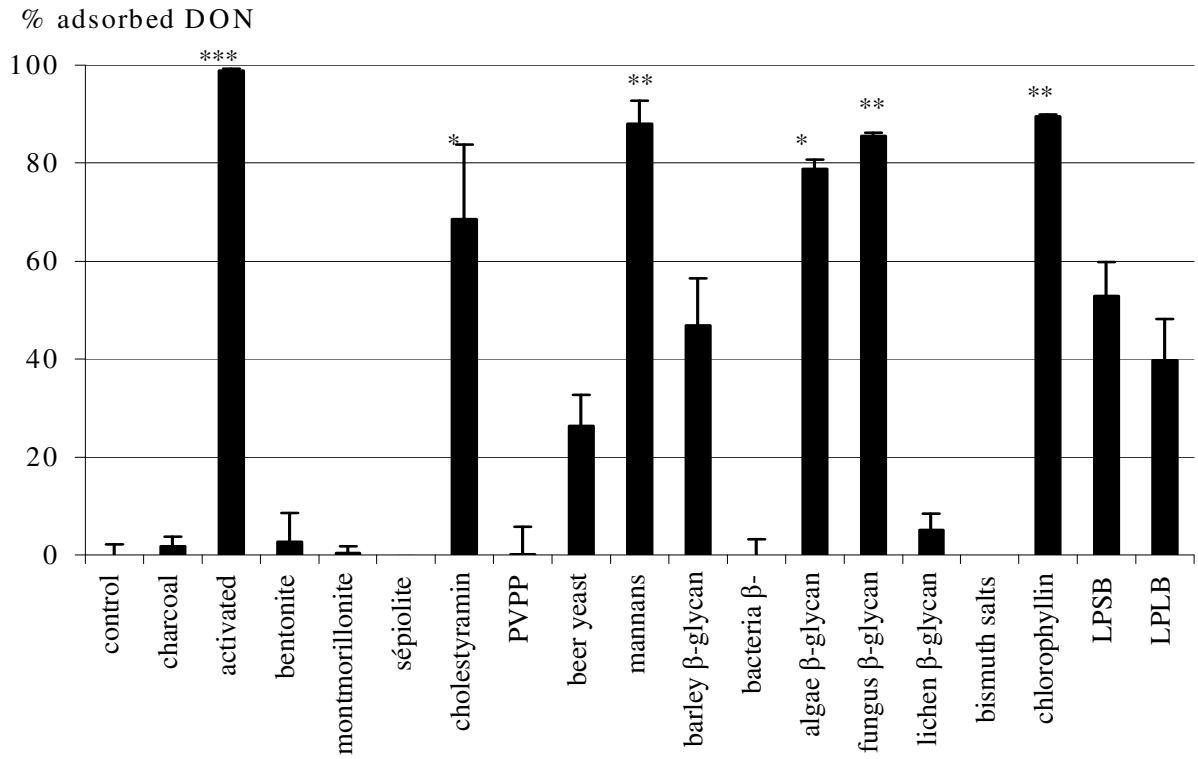

Figure 1. Adsorbed deoxynivalenol part in pH7 buffer for 2 toxin concentrations (1 µM (a.) and 2 µM (b.)) depending on adsorbent material.(LPSB= leguminous plant short bladed; LPLB= Leguminous plant long bladed)

* statistically different from control, $p < 0.05$ ** statistically different from control, $p < 0.01$
*** statistically different from control, $p < 0.001$

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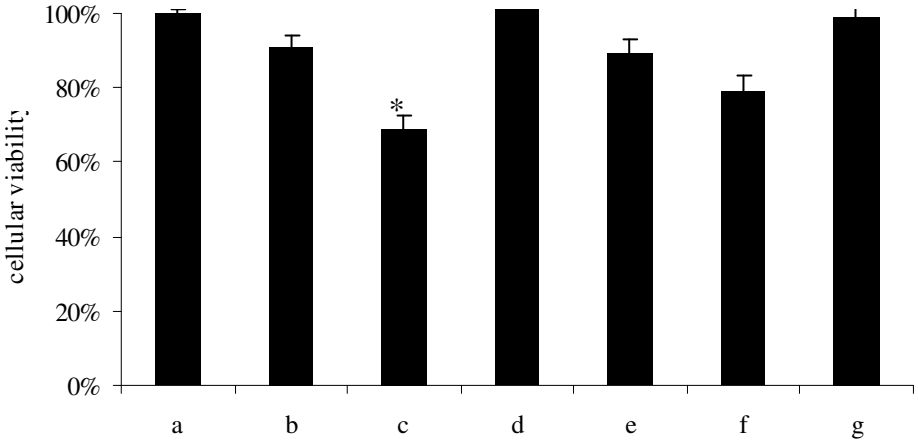

a: control b: activated charcoal c: cholestyramin
d: mannans e: algae β-glycan f: fungus β-glycan
g: leguminous plant short bladed

Figure 2. Adsorbent effects on cellular viability of proliferative Caco-2 cells.

Data are mean values ± SD (n=6) expressed as percentage of control response.

* statistically different from control cell monolayers, $p < 0.05$.

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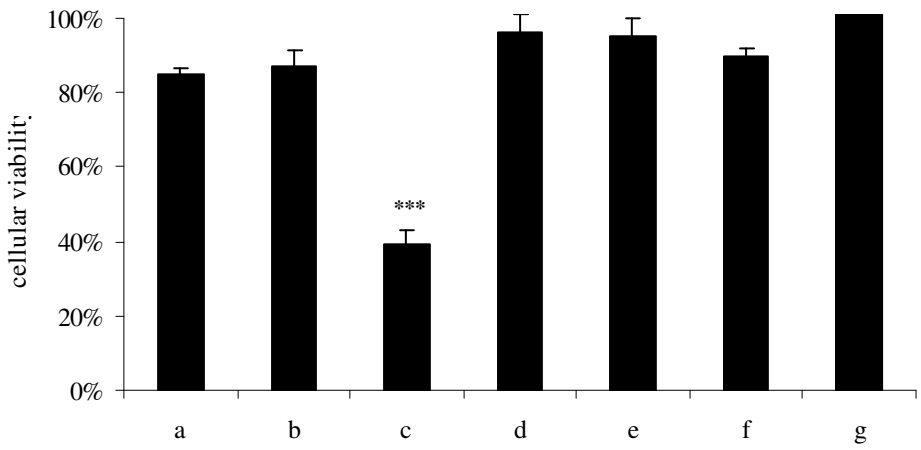
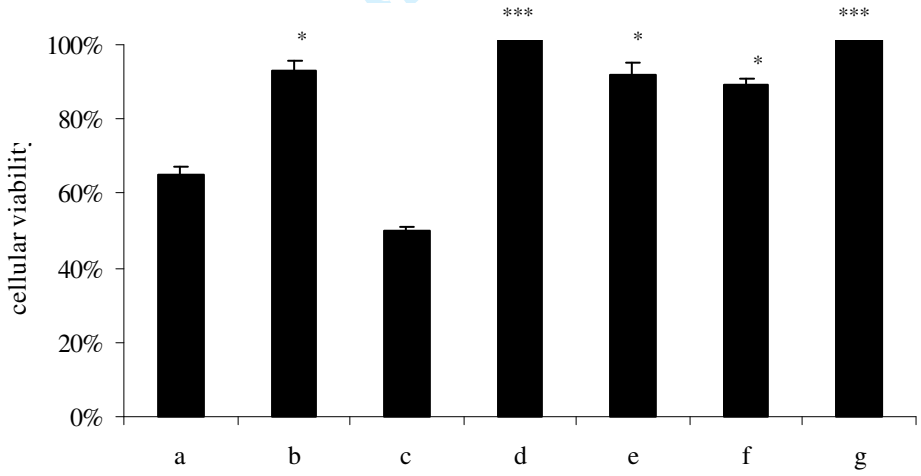
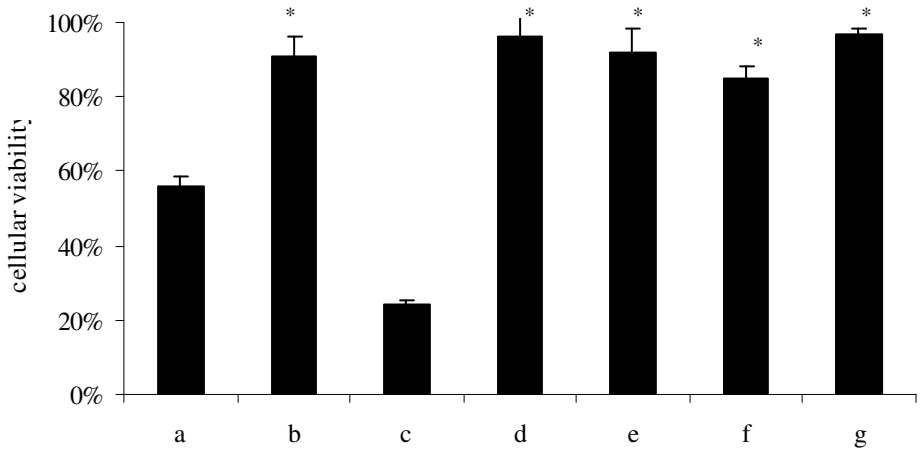
1 μM



10 μM



50 μM



a: control b: activated charcoal c: cholestyramin
d: mannans e: algae β-glycan f: fungus β-glycan
g: leguminous plant short bladed

Figure 3. Adsorbent effects on cellular viability of proliferative Caco-2 cells exposed to different concentrations of DON (1, 10 and 50 µM).

Data are mean values ± SD (n=6) expressed as percentage of control response.

* statistically different from control cell monolayers, $p < 0.05$.

** statistically different from control cell monolayers, $p < 0.01$.

*** statistically different from control cell monolayers, $p < 0.001$.

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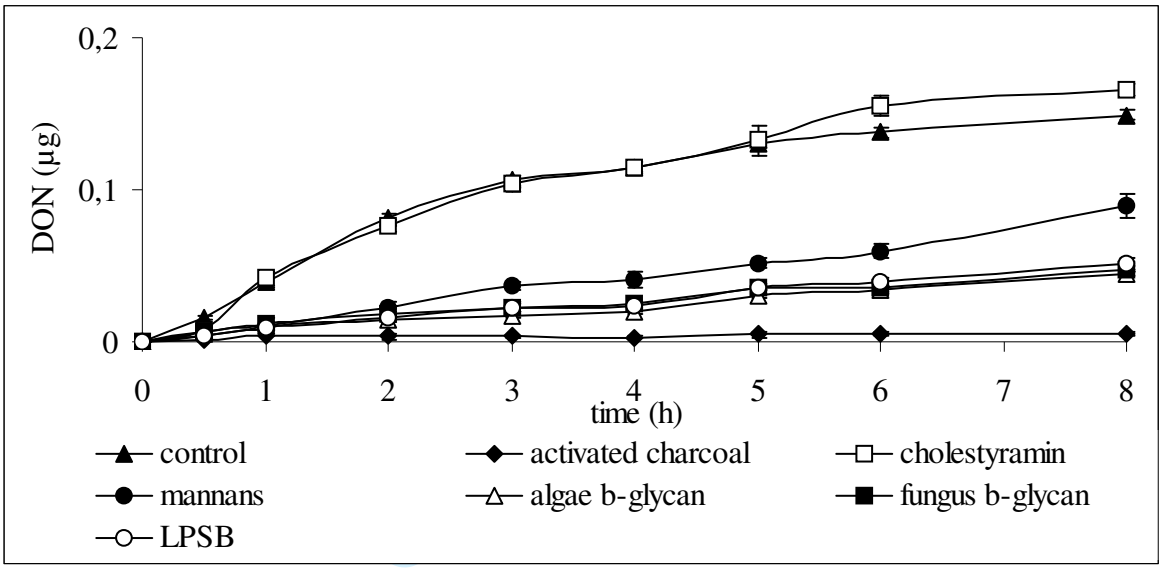

Figure 4. DON transfer in basolateral compartment after an apical exposure (10 µM) with or without adsorbent (1mg/ml). (n = 4)

Note: LPSB=leguminous plant short bladed.

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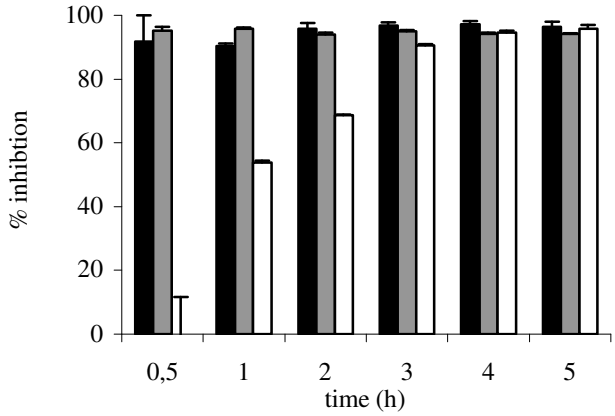

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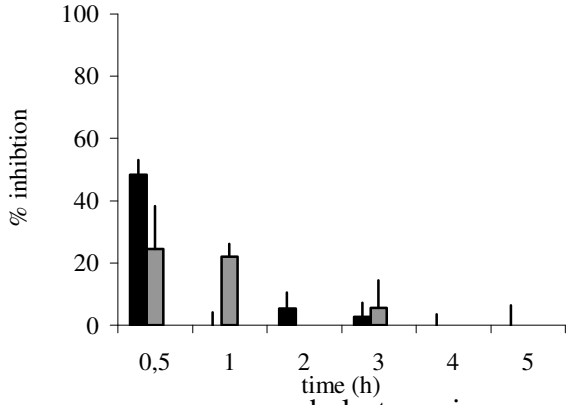
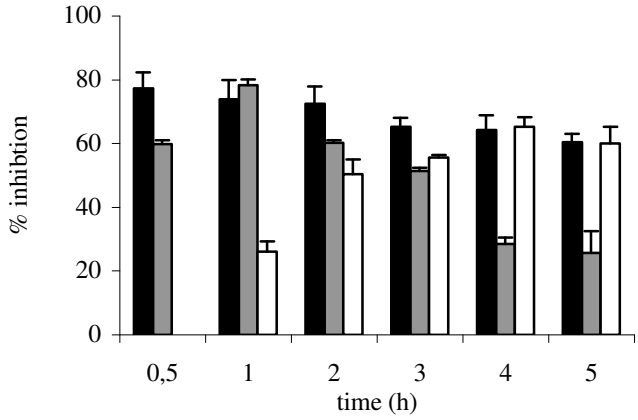
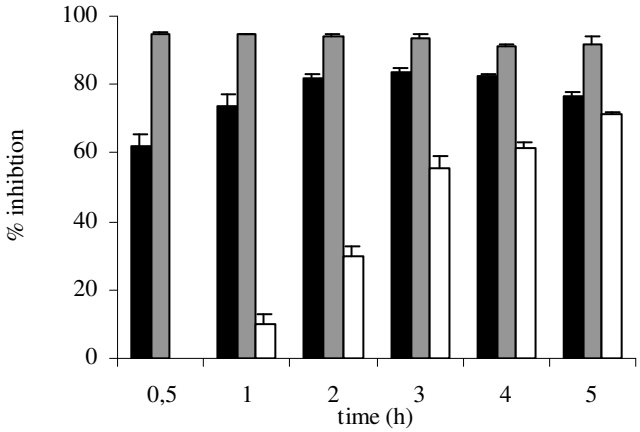


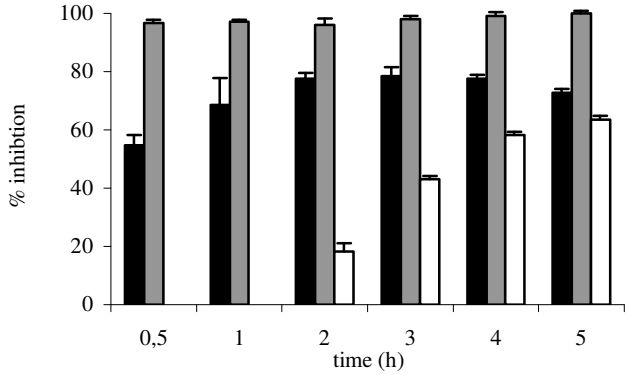
cholestyramin



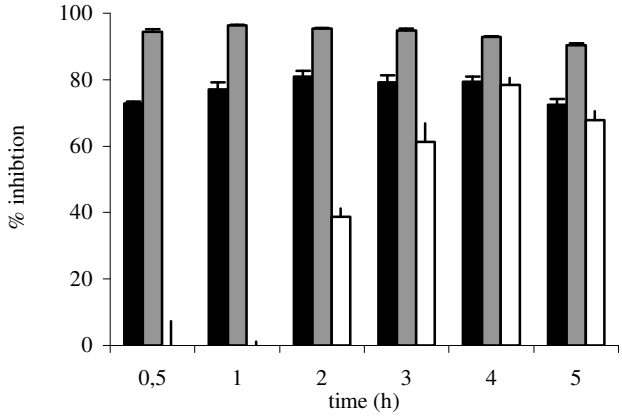
mannans



algae β-glycan



fungus β-glycan



LPSB

Figure 5: Inhibition percentages of deoxynivalenol (10 µM) transfer in Caco-2 cell monolayers when adding different adsorbents (1mg/ml) according to 3 modalities: simultaneous exposure (■), adsorbent+deoxinivalenol incubation 1 h before cell exposure (preincubation: ■) or adsorbent addition 1h after cell exposure to DON (post-exposure: □). (n=4).

Note: LPSB=leguminous plant short bladed.

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