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Determination of DON adsorbents and characterisation of their efficacy using complementary in vitro tests.

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3 1 **Assessment of deoxynivalenol (DON) adsorbents and characterisation of**
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6 2 **their efficacy using complementary *in vitro* tests.**
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12 4 S. Cavret^{*}, N. Laurent, B. Videmann, M. Mazallon and S. Lecoer
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23
24

25 9
26
27 10 **Abstract**
28

29
30 11 Deoxynivalenol (DON) is a prevalent and resistant mycotoxin found in cereals and their
31
32 12 related products. Adsorbents appear to provide an opportunity to decrease DON absorption in
33
34 13 animals, but it is very difficult to evaluate their real efficacy due to their specificity. It is often
35
36 14 senseless to extrapolate results obtained with one mycotoxin to another one and even to
37
38 15 extrapolate results obtained *in vitro* in buffer to *in vivo* situation. We have carried out
39
40 16 experiments to characterize the properties of potential DON adsorbents. Tests in buffer pH 7
41
42 17 first allowed us to focus on six adsorbents: activated charcoal, cholestyramin, *saccharomyces*
43
44 18 *cerevisiae* mannans, algae β -glycan, fungus β -glycan and leguminous plant. The use of
45
46 19 equilibrium sorption models suggested non-saturated phenomenon and involved variable
47
48 20 mechanisms according to the specific material. Then, tests with Caco-2 cell model showed a
49
50 21 high reduction of DON cytotoxicity on proliferative intestinal cells and of DON absorption by
51
52 22 differentiated intestinal cells, when adsorbent was added (except for cholestyramin).
53
54 23 Otherwise, values were not always in accordance with those obtained in buffer. Our work
55
56 24 allowed us to point out five potential DON adsorbents and to propose a complementary *in*
57
58 25 *vitro* test allowing the enhancement of the determination of adsorbent properties.
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1

2 **Keywords:** mycotoxins; deoxynivalenol; adsorption; intestinal transfer

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5

6 **Introduction**

7 Fusariotoxins are secondary metabolites from certain species of the *Fusarium* genus,
8 present in soil which probably produces the most important quantity of toxins in temperate
9 Northern hemisphere (North America, Europe or Asia) (Placinta 1999). Among fusariotoxins,
10 deoxynivalenol (DON) is the most frequently detected (Placinta et al. 1999; Bennett and
11 Klich 2003; Mac Donald et al. 2004) and is commonly found in cereals and their related
12 products, and cereal components constituting an important part of animal feed (Miraglia
13 1996; Bennett and Klich 2003; Sudakin 2003). Studies measuring plasmatic concentration of
14 DON after oral administration demonstrated that the toxin is effectively absorbed by the
15 organism (Prelusky et al. 1986; Prelusky et al. 1988; Azcona-Olivera et al. 1995; Meko et al.
16 2003). In monogastric animals, and particularly in pigs, this toxin often causes a decrease of
17 alimentary uptake, digestive perturbations, lesions, and sometimes even the animal's death
18 (Larsen et al. 2004; Eriksen and Pettersson 2004; Sklan et al. 2003). Economic loss can have
19 an important impact on breeding and the question of a potential transfer of the toxin or some
20 residue to animal products has not yet been solved.

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

1 decreasing intestinal absorption, blood concentration and distribution to target organs.
2
3 Adsorbents are characterised by their affinity, their adsorption capacity and their action
4
5 mechanisms (Huwig et al. 2001). Substrate properties (polarity, solubility...) intervene too
6
7 and it is very difficult to evaluate the efficacy of any one adsorbent on any one substrate.
8
9 Many studies have already dealt with the characterisation of mycotoxin adsorbents, but
10
11 adsorbent properties appear to be closely dependent on the molecule to be bound. As
12
13 published data principally focuses on aflatoxins, few results are available for DON or
14
15 fusariotoxins. All activated or not activated charcoals prove to bind most mycotoxins, and
16
17 notably trichothecenes although their adsorption capacity may change (Galvano et al. 1998;
18
19 Avantaggiato et al. 2004). Among aluminosilicate minerals, only montmorillonite, bentonite
20
21 and sepiolite seem capable to partially adsorb a fusariotoxin, namely zearalenone (ZEA)
22
23 (respectively 192 µg/g, 112 µg/g and 74 µg/g) (Ramos et al. 1996). Avantaggiato et al. (2004)
24
25 demonstrated a small but detectable adsorption of fusariotoxins using resins (poly-
26
27 (vinylpolypyrrolidone) (PVPP) and cholestyramin). It is also more and more common to
28
29 envisage microorganisms and their cellular wall as mycotoxin adsorbents too, many studies
30
31 focusing on cell wall components like mannans or β-glycans, capable of binding some
32
33 mycotoxins *in vitro* (Yiannikouris and Jouany, 2002; Huwig et al., 2001). Other materials are
34
35 sometimes cited as potential mycotoxin adsorbents and may be of interest: chlorophyllin,
36
37 Bismuth salts, leguminous plants (Versantvoort et al. 2004; Mata et al. 2004; Scott 1998;
38
39 Strangoom and Smith 1984).

40
41
42 Moreover, many studies concern *in vitro* binding between adsorbents and mycotoxins
43
44 but *in vitro* efficacy is not always a reliable indicator of their actual efficacy *in vivo* (Ledoux
45
46 et al. 2003). In parallel, *in vivo* tests remain quite rare and hardly convincing as many
47
48 variation factors can influence the animal's response to mycotoxin presence in feed (age,
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50 weight, sex, breeding conditions). It is then very difficult to designate effective adsorbents, on
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52 the basis of published results. Models simulating conditions in gastrointestinal tract have been
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3 1 related (Avantaggiato et al. 2003; Sabater-Vilar et al. 2007) but it would be of great interest
4
5 2 to test adsorbent properties in the presence of intestinal cells in order to focus on what
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7 3 happens at the intestinal level. Indeed, the greater part of DON appears to be absorbed at the
8
9 4 level of the first intestinal segments (Eriksen et al. 2003; Danicke et al. 2004). The use of a
10
11 5 model simulating pig gastrointestinal tract confirmed that DON absorption principally
12
13 6 occurred at the small intestinal level, and specified jejunum preponderance (Avantaggiato et
14
15 7 al. 2004).

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19
20 8 Our purpose was first to determine those adsorbents that effectively bind DON with
21
22 9 adsorption/desorption tests. After a screening of 18 potential adsorbents, 6 were selected and
23
24 10 their properties for DON characterised. Then, in order to focus on effects at the intestinal
25
26 11 level, intestinal cells were used to better describe consequences of the presence of adsorbents
27
28 12 in response to 2 questions. On the one hand, our experiments aimed at evaluating an eventual
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30 13 protector role of adsorbents against DON cytotoxicity on proliferative intestinal cells. On the
31
32 14 other hand, we studied the adsorbent impact on DON transfer through intestinal epithelium in
33
34 15 order to specify an eventual effect on DON bioavailability at intestinal level. These
35
36 16 experiments were carried out on the enterocyte model Caco-2. Cultures of Caco-2 cell
37
38 17 monolayers, isolated from a human colon carcinoma, are commonly used as a model system
39
40 18 to study intestinal uptake and transport processes. Differentiated post-confluent Caco-2 cells
41
42 19 exhibit well-developed microvilli and, grown on semi-permeable supports, form tight
43
44 20 monolayers with a polarized distribution of brush border enzymes (Artursson et al. 2001).

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51 21

52 53 54 22 **Material and methods**

55
56 23 Chemicals. charcoal, bentonite, sepiolite, *Saccharomyces cerevisiae* and short and
57
58 24 long bladed leguminous plant were kindly supplied by NEAR (Nutrition Experimentation
59
60 25 Advisers Research) company (Orvault, France). Barley, bacteria, algae, fungus and lichen β -
26 26 glycans came from Carbomer (San Diego, USA). DON (CAS Number: 51481-10-8) and all

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2
3 1 other chemicals were purchased from Sigma Aldrich (St Quentin Fallavier, France). DON
4
5 2 was diluted in ethanol (0.3 M).
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10 **3.1. Adsorption/desorption tests in aqueous buffer**

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12 4 *Screening.*

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15 5 18 adsorbents were tested, corresponding to the principal adsorbent categories (table
16
17 1). Adsorption studies were carried out in triplicate, at pH 7 (0.05M phosphate buffer) and pH
18
19 3 (0.1M HCl), for 2 DON concentrations (1 and 2 μM), either with or without a constant
20
21 7 amount of adsorbent (1 mg/ ml). Incubation was performed in 5 ml centrifugation tubes in a
22
23 8 metabolic shaker at 37°C for 90 min. The adsorbent-DON suspension was then centrifuged
24
25 9 for 10 min at 10000 rpm. The concentration of unadsorbed DON in supernatant was measured
26
27 10 by GC analysis described later. The amount of DON adsorbed by each testing material was
28
29 11 calculated according to the difference between the initial and final concentrations of DON in
30
31 12 the testing solution.
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38 14 *Adsorption and desorption experiments.*

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40
41 15 Screening allowed us to determine 6 potential DON adsorbents. Previously
42
43 16 described experiments were repeated with buffer solution (pH 7 and 3) containing increasing
44
45 17 concentrations of DON (1, 2, 6, 8 10 and 12 μM). Residues of samples obtained at pH 3 were
46
47 18 re-suspended in phosphate buffer pH 7, homogenised, centrifuged and supernatants were
48
49 19 analysed to highlight an eventual desorption phenomenon.
50
51

52 20 *Use of equilibrium sorption models.*

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54
55 21 Sorption data was tested against the linear, Freundlich and Langmuir adsorption
56
57 22 isotherm models.

58
59 23 Linear isotherm equation was:

60
24
$$C_a = a + b C_e$$

1 where: C_a =DON amount adsorbed at equilibrium ($\mu\text{g/ml}$); C_e =DON concentration in buffer
2 at equilibrium ($\mu\text{g / ml}$); a, b=constants determined from the slope and intercept calculated
3 using linear regression (Microsoft Excel function, Excel:PC 2003).

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

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

7 where: C_a =DON amount adsorbed at equilibrium ($\mu\text{g / ml}$); C_e =DON concentration in buffer
8 at equilibrium ($\mu\text{g / ml}$); K_f , n = Freundlich constants determined from the slope and intercept
9 calculated using linear regression (Microsoft Excel function, Excel:PC 2003).

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

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

12 where: C_a =DON amount adsorbed at equilibrium ($\mu\text{g / ml}$); C_e =DON concentration in buffer
13 at equilibrium ($\mu\text{g / ml}$); K_l , b = Langmuir constants determined from the slope and intercept
14 calculated using linear regression (Microsoft Excel function, Excel:PC 2003).

162. *Efficacy tests on Caco-2 cells*

17 *Cell culture.*

18 The Caco-2 cell line was obtained from American Tissue Culture Collection (ATCC
19 N^oHTB37) and was used between passage 24 and 34. The cells were maintained in
20 Dulbecco's Modified Eagle's Medium containing 10 % heat-inactivated foetal bovine serum,
21 1 % non-essential amino acids, 5 % penicillin (100 U/ml) and streptomycin (100 mg/ml) in a
22 humidified atmosphere of 95 % air and 5 % CO₂ at 37°C. Cells were split every 7 days at a
23 density of 10⁶ cells/flask. For transport studies, cells were harvested with trypsin-EDTA and
24 seeded on polycarbonate membrane inserts (0.2 μm pore diameter, 25 mm diameter; NUNC,
25 Roskilde, Denmark) at a density of 2 x 10⁵ cells/insert. Culture medium was changed 3 times

1
2
3 1 a week and cells were used for experiments at day 21 post-seeding, when morphological
4
5 2 differentiation was reached (Pinto et al. 1983). Cell culture media and reagents came from
6
7
8 3 Invitrogen (Cergy Pontoise, France). Foetal bovine serum came from Perbio Science
9
10 4 (Brebieres, France). Culture flasks, and plates came from Falcon (VWR international,
11
12 5 Strasbourg, France).

13
14
15 6 *Cytotoxicity tests.*

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17 7 MTT assays were provided by Sigma Aldrich (St Quentin Fallavier, France) (“In vitro
18
19 8 toxicology assay kit, MTT based”). Cells were seeded on 96-well plates at a density of 4×10^4
20
21 9 cells/well. MTT tests were carried out on proliferative cells at day 3 after seeding: cultures
22
23 10 were incubated 24h with DON (0, 1, 10 and 50 μM) either with or without adsorbent material
24
25 11 (1 mg/ml), and treated according to the manufacturer’s recommendations. Acidified formazan
26
27 12 crystals formation was measured at 570 nm, with a reference wavelength of 690 nm. The
28
29 13 amount of dye produced was proportional to the number of metabolically active live cells. 6
30
31 14 repetitions were performed.

32
33
34
35
36 15 *Transport studies.*

37
38 16 The Caco-2 cell monolayers were rinsed twice with phosphate buffered saline and pre-
39
40 17 incubated for 30 min at 37°C in Hank’s balanced salt solution (HBSS). Apical (AP) and
41
42 18 basolateral (BL) chamber volumes were maintained respectively at 1.5 and 2 ml.
43
44 19 Transepithelial transport was assayed at day 21 after seeding by adding DON (10 μM) to the
45
46 20 apical compartment containing or not adsorbent material (1 mg/ml) and measured for 8 hours.
47
48 21 DON was diluted in ethanol so that final volume of ethanol added to the donor compartment
49
50 22 did not exceed 0.5 %. At each sampling time, 200 μl of transport medium were withdrawn
51
52 23 from the receiver basolateral compartment and replaced by fresh buffer. Four repetitions were
53
54 24 carried out.

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56
57
58
59 25 The same experiments were repeated pre-incubating DON and adsorbent 1 h before
60
26 exposing cells or incorporating adsorbent material in apical transport medium 1 h after DON

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

7 ***DON analysis***

8 For the analysis of BL medium in transport experiments, each sample was heated 10
9 min at 90°C and then centrifuged at 1600 g at room temperature. Every analysed sample was
10 evaporated to dryness under a stream of nitrogen at 60°C (Videmann et al., 2007). The dry
11 residue was derivatized in 25 μl Trisil-TBT with 75 μl hexane, shaken and heated at 60°C for
12 30 min. It was then extracted with twice 1 ml hexane and phosphate buffer, pH 7 (22%
13 NaH_2PO_4 , H_2O 0.2M, pH 4.8, 32% Na_2HPO_4 , H_2O 0.2M pH 9.4). Hexane phases were
14 combined and evaporated to dryness under a stream of nitrogen at room temperature. 200 μl
15 of 1,1-bis(p-chlorophenyl)-2,2-dichloroethylene (DDE 20 ng/ml in hexane) was added to the
16 residue as an internal standard. Samples were analyzed by GC-ECD (Hewlett Packard 5890
17 set II) using a capillary column Rtx-5 (length: 60m, internal diameter: 0.25mm, film
18 thickness: $0.25\mu\text{m}$). GC conditions: carrier gas linear velocity, 1.0 ml/min; injector
19 temperature, 250°C ; splitless, 0.75 min; detector temperature (^{63}Ni -ECD), 300°C ; column
20 oven temperature, hold at 50°C (1 min), programmed 50 - 250°C at $30^\circ\text{C}/\text{min}$, 270 - 290°C at
21 $15^\circ\text{C}/\text{min}$, hold at 290°C (12 min), 290 - 300°C at $30^\circ\text{C}/\text{min}$ and hold at 300°C (3 min). The
22 detection limit and the quantification limit were $0.01 \mu\text{M}$ and $0.03 \mu\text{M}$ respectively.

58 **244. *Statistical analysis.***

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

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3 1 Cytotoxicity data are expressed as a mean of 6 experiments \pm S.D. Transport data are
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6 2 expressed as a mean flux of 3 independent experiments \pm S.D. Statistical analysis was
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8 3 performed using one-way analysis of variance (ANOVA), and a p-value of < 0.05 was
9
10 4 considered significant.
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12

13 5 14 15 16 6 **Results**

17 18 19 7 *Screening* (Figure 1).

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21 8 Six adsorbents significantly decreased DON concentration in phosphate buffer,
22
23 9 regardless of the level of DON concentration (0.3 and 0.6 mg DON/g adsorbent): activated
24
25
26 10 charcoal (respectively $99.9 \pm 0.1\%$ and $98.4 \pm 0.4\%$), cholestyramin ($97.8 \pm 1.2\%$ and 68.6 ± 15
27
28 11 %), *saccharomyces cerevisiae* mannans ($84.6 \pm 1\%$ and $88.1 \pm 4.7\%$), algae β -glycan
29
30 12 ($81.1 \pm 2.3\%$ and $78.8 \pm 2\%$), fungus β -glycan ($80.0 \pm 1.9\%$ and $85.6 \pm 0.7\%$) and chlorophyllin
31
32 13 ($85.1 \pm 3.1\%$ and $89.6 \pm 0.4\%$). Leguminous plant samples presented such heterogeneity that
33
34 14 obtained values could show dramatic variations. Short bladed material showed better results
35
36 15 than long bladed material ($47.3 \pm 7.3\%$ and $52.8 \pm 7\%$ against $32.3 \pm 6.5\%$ and $39.8 \pm 8.4\%$).
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41 16 *DON adsorption at pH 7 and pH 3* (Tables 2 and 3).
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44 17 Whatever the pH and concentration, activated charcoal led to a decrease of more than
45
46 18 95% of DON concentration in solutions. Cholestyramin reduced DON levels in buffer by
47
48 19 about 60% at pH 7. The amount of DON adsorbed by *Saccharomyces cerevisiae* mannans
49
50 20 decreased when the DON concentration increased at pH 7. This phenomenon was not
51
52 21 observed at pH 3. DON levels in buffers decreased by between 70 and 90% in the presence of
53
54 22 algae or fungus β -glycans. DON adsorption by short-bladed leguminous plants remained
55
56 23 constant at about 50% at pH 7 but this material hardly decreased DON concentration at pH 3.
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58
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60 24 Desorption tests never demonstrated a DON desorption phenomenon when residues
25
obtained at pH 3 were re-suspended at pH 7 (data not shown). Linear regression of the linear,

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3 1 Freundlich and Langmuir adsorption isotherms for DON at pH 7 and pH 3 lead to their
4
5 2 constant determination (Tables 4 and 5). Isotherm adsorption models are usually used to
6
7 3 describe pesticide, radionuclide, ZEA adsorption (Avom et al. 2001; Hussein et al. 2004,
8
9 4 Ramos et al. 1996). Here, they allowed for a better characterisation of the binding reactions
10
11 5 involved. They provided a relation between DON concentration in buffer and the amount of
12
13 6 DON absorbed per mg of adsorbent for a given temperature (37°C). The linear model is
14
15 7 available for highly diluted medium and stationary experimental conditions. It is based on the
16
17 8 hypothesis of unlimited adsorption capacity and constant affinity. The Freundlich model does
18
19 9 not need any specific knowledge about adsorbent material and supposes an unlimited number
20
21 10 of adsorption sites. Kf constant obtained is proportional to adsorption capacity of the
22
23 11 adsorbent and n corresponds to its affinity (Avom et al., 2001; Horsfall and Spiff, 2004). It is
24
25 12 believed that adsorption is significant as soon as 1/n is lower than 1. The Langmuir model
26
27 13 supposes only one surface site type, an instantaneous and reversible binding, then a dynamic
28
29 14 equilibrium, a limited number of fixation sites and an adsorption that reaches a steady state.
30
31 15 The lower b is, the higher the adsorbent affinity (Hussein et al., 2004). K1 corresponds to the
32
33 16 energy needed for binding.
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17 *Adsorbent effects on DON cytotoxicity on proliferative Caco-2 cells.*

18 On the one hand, results obtained with an adsorbent alone showed no significant
19 cytotoxic effects on proliferative intestinal cells, except with cholestyramin where cell
20 viability decreased by $31 \pm 3.5\%$ after a 24 h exposure (Figure 2). On the other hand, after
21 DON exposure, cell viability slightly decreased by $15 \pm 1\%$ for a DON concentration of $1 \mu\text{M}$
22 and was very significantly diminished for higher concentrations (respectively 35 ± 1 and
23 $44 \pm 2\%$ for 10 and $50 \mu\text{M}$) (Figure 3). With the exception of cholestyramin, every adsorbent
24 material succeeded in maintaining at least a higher cell viability than the control, for every

1
2
3 1 DON concentration. Each result obtained was not significantly different to control samples
4
5
6 2 without DON.

7
8
9 3 *Adsorbent effects on DON transfer in Caco-2 cells.*

10
11 4 Even in wells exposed to cholestyramin, TEER values reached $400 \pm 10 \Omega \cdot \text{cm}^2$ at the
12
13 5 beginning of the transfer experiment and $450 \pm 13 \Omega \cdot \text{cm}^2$ after. These measurements indicated
14
15 6 no diminution of epithelium integrity during transfer experiments, assessing confluent and
16
17 7 viable cells. Cholestyramin no longer prevented DON transfer after as little as 1 h. The
18
19 8 results showed that the presence of another adsorbent in apical medium leads to a decrease of
20
21 9 DON transfer in basal medium (Figure 4). The pre-incubation of activated charcoal,
22
23 10 cholestyramin or mannans with DON did not influence DON transfer in intestinal cells
24
25 11 (Figure 5). However, incubating leguminous plant, algae or fungus β -glycans with DON 1 h
26
27 12 before cell exposure led to a higher inhibition of DON transepithelial transfer.

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33 13 After 1 h exposure, DON transfer (without adsorbent) was about 5% of the total
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35 14 amount transferred in 6 h. Addition of an adsorbent after 1 h exposure limited DON transfer
36
37 15 but it needed 3 h to reach levels similar to those obtained when DON and adsorbent were
38
39 16 added together. The presence of cholestyramin in apical medium 1 h after DON
40
41 17 contamination had no consequence on the toxin transfer in epithelium.

42 43 44 45 46 47 48 19 **Discussion**

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50
51 20 We confirmed with this study that it is difficult to extrapolate adsorption results
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53 21 obtained with one mycotoxin to another one, notably because of adsorbent specificity
54
55 22 (Phillips 1999). Our work, combining different *in vitro* tests, has permitted us to increase our
56
57 23 understanding of DON adsorption and to corroborate or refute different hypotheses presented
58
59 24 in the literature. Screening concentrations were chosen according to presumed norms in
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25 Europe for DON in cereals (1.25 to 1.75 mg/kg) and the fact that adsorbent rate in animal

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2
3 1 feed can vary from 1 to 5 kg/t. We obtained a rate between DON and adsorbent of about 0.25
4
5
6 2 to 1.7 mg/g, corresponding to about 0.75 to 5.1 μM DON with our dilutions. Screening
7
8 3 allowed us to identify 6 potential DON adsorbents, characteristics of which remained to be
9
10 4 specified. At studied doses, DON concentration did not show significant influence on
11
12 5 adsorption.
13
14

15 6 Our results supported the hypothesis that DON adsorption by activated charcoal would
16
17 7 be of about 90% for DON concentrations lower than 2 mg/g and where the pH was between 3
18
19 8 and 8 (Avantaggiato et al. 2004; Galvano et al. 1998; Galvano et al. 2001). Variations of
20
21 9 adsorbed DON were maybe principally due to the charcoal used. However, Avantaggio et al.
22
23 10 (2004) described a 55% DON diminution for a concentration of 10 mg/g, suggesting that
24
25 11 DON linkage to charcoal might be a saturable mechanism for very high toxin concentrations.
26
27 12 Activated charcoal combined a high adsorption capacity and an important efficacy whatever
28
29 13 the pH was, according to the Freundlich adsorption isotherm. Charcoal did not seem to
30
31 14 significantly bind DON when not activated.
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37 15 We demonstrated, as did Avantaggiato et al. (2004) in a previous study, the significant
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39 16 effect of cholestyramin on DON concentration in buffer at pH 7. Using adsorption isotherms,
40
41 17 the resin showed average efficacy and capacity. However, the previous work showed an
42
43 18 adsorption of $10 \pm 1 \%$ and $4 \pm 7 \%$ for concentrations of 2 and 10 mg/g, at pH 8, whereas
44
45 19 our results gave a rate of about 60 % at pH 7 for concentrations corresponding to 0.3 to 3.6
46
47 20 mg/g. DON adsorption by cholestyramin could then be a saturable phenomenon. It seemed
48
49 21 that pH had a great influence on cholestyramin efficacy too as the amount of DON adsorbed
50
51 22 remained dramatically low at pH 3. It is believed that mannans can adsorb many
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53 23 mycotoxins, notably fusariotoxins, and to a better extent than the other categories of
54
55 24 adsorbents (Devegowda and Castaldo, 2000, cited by Yiannikouris and Jouany, 2002). Our
56
57 25 study confirmed that *Saccharomyces cerevisiae* mannans could bind DON and specified that
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3 1 DON adsorption rate decreased as DON concentration increased. Adsorption isotherms
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5 2 suggested they have an average efficacy and capacity at both pH.
6
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8 3 Many recent studies are based on cell wall components, and β -glycans in particular.
9
10 4 We tested different β -glycans and it appeared that bound type (1-3 or 1-6) or ramification
11
12 5 might not be sufficient criteria to characterise their adsorption properties. Among the glycans
13
14 6 tested, algae and fungus ones showed a better and quite efficient adsorption of DON. pH
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16 7 appeared to have an impact on these 2 β -glycans properties as they presented a low efficacy
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18 8 but a high adsorption capacity at pH 7, and the inverse at pH 3.
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22 9 Chlorophyllin showed a highly significant effect on DON but its prohibited use in
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24 10 animal feed makes it of no interest. However, as it is composed of chlorophyll and Cu, it
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26 11 could be suggested that chlorophyll has an adsorption action on DON. In parallel, we
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28 12 expected long-bladed leguminous plants to be more efficient than short-bladed ones as
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30 13 Strangoom and Smith (1984) had observed that the fibrous fraction of alfalfa could neutralise
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32 14 negative ZEA effects on growth by rats, but we observed that short-bladed material showed a
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34 15 better adsorption rate than long-bladed material. Adsorption mechanisms in leguminous plant
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36 16 might involve chlorophyllin more than fibre. These results corroborated the hypothesis
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38 17 concerning the potential of chlorophyllin in DON adsorption.
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44 18 The use of the three equilibrium sorption models permitted a better understanding of
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46 19 the mechanisms involved in DON adsorption in relation to the considered material and gave
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48 20 results in line with DON adsorption rate in buffer. Whatever the material and the pH, the
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50 21 linear model appeared the least adapted. Moreover, it was sometimes difficult to discern
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52 22 which of the Freundlich or the Langmuir model was the most suitable, maybe because used
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54 23 concentrations did not allow us to reach the final steady-state if fixation sites were limited.
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56 24 This suggested that phenomenon of adsorption was not saturated for any material at the given
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58 25 concentration. Constants of the Freundlich model demonstrated that mechanisms involved in
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60 26 DON adsorption were different according to the considered material.

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3 1 In contrast, pH showed itself to be a determinant factor, the rate of DON adsorbed
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5 2 decreasing with pH. Some material like cholestyramin or leguminous plant no longer bound
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8 3 DON at pH 3. Considering pH, tests in pH 3 buffer reflected stomach condition and tests in
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10 4 pH 7 buffer, those, approximatively, of the small intestine. Adsorbents may then be more
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12 5 effective at the intestinal level, the principal absorption site.

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14 6 These results could not be extrapolated to an estimation of *in vivo* adsorption but it is
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16 7 possible to foresee a real decrease of DON absorption. To better comprehend the
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18 8 consequences of adding an adsorbent to animal feed on DON effects, it appeared determinant
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20 9 to specify what happens at the intestinal epithelium level. Caco-2 cell model first allowed
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22 10 assessing consequence of adsorbent use on proliferative intestinal cell viability. It indicated
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24 11 that only cholestyramin decreased cell viability and then might provoke lesions at the level of
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26 12 intestinal integrity.

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29 13 DON showed a growing cytotoxicity on proliferative intestinal cells as its
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31 14 concentrations increased. Except for cholestyramin, the presence of adsorbent material
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33 15 permitted to highly decrease, indeed to cancel, DON cytotoxicity on proliferative intestinal
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35 16 cells. Then, we focused on adsorbent effects on intestinal transepithelial transfer of DON.
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37 17 DON transfer decrease remained quite stable except for cholestyramin and mannans which
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39 18 showed a more and more increasing transfer, suggesting adsorption mechanisms depending
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41 19 on a thermodynamic equilibrium function of DON concentration in medium. Comparing to
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43 20 experiments in pH 7 buffer at same toxin concentration, activated charcoal and algae β -glycan
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45 21 showed an inhibition of DON transfer in Caco-2 cells similar to their DON adsorption in
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47 22 buffer. Otherwise, cholestyramin and in a lesser extent fungus β -glycan presented a decrease
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49 23 of transferred DON less important than adsorption rates in buffer. In parallel, leguminous
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51 24 plant appeared more effective to diminish DON transfer than its adsorption tests could
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53 25 predict. These results were in concordance with the impossibility to increase the scope of
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55 26 buffer tests to *in vivo* reality. Intestinal cell absorption decreased apical DON concentration

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3 1 and made then varying apical medium conditions, phenomenon that could have repercussions
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5 2 on transferred DON amount. It is also possible that competition mechanisms were involved
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8 3 between absorption and adsorption sites.
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10 4 Adsorbent effects could also depend on the way they are administered. It was then of
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12 5 great interest to test, on the one hand how pre-incubation of DON and adsorbent (as in animal
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14 6 feed) or, in the other hand, the addition of adsorbent after DON exposure (evaluating their
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16 7 efficacy subsequent to a contamination) could influence the toxin transfer in Caco-2 cells.
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18 8 These experiments could also allow us to better interpret the mechanisms involved. Results
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20 9 demonstrated that pre-incubation allowed a more efficient adsorption for leguminous plant,
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22 10 algae and fungus β -glycans. DON adsorption seemed thus to need an interval of time for these
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24 11 adsorbents. Pre-incubation globally led to a rapid and better diminution of DON absorption *in*
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26 12 *vitro*. This hypothesis should be tested *in vivo* to assess if the addition of adsorbents to feed
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28 13 could enhance diminution of DON bioavailability. In mice, it was demonstrated that smectite
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30 14 (2g/kg) did not succeed in preventing T-2 toxin (1mg/kg) affecting intestinal transit, but a 24h
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32 15 pre-incubation period inhibited all the toxin effects (Fioramont et al., 1987). Otherwise, the
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34 16 addition of an adsorbent after DON exposure could limit DON absorption, but only after at
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36 17 least a 3h delay. We could extrapolate that in case of ingestion of contaminated feed,
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38 18 adsorbent administration could reduce DON absorption at the intestinal level. The acceptable
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40 19 delay could not be determined here, but as it seems obvious that adsorbent efficacy weakens
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42 20 with time, as then the delay has to be as short as possible.
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53 22 **Conclusion**

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55 23 Due to the specificity of adsorbents, it is more often difficult to extrapolate adsorption
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57 24 results obtained with one mycotoxin to another one (Phillips, 1999). Here, interesting DON
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59 25 adsorbents were not always those already referenced for other mycotoxins, even for
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26 fusariotoxins. It was then of great interest to carry out specific tests with DON. Furthermore,

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3 1 it appeared difficult to simply evaluate the efficacy of an adsorbent using only one mycotoxin
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5 2 because neither *in vitro* nor *in vivo* tests described in literature prove satisfactory. We
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7 3 proposed here experiments that allowed for a better evaluation of the efficacy of an adsorbent,
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9 4 even if results remain to be tested *in vivo*.

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12 5 It appeared difficult to classify DON adsorbents because of their heterogeneity. But it
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14 6 seems conceivable that a mix of different adsorbents would allow an additivity of their
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16 7 efficacy, or even a synergy due to their specific characteristics. Our results can help to
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18 8 elaborate not the adsorbent but a mix of adsorbents usable in animal feed. It would be all the
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20 9 more interesting to explore this as multi-contamination is often observed in cereals and
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22 10 adsorbent material has to prove efficient at dealing with several mycotoxins.
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29 12 **Acknowledgments**

30
31 13 We thank NEAR Company for its participation in this work.
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36 15 **References**

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	Adsorbent material	provider
Charcoals	Charcoal	NEAR
	Activated charcoal (3500 m ² /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 (<i>Saccharomyces cerevisiae</i>)	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.

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

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

	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)

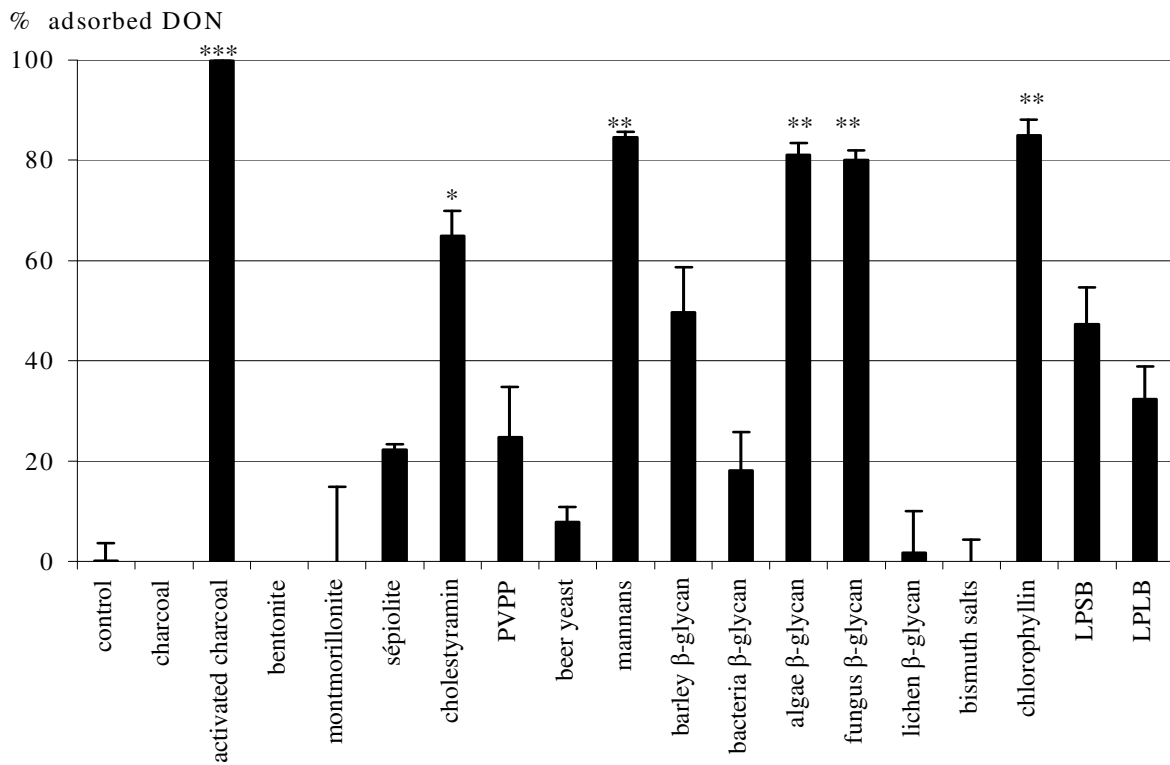
Note: LPSB=leguminous plant short bladed.

	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

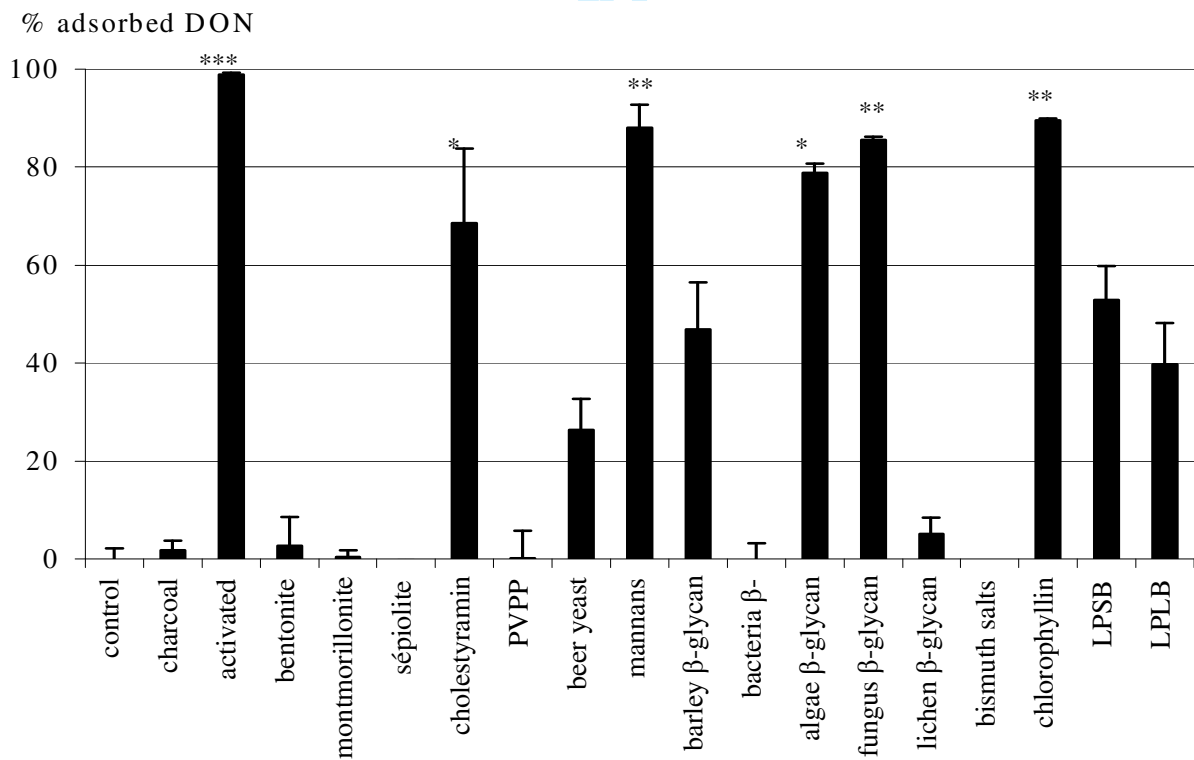
Table 5. Linear, Freundlich and Langmuir constants obtained from linear regression of DON adsorption isotherms at pH 3. (n = 18)

Note: LPSB=leguminous plant short bladed.

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2 b.

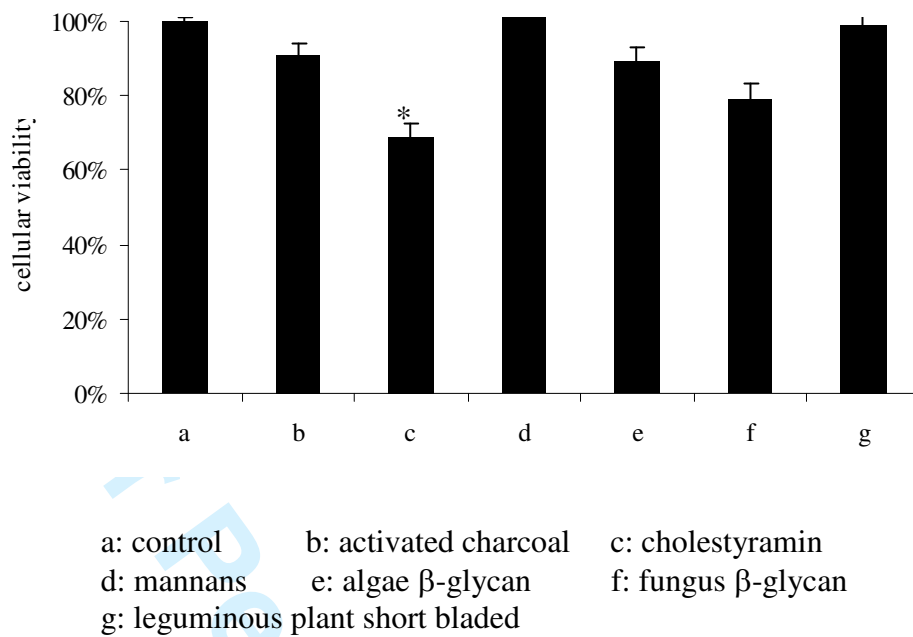


3 Figure 1. Adsorbed deoxynivalenol part in pH7 buffer for 2 toxin concentrations (1 µM (a.)
 4 and 2 µM (b.)) depending on adsorbent material.(LPSB= leguminous plant short bladed;
 5 LPLB= Leguminous plant long bladed)

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

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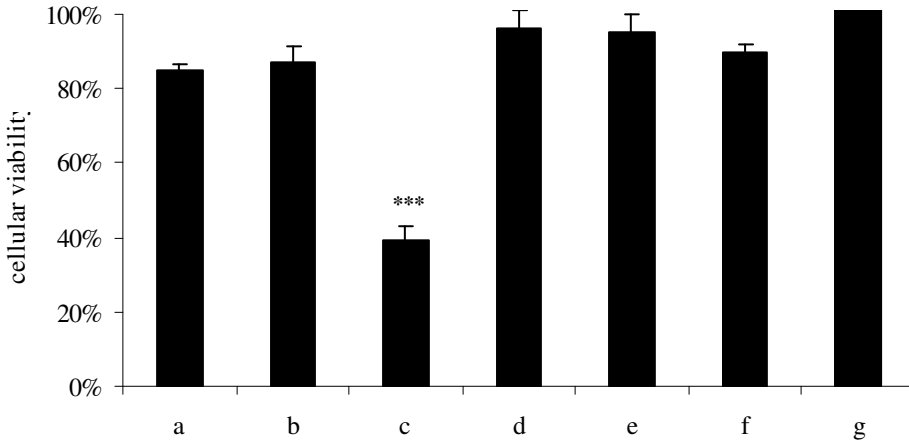
7 Figure 2. Adsorbent effects on cellular viability of proliferative Caco-2 cells.

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

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

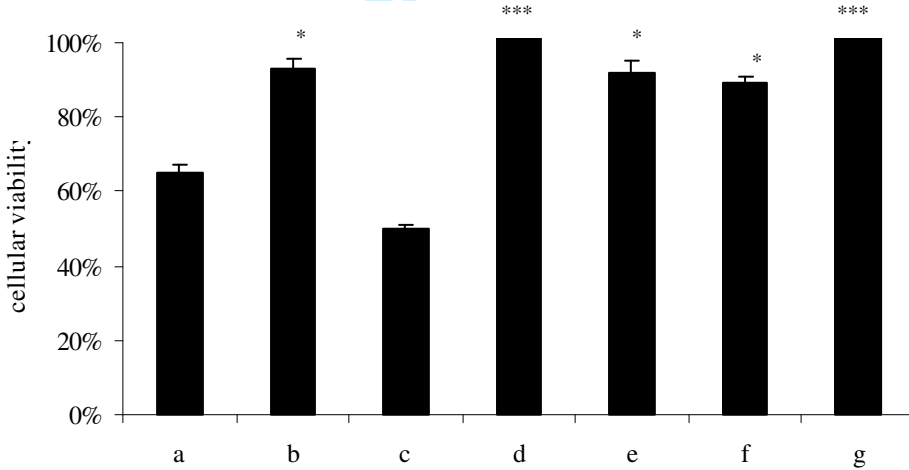
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1 1 μM



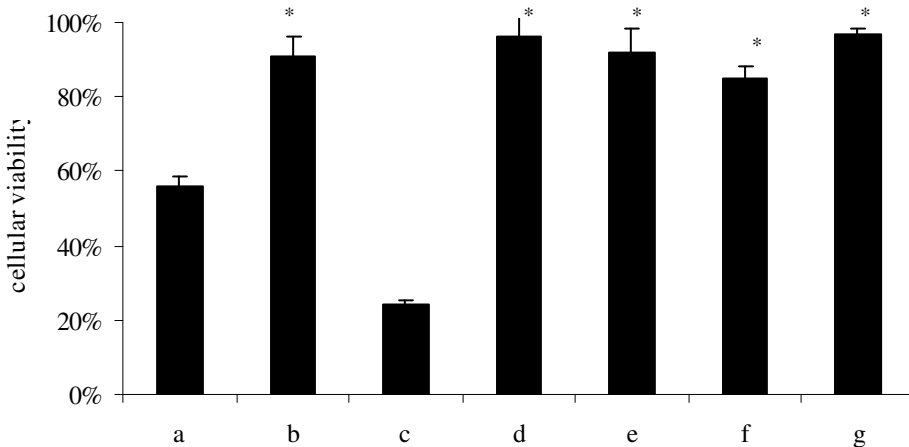
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3 10 μM



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5 50 μM



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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

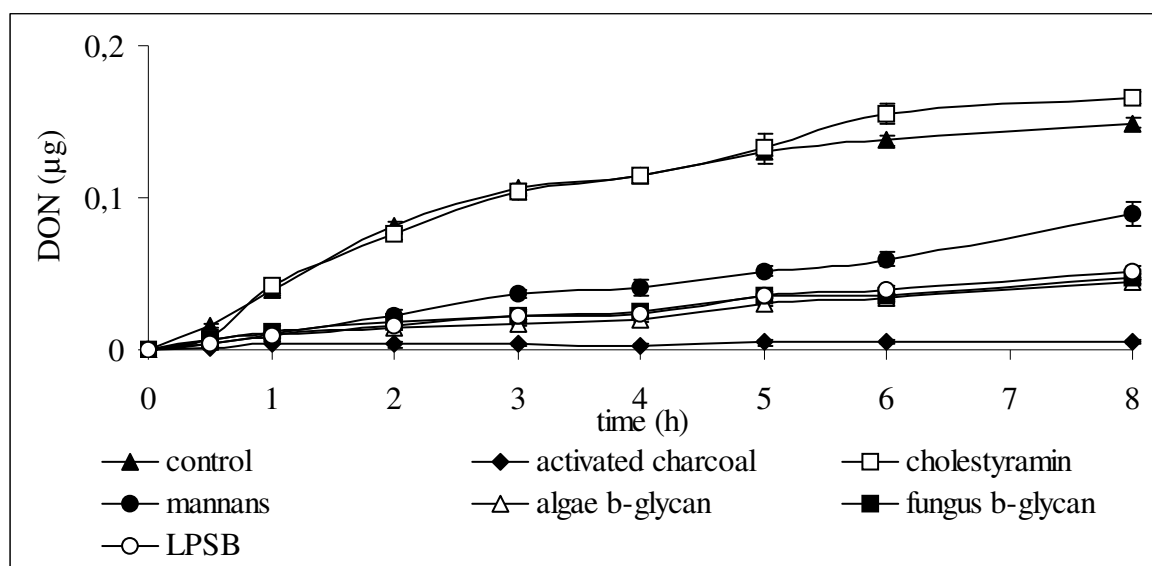
Figure 3. Adsorbent effects on cellular viability of proliferative Caco-2 cells exposed to different concentrations of DON (1, 10 and 50 μ M).

Data are mean values \pm 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, $p < 0.001$.

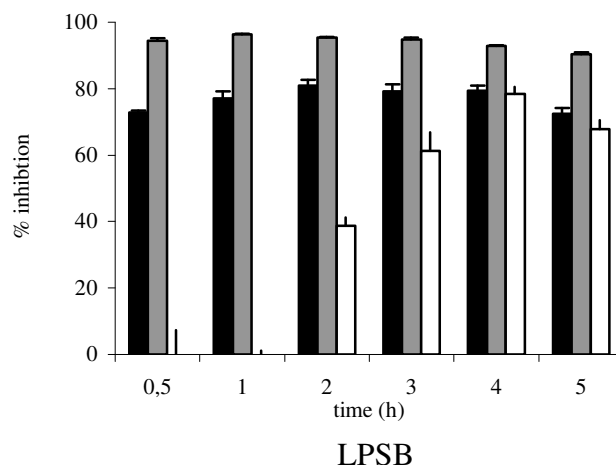
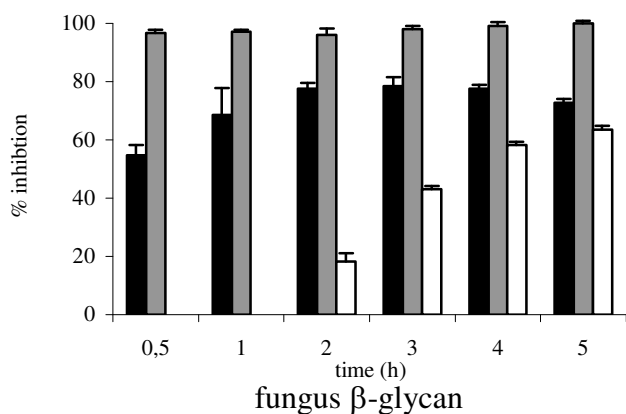
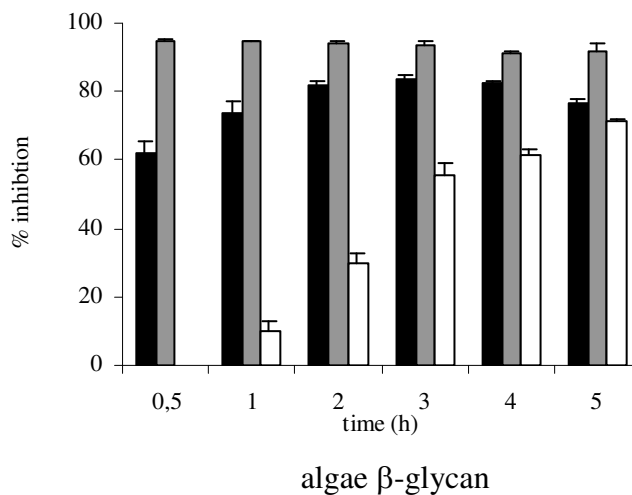
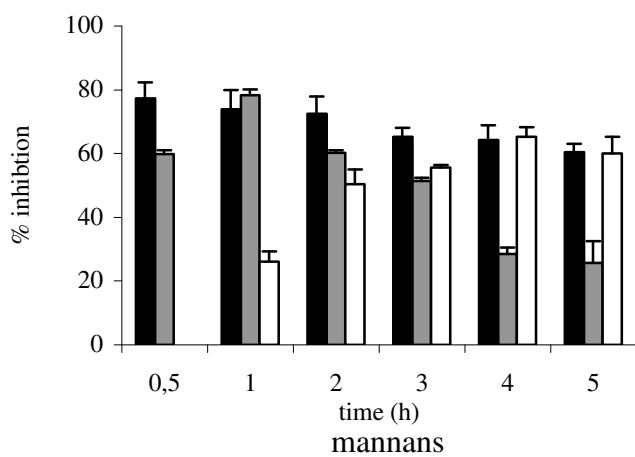
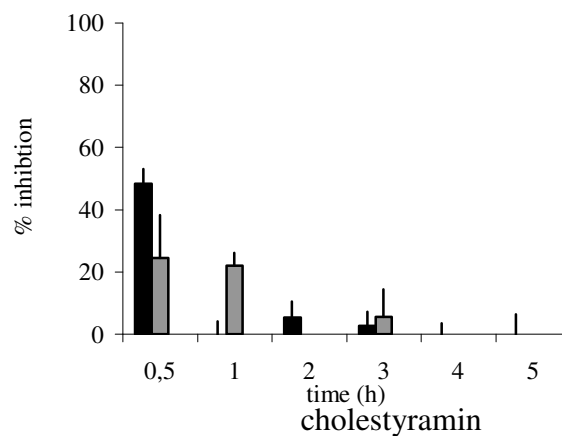
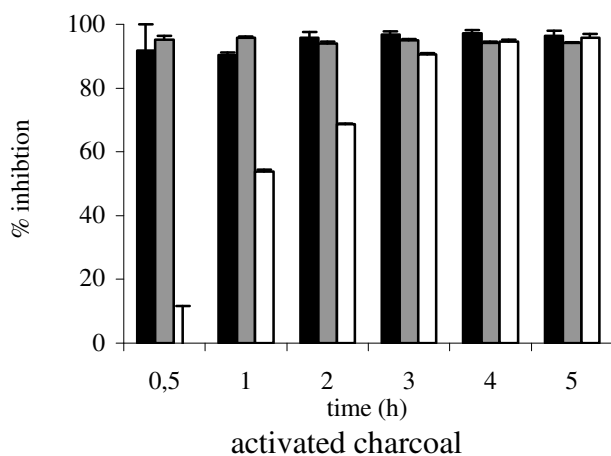


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Figure 4. DON transfer in basolateral compartment after an apical exposure (10 μ M) with or without adsorbent (1 mg/ml). (n = 4)

Note: LPSB=leguminous plant short bladed.

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3 Figure 5: Inhibition percentages of deoxynivalenol (10 μ M) transfer in Caco-2 cell
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5 monolayers when adding different adsorbents (1mg/ml) according to 3 modalities:
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7 simultaneous exposure (■), adsorbent+deoxinivalenol incubation 1 h before cell exposure
8
9 (preincubation: ■) or adsorbent addition 1h after cell exposure to DON (post-exposure: □).
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13 (n=4).
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15 Note: LPSB=leguminous plant short bladed.
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