Efficacy and safety testing of mycotoxin-detoxifying agents in broilers following the European Food Safety Authority guidelines

A. Osselaere,*[1] M. Devreese,* A. Watteyn,* V. Vandenbroucke,* J. Goossens,* V. Hautekiet,† M. Eeckhout,‡ S. De Saeger,§ S. De Baere,* P. De Backer,* and S. Croubels*

*Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; †Sanluc International NV, Schoolstraat 49, 9860 Gijzenzele, Belgium; ‡Department of Food Science and Technology, Faculty of Applied Bioscience-engineering, Ghent University College, Schoonmeersstraat 52, 9000 Ghent, Belgium; and §Department of Bio-analysis, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium

ABSTRACT Contamination of feeds with mycotoxins is a worldwide problem and mycotoxin-detoxifying agents are used to decrease their negative effect. The European Food Safety Authority recently stated guidelines and end-points for the efficacy testing of detoxifiers. Our study revealed that plasma concentrations of deoxynivalenol and deepoxy-deoxynivalenol were too low to assess efficacy of 2 commercially available mycotoxin-detoxifying agents against deoxynivalenol after 3 wk of continuous feeding of this mycotoxin at concentrations of 2.44 ± 0.70 mg/kg of feed and 7.54 ± 2.20 mg/kg of feed in broilers. This correlates with the poor absorption of deoxynivalenol in poultry. A safety study with 2 commercially available detoxifying agents and veterinary drugs showed innovative results with regard to the pharmacokinetics of 2 antibiotics after oral dosing in the drinking water. The plasma and kidney tissue concentrations of oxytetracycline were significantly higher in broilers receiving a biotransforming agent in the feed compared with control birds. For amoxicillin, the plasma concentrations were significantly higher for broilers receiving an adsorbing agent in comparison to birds receiving the biotransforming agent, but not to the control group. Mycotoxin-detoxifying agents can thus interact with the oral bioavailability of antibiotics depending on the antibiotic and detoxifying agent, with possible adverse effects on the health of animals and humans.

Key words: antibiotic, deoxynivalenol, detoxifying agent, efficacy and safety testing, broiler

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INTRODUCTION

Food safety is an important issue receiving a lot of scientific attention worldwide. The presence of mycotoxins, produced as secondary metabolites of toxigenic molds, in food and feed is one of the most important concerns. Also climate changes, characterized by an increase of temperature and relative humidity have a great impact on plants and their pathogens (Ingram, 1999; Miraglia et al., 2009). Both parameters, in combination with the rising level of CO_2 in the atmosphere, have an influence on the mycotoxin production. The changed production of mycotoxins is correlated mainly to the predominance of *Fusarium graminearum*, a fungus responsible for the production of zearalenone and of mycotoxins belonging to the class of trichothecenes (Paterson and Lima, 2010).

Consequences of climate changes for the food system comprise all the stages from 'farm to fork' and thus not only the preharvest conditions, but also other parameters are important such as quality and temperature of the grain storage facilities. Poor management can lead to microbial activity and loss of quality, for this reason, Good Agricultural Practice (**GAP**) is essential to minimize mycotoxin development (Magan and Aldred, 2007). Mycotoxins are very resistant to all kinds of production steps, even brewing, malting, and extrusion-processes are not a threat for these contaminants (Scudamore et al., 2008).

Mycotoxins exert several direct adverse effects on human and animal health, while the excretion of some mycotoxins in animal products such as milk can also contribute to the consumers' exposure (Fink-Gremmels, 2008). Apart from the health problem, mycotoxins can

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[1]Corresponding author: Ann.Osselaere@ugent.be

cause important economic losses. Their toxic properties depend on the particular mycotoxin and its dose. Deoxynivalenol (**DON**), for example, which is one of the most prevalent mycotoxins (Monbaliu et al., 2010) mainly produced by *F. graminearum*, causes vomiting and diarrhea when ingested in high doses, especially in pigs. In lower concentrations, it induces weight loss and feed refusal (Miller et al., 2001). Poultry, on the other hand, are very resistant to DON; for example, concentrations above 5 mg/kg of diet are necessary to cause detrimental effects (Awad et al., 2006).

One of the most recent methods to counteract mycotoxicosis in animals is the use of mycotoxin-detoxifying agents in feed. These detoxifiers are added to animal feed and act in the digestive tract of the animal. The additives can consist of different components and, depending on their mode of action, they are 'adsorbing' or 'biotransforming' agents. The first class is able to bind mycotoxins and to reduce their gastro-intestinal absorption, the second one degrades mycotoxins to less or nontoxic metabolite(s) (Anonymous, 2010). Research has already been performed on the efficacy of these feed additives using both in vitro and in vivo assays (Galvano et al., 2001; Avantaggiato et al., 2004; Avantaggiato et al., 2005). Testing under laboratory circumstances is difficult because the intestinal conditions are almost impossible to reproduce. Adsorption isotherms and different models, such as intestinal cell lines and Ussing chambers using intestinal tissues, can be used to study these detoxifiers in vitro (Lemke et al., 1998; Clarke, 2009). Although in vitro trials are a rapid screening method to choose which agents can be further tested in vivo, animal trials remain essential.

The set up of in vivo trials is an important point of discussion. The European Food Safety Authority (**EFSA**) has recently published a scientific opinion about the efficacy and safety testing of mycotoxin-detoxifying agents and focusing on the experimental design of in vivo studies (Anonymous, 2010). The EFSA has also pointed out several relevant end-points for the different mycotoxins. For DON, the analysis of this mycotoxin and its metabolite deepoxy-deoxynivalenol (**DOM-1**) in blood has been indicated as the most relevant endpoint. On the other hand, EFSA also pays importance to the safety testing of mycotoxin-detoxifying agents. Possible interactions with respect to nutrients or veterinary drugs absorption should also be investigated (Anonymous, 2010).

The first aim of this present study was to test whether the guidelines published by EFSA can be applied for the in vivo efficacy testing of 2 commercially available mycotoxin-detoxifying agents with respect to DON in broilers. We performed an in vivo trial with broilers according to these guidelines and tested the most relevant endpoints for the mycotoxin DON as stated by EFSA. A second aim of this study was to test if the 2 selected mycotoxin-detoxifying agents can have an influence on the pharmacokinetics and tissue residues of commonly used antibiotics in poultry medicine.

MATERIALS AND METHODS

Materials

Deoxynivalenol, used for artificial contamination of the feed, was produced with a reference strain obtained from the Mycothèque de l'Université Catholique de Louvain, Belgium (**MUCL**). Both antibiotics, oxytetracycline (Oxytetracycline 80%) and amoxicillin (Amoxicilline 70%), were obtained from Kela Veterinaria (Sint-Niklaas, Belgium). Blood was collected in heparinized tubes (Venoject) obtained from Terumo Corp. (Tokyo, Japan). Standards used for analytical experiments (DON and DOM-1) were obtained from Sigma-Aldrich (Bornem, Belgium) and stored at $\leq -15°C$. The internal standard $^{13}C_{15}$-DON was purchased as a 25 μg/mL solution in acetonitrile from Biopure (Tulln, Austria). Water, methanol and acetonitrile were of HPLC grade, whereas ammonium acetate, ethyl acetate, and glacial acetic acid were of analytical grade. All these reagents were obtained from VWR (Leuven, Belgium). Oasis HLB solid-phase extraction (SPE) columns (60 mg/3 cc) were obtained from Waters (Zellik, Belgium). Millex-GV PVDF filter units (0.22 μm) were obtained from Millipore (Brussels, Belgium).

Experimental Design for Birds and Diets

Efficacy Testing. Sixty-four 1-d-old Ross broiler chicks, in an equal number of both sexes, were obtained from a commercial hatchery. During an acclimatization period of 10 d, the birds were fed the control feed. Males and females were housed separately. Afterward, the chickens were randomly assigned to 1 of 8 dietary groups, each consisting of 8 animals (4 female and 4 male). The birds were housed in 8 cages with a floor area of at least 1 m^2. The concrete floor was covered with wood shavings. During the 3 wk of experimental feeding, the birds received different diets, as illustrated in Table 1. The concentrations of the different mycotoxins as shown in Table 1 were determined by a multi-mycotoxin LC-MS/MS method (Monbaliu et al., 2010). The feed of groups 3, 4, and 5 was the same feed as group 1 (blank feed) but it was artificially contaminated with DON in the laboratory. The DON was produced with the reference strain MUCL 6131. This strain was grown in liquid **GCY** medium (glucose 10 g/L, yeast extract 1 g/L, peptone 1 g/L) together with 50 μ*M* hydrogen peroxide (H_2O_2) which induces an oxidative stress in the fungus that stimulates the production of DON (Audenaert et al., 2010). After 14 d of cultivation, with supplementation of H_2O_2 every 2 d, the culture was filtered and freeze-dried and mixed into the blank feed to have a final DON concentration of 2.44 ± 0.70 mg/kg of feed, thus below the maximum level of 5 mg/kg as recommended by the EFSA (Anonymous, 2006). For the last 3 groups (6, 7, and 8), a naturally contaminated feed was prepared out of contaminated maize. The concentration of DON in this feed (7.54 ±

Table 1. Composition of 8 dietary groups for the efficacy testing[1]

Group[2]	DON[3]	NIV[3]	3-aDON[3]	15-aDON[3]	FB1[3]	FB2[3]	FB3[3]	Detoxifying agent
1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	no
2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	adsorbing (1.5 kg/tonne)
3	2.439 ± 0.70	0.086 ± 0.030	0.571 ± 0.22	2.051 ± 0.80	<LOD	<LOD	<LOD	no
4	2.439 ± 0.70	0.086 ± 0.030	0.571 ± 0.22	2.051 ± 0.80	<LOD	<LOD	<LOD	adsorbing (1.5 kg/tonne)
5	2.439 ± 0.70	0.086 ± 0.030	0.571 ± 0.22	2.051 ± 0.80	<LOD	<LOD	<LOD	biotransforming (1.5 kg/tonne)
6	7.540 ± 2.20	<LOD	1.481 ± 0.57	<LOD	0.700 ± 0.08	0.201 ± 0.02	0.207 ± 0.08	no
7	7.540 ± 2.20	<LOD	1.481 ± 0.57	<LOD	0.700 ± 0.08	0.201 ± 0.02	0.207 ± 0.08	adsorbing (1.5 kg/tonne)
8	7.540 ± 2.20	<LOD	1.481 ± 0.57	<LOD	0.700 ± 0.08	0.201 ± 0.02	0.207 ± 0.08	biotransforming (1.5 kg/tonne)

[1]Mycotoxin levels (mg/kg of feed) in the diets fed to the broilers for 3 wk after the acclimatization period.
[2]Groups 3 to 5 received artificially DON-contaminated feed. Groups 6 to 8 received naturally contaminated feed.
[3]DON = deoxynivalenol [limit of detection (LOD) = 11.09 µg/kg], NIV = nivalenol (LOD = 66.26 µg/kg), 3-aDON = 3-acetyldeoxynivalenol (LOD = 8.96 µg/kg), 15-aDON = 15-acetyldeoxynivalenol (LOD = 5.62 µg/kg), FB1 = fumonisin B1 (LOD = 58.24 µg/kg), FB2 = fumonisin B2 (LOD = 44.57 µg/kg), FB3 = fumonisin B3 (LOD = 42.40 µg/kg).

2.20 mg/kg of feed) was higher than the recommended maximum level (Anonymous, 2006).

Two different kinds of commercially available mycotoxin-detoxifying agents were supplemented to the diets. The adsorbing agent was a smectite-type clay mineral (illite-ambrosite). The biotransforming agent, as classified by the EFSA (Anonymous, 2009), was a bentonite-montmorillonite upgraded with a yeast. The yeast is claimed to be able to detoxify DON in vitro by opening the C-12,13 epoxide ring. Group 2 received blank feed supplemented with the adsorbing agent to test possible effects of the detoxifier. Water and feed were given ad libitum to all broilers. A light regimen of 20 h of light and 4 h of darkness was applied. The experimental procedures conducted with the chickens were in accordance with the European guidelines for the care and use of animals for research purposes. The protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University) (EC 2010/064 and EC 2010/076).

Safety Testing. Forty-eight 1-d-old Ross broiler chicks, in an equal number of both sexes, were obtained from a commercial strain. During an acclimatization period of 7 d, the birds were fed blank feed. Males and females were housed separately. Afterward, the chickens were randomly assigned to 1 of 6 dietary groups, each consisting of 8 birds (4 female and 4 male) and received experimental diets for 3 wk. The birds were housed in 6 cages with a floor area of at least 1 m^2 covered with wood shavings. The composition of the dietary groups is shown in Tables 2 and 3. The blank feed did not contain any mycotoxin above the limit of detection of a multi-mycotoxin LC-MS/MS method analyzing 23 mycotoxins (Monbaliu et al., 2010). This uncontaminated feed was useful for this study, as an interaction with naturally occurring mycotoxins had to be avoided. Both mycotoxin detoxifying agents, the same as used during the efficacy study, were added in a dosage of 1.5 kg/tonne of feed. Water and feed were given ad libitum to the broilers. A light regimen of 20 h of light and 4 h of darkness was applied. From d 28 onwards, 2 different antibiotics, frequently used in poultry industry against bacterial diseases, were administered. Oxytetracycline is a broad-spectrum antibiotic that interacts with the ability of bacteria to produce proteins. On the other hand, amoxicillin, a moderate-spectrum antibiotic with a bacteriolytic function, was tested. The antibiotics were administered to the birds as stated in Table 2 for oxytracycline and in Table 3 for amoxicillin. For the oral bolus study on d 28, the antibiotics were dissolved in tap water and administered directly in the crop with a tube to conduct a pharmacokinetic study. Therefore, blood was collected from the leg vein in heparinized tubes at the following time points: 0′ (before administration) and 20′, 40′, 60′, 90′, 2 h, 2.5 h, 3 h, 5 h, 8 h, 12 h postadministration (**p.a.**) for oxytetracycline and 0′ (before administration), 5′, 10′, 20′, 30′, 40′, 50′, 1 h, 1 h15′, 1 h30′, 1 h45′, 2 h p.a. for amoxicillin. The blood samples were immediately centrifuged at 2,095 × *g* for

Table 2. Timetable of the critical events of the bird experiment with oxytetracycline

Time	d 8–d 35	d 28	d 29–d 33	d 34	d 35
8 a.m.	Administration of the feed to 3 different dietary groups: - Blank feed (n = 8) - Blank feed supplemented with an adsorbing agent (n = 8) - Blank feed supplemented with a biotransforming agent (n = 8)	Oral bolus (100 mg/kg BW) followed by a kinetic study during 24 h	Oral bolus (50 mg/kg BW) Oxytetracycline in water (100 mg/kg BW)	Oral bolus (50 mg/kg BW) Refreshing of the medicated water (100 mg/kg BW)	Euthanasia 16 h after withdrawal of medicated water
8 p.m.			Refreshing of the medicated water (100 mg/kg BW)	Unmedicated water	

10 min at 4°C and plasma was stored at −70°C until analysis. Next, the antibiotics were added to the drinking water during 5 d for amoxicillin and 7 d for oxytetracycline, as stated by the leaflet. To be sure that every bird received the required dose, an extra oral bolus at half the dosage was administered every morning. On d 35 of the experiment with oxytetracycline, the birds were euthanized 16 h after withdrawal of the antibiotic by cervical dislocation and liver and kidneys were collected. For amoxicillin, the birds were euthanized on d 33, 12 h after the last antibiotic administration, and the same organs as described for oxytetracycline were collected. The concentration of both antibiotics in plasma and tissues was determined by validated LC-MS/MS methods (based on Reyns et al., 2006; Reyns et al., 2008 for amoxicillin, based on Cherlet et al., 2003; Cherlet et al., 2006 for oxytetracycline). Plasma pharmacokinetic parameters such as area under the plasma concentration-time curve ($AUC_{0-\infty}$ and AUC_{0-t}), maximum plasma concentration (C_{max}), time to reach maximum plasma concentration (T_{max}), and elimination half-life ($T_{1/2el}$) were calculated using WinNonlin 6.2.0 (Pharsight Corporation, Mountain View, CA). The protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University) (EC 2010/063 and EC 2011/002).

Experimental Parameters Measured During the Efficacy Testing Study

BW, Feed Consumption, and Live Weight Gain. The chickens were weighed individually 3 times a week and feed consumption for each group was measured daily during the whole experiment. Live weight gain was calculated according to the formula: (BW d 32 – BW d 11)/n, with n equal to the number of days of feeding the experimental diet.

Analysis of Mycotoxins in Plasma, Bile, and Tissues. After the first week (d 18) of feeding the experimental diets, blood was taken from the leg vein in heparinized tubes. For animal welfare, it was not appropriate to take enough blood from all the birds to perform the LC-MS/MS analysis for DON and DOM-1 for this particular time point, so the blood of 2 times 4 birds was pooled to analyze plasma concentrations of DON and DOM-1 for each group. The second week (d 25), blood was taken from the leg vein of all the birds, and plasma was separated after centrifugation at 2,095 × *g* at 4°C for 10 min. The birds were euthanized after the third week (d 32). Blood, liver, and kidneys were collected. The bile fluid was collected from each bird and pooled per group. All samples were frozen at ≤−20°C until assayed. Prior to analysis, the tissue samples were minced and homogenized using a Moulinette mixer (Moulinex, Paris, France). The concentration of DON and deepoxy-deoxynivalenol (DOM-1) was determined in plasma and bile by a validated LC-MS/MS method (De Baere et al., 2011).

For the quantitative determination of DON and DOM-1 in liver and kidney samples, the method of De Baere et al. (2011) was used with minor modifications: 112.5 μL of the internal standard working solution ($^{13}C_{15}$-DON, 1 μg/mL) was added to 2.0 g of tissue sample. After vortex mixing, the sample was left at

Table 3. Timetable of the critical events of the bird experiment with amoxicillin

Time	d 8–d 33	d 28	d 29–d 31	d 32	d 33
8 a.m.	Administration of the feed to 3 different dietary groups: - Blank feed (n = 8) - Blank feed supplemented with an adsorbing agent (n = 8) - Blank feed supplemented with a biotransforming agent (n = 8)	Oral bolus (20 mg/kg BW) followed by a kinetic study during 2 h	Oral bolus (10 mg/kg BW) Refreshing of the medicated water (20 mg/kg BW)	Oral bolus (10 mg/kg BW) Refreshing of the medicated water (20 mg/kg BW)	Euthanasia 12 h after withdrawal of medicated water
8 p.m.		Amoxicillin in water (20 mg/kg BW)	Refreshing of the medicated water (20 mg/kg BW)	Unmedicated water	

room temperature for 5 min. Thereafter, 5 mL of a water/acetonitrile mixture (10/90, vol/vol) was added, followed by a vortex mixing step (15 s). The sample was extracted on a homemade rotary apparatus for 20 min, followed by a centrifugation step (10 min, 2,095 × *g*). The supernatant was transferred to another extraction tube and 8 mL of a solution of 7% acetic acid in ethyl acetate was added. After vortex mixing, the sample was extracted for 20 min, followed by a 10-min centrifugation step (2,095 × *g*). The organic phase was transferred to another extraction tube and evaporated using a gentle stream of N_2 (~45°C). The dry residue was reconstituted in 1 mL of a water/methanol (90/10, vol/vol) solution and vortex mixed for 15 s. The sample was further purified using an Oasis HLB column (same procedure as for plasma and bile analysis). The dry residue was reconstituted in 150 μL of a 70/30 (vol/vol) mixture of mobile phase A/B [A: 5 m*M* ammonium acetate in water/methanol/acetic acid (94/5/1, vol/vol/vol); B: 5 m*M* ammonium acetate in methanol/water/acetic acid (97/2/1, vol/vol/vol)] and filtered through a Millex-GV PVDF filter unit (0.22 μm; Millipore, Brussels, Belgium). An aliquot (10 μL) was injected onto the LC-MS/MS instrument. The HPLC settings were as follows: gradient elution: 0–2 min (70% A, 30% B), 6 min (linear gradient to 30% A), 6–14 min (30% A, 70% B), 15 min (linear gradient to 70% A), 15–20 min (70% A, 30% B), flow-rate: 200 μL/min, column temperature: 35°C, column oven temperature: 5°C. The LC-MS/MS instrument and the MS/MS conditions were the same as for plasma and bile analysis (De Baere et al., 2011).

Statistical Analysis

The data were analyzed using ANOVA (SPSS 17.0 software for Windows, IBM) to address the significance of difference between the mean values, with significance level set at $P < 0.05$.

RESULTS

Efficacy Testing

Zootechnical Parameters: BW, Feed Consumption, and Live Weight Gain. Diets with or without different concentrations of DON, with or without the supplementation of detoxifying agents, had no significant effect on the BW and live weight gain of broilers after 3 wk of feeding ($P > 0.05$). The feed intake was also not affected ($P > 0.05$).

EFSA End-Points: DON and DOM-1 in Plasma. Plasma levels were measured above the limit of quantification (LOQ = 1.25 ng/mL) in the 3 groups that received feed naturally contaminated at a level of 7.54 ± 2.20 mg of DON/kg of feed. This means that plasma concentrations could not be detected during feeding a contamination level of 2.44 ± 0.70 mg of DON/kg of feed. The plasma levels of the birds that received naturally contaminated feed with or without supplementation of detoxifying agents are shown in Figure 1. For group 6 (naturally contaminated feed without supplementation), a trend was observed: the plasma concentrations were higher after one week of feeding than after 2 wk. At the end of the experiment, after 3 wk of feeding, even no plasma concentrations were detected for this group. For group 7, which received the same feed but with an adsorbing agent, the plasma concentrations were higher than in group 6, but the same decreasing trend was observed and also after 3 wk of feeding, no plasma levels could be detected. The group supplemented with a biotransforming agent (group 8) also reached higher plasma concentrations than group 6, but the decreasing trend was not so obvious for this group within the first 2 wk of feeding. After 3 wk of feeding also for group 8, no plasma concentrations could be detected. The metabolite DOM-1 was also analyzed in the plasma samples, but no levels above the LOQ (LOQ = 1.25 ng/mL) could be measured in any of the groups.

DON and DOM-1 in Tissues and Bile Fluid. No residues above the LOQ (LOQ = 1.25 ng/g) could be detected in the liver and kidneys of the birds of the 8 different groups. The results of the analyses of bile fluid are shown in Table 4. The DON was only detected in the bile fluid of the 3 groups that received naturally contaminated feed containing 7.54 ± 2.20 mg of DON/kg of feed. The metabolite DOM-1 was detected in all the groups that received contaminated feed. However, the concentration of DOM-1 in the groups that received artificially contaminated feed (groups 3, 4, and 5) was significantly lower than the 3 groups fed naturally contaminated feed (groups 6, 7, and 8; $P < 0.05$). Supplementation of a detoxifying agent did not have a significant influence on the concentration in the bile fluid of any measured analytes ($P > 0.05$).

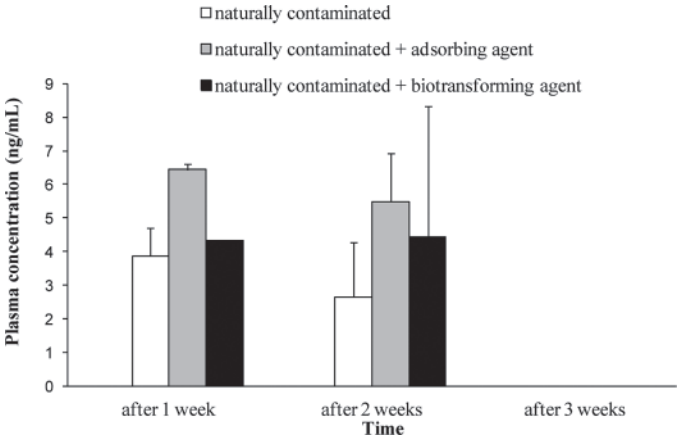

Figure 1. Plasma concentrations of deoxynivalenol (DON) in broilers after 1, 2, and 3 wk of feeding naturally contaminated feed with DON in a concentration higher than the maximum guidance level of 5 ppm. The effect of the supplementation of an adsorbing or a biotransforming agent was evaluated. Results are presented as mean values + SD.

Table 4. Concentration of deoxynivalenol (DON) and deepoxy-deoxynivalenol (DOM-1) in the pooled bile fluid of broilers (n = 8/ group) after 3 wk of feeding (un)contaminated feed with DON

Group	DON (ng/mL)[1]	DOM-1 (ng/mL)[1]
Group 1 (control)	ND	ND
Group 2 (control + adsorbing agent)	ND	ND
Group 3 (artificial DON contaminated)	ND	10.0
Group 4 (artificial DON contaminated + adsorbing agent)	ND	12.0
Group 5 (artificial DON contaminated + biotransforming agent)	ND	11.5
Group 6 (naturally contaminated)	7.5	51.6
Group 7 (naturally contaminated + adsorbing agent)	8.6	57.4
Group 8 (naturally contaminated + biotransforming agent)	5.7	52.7

[1]ND = not detected, limit of quantification of DON = 1 ng/mL, DOM-1 = 2.5 ng/mL.

Safety Testing

Influence of 2 Different Detoxifying Agents on the Pharmacokinetics of Oxytetracycline and Amoxicillin. The plasma concentration-time profiles for oxytetracycline in the control and 2 detoxifying agents groups are shown in Figure 2. Remarkably, the area under the curve ($AUC_{0\rightarrow 12h}$) is significantly higher for the group fed a diet with a biotransforming agent (32·70 h.μg/mL) compared with the group without detoxifying agent (18.12 h·μg/mL) and the group fed an adsorbing agent (24.72 h·μg/mL; $P < 0.05$). The C_{max} of the group with the biotransforming agent (5.49 μg/mL) was also significantly higher than for the adsorbing agent and the control group (2.37 μg/mL and 3.04 μg/mL, respectively). No significant differences in T_{max} ($P = 0.114$) and $T_{1/2el}$ ($P = 0.305$) were noticed between the groups.

Results for the antibiotic amoxicillin are presented in Figure 3. For this antibiotic, the group that received the adsorbing agent reached higher plasma concentrations compared with the other groups. The area under the curve ($AUC_{0\rightarrow 2h}$) for the group with the adsorbing agent (608.26 min·μg/mL) was significantly different with the one with the biotransforming agent (414.26 min·μg/mL; $P < 0.05$), but not with the control group (507.52 min·μg/mL). The C_{max} of the group receiving the adsorbing agent (7.00 μg/mL) was significantly higher than for the group with the biotransforming agent (4.19 μg/mL), but no significant differences were noticed with the control group (5.20 μg/mL). No significant differences in T_{max} ($P = 0.597$) and $T_{1/2el}$ ($P = 0.915$) were observed between the groups.

Influence of 2 Different Detoxifying Agents on the Tissue Residues of Oxytetracycline and Amoxicillin. The results of the analysis of oxytetracycline in liver and kidney tissues are shown in Figure 4. The residues of oxytetracycline in the liver were not significantly different between groups. For the kidneys, the concentration of oxytetracycline was much higher in the group that received feed supplemented with a biotransforming agent compared with the control group ($P < 0.05$). This is in agreement with the results obtained in plasma (Figure 2). For amoxicillin, on the other hand, the residue levels in kidneys and liver were lower than the limit of quantification for all the birds (LOQ = 12.5 ng/g; data not shown).

DISCUSSION

This study is, to our knowledge, the first one that fully complies with recent EFSA guidelines for in vivo efficacy testing of mycotoxin-detoxifying agents in broilers. Zootechnical parameters, such as feed intake

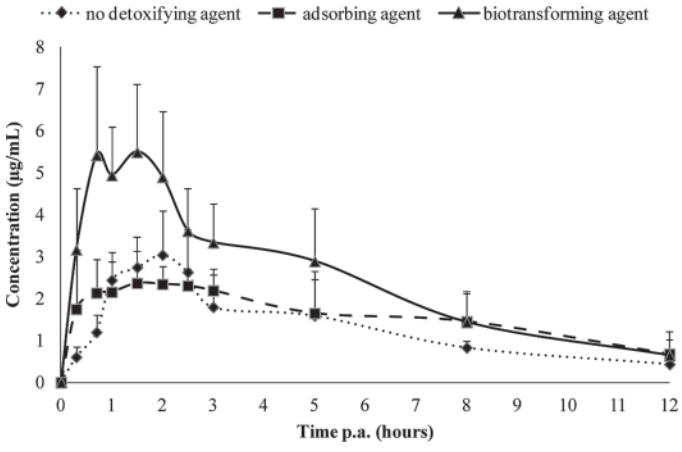

Figure 2. Effects of 3 wk of feeding detoxifying agents on the plasma concentration versus time profile of oxytetracycline, administered as a single oral bolus of 100 mg/kg of BW in broilers (n = 8, mean + SD).

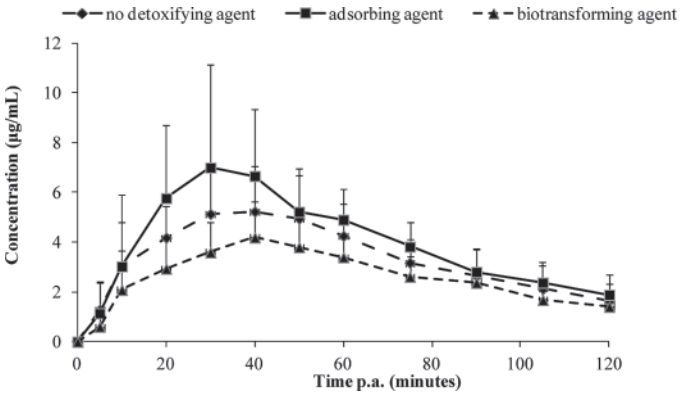

Figure 3. Influence of 3 wk of feeding detoxifying agents on the plasma profile of amoxicillin, administered as a single oral bolus of 20 mg/kg of BW in broilers (n = 8, mean + SD).

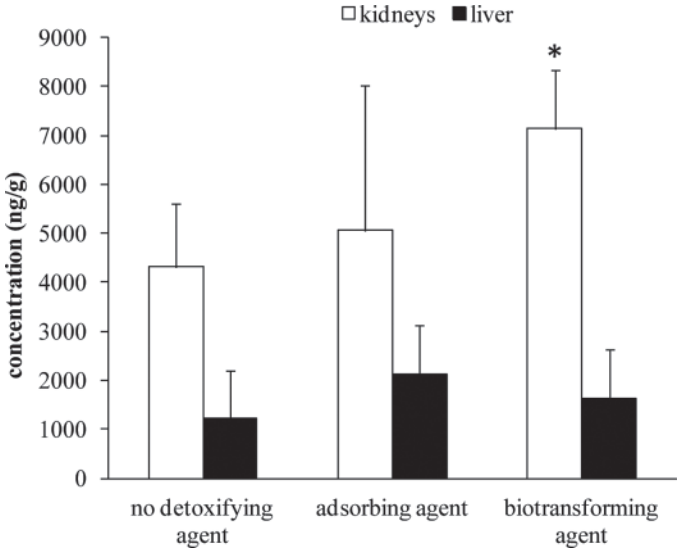

Figure 4. Influence of 3 wk of feeding of detoxifying agents on residues of oxytetracycline in broiler tissues, obtained 16 h after withdrawal from medicated drinking water (n = 8, mean + SD; * = significant difference).

and live weight gain, could not be used to demonstrate efficacy of the tested detoxifiers for DON in broilers because no significant differences were seen between groups. Nevertheless, the same biotransforming agent showed potential in diminishing deleterious effects of DON on growth performance and other nonspecific parameters in pigs (Plank et al., 2009) and of T-2 toxin in broiler chickens (Diaz et al., 2005). Moreover, Dänicke et al. (2003) stated that this detoxifying agent even decreased the performance of broilers independently of the dietary mycotoxin concentration. Negative effects on the performance of the broilers however were not noticed during this trial.

Analysis of DON in plasma only resulted in quantifiable levels in those birds that received DON in a concentration of 7.54 ± 2.20 mg/kg of feed, which is higher than the guidance level of 5 mg/kg in feed, during a 3-wk feeding period (Anonymous, 2006). It was remarkable that supplementation of the naturally contaminated feed with mycotoxin-detoxifying agents even slightly increased the plasma levels of DON within the first 2 wk of feeding. Devreese et al. (2012) performed in vivo efficacy testing of detoxifying agents in broilers with different oral bolus models. A single oral bolus of DON with or without a detoxifying agent in broilers also revealed a significantly higher $AUC_{0\rightarrow\infty}$, C_{max} and relative oral bioavailability in the group with a detoxifying agent compared with the control group. Possible hypotheses for the increased oral bioavailability of DON include unspecific effects such as an increased ratio of villus height-crypt depth due to feeding detoxifying agents (de los Santos et al., 2007; Baurhoo et al., 2009; Star et al., 2009), but further investigations are needed to confirm this. Other nonspecific effects, such as increased protein digestibility, were also attributed to detoxifying agents and should be investigated, as they can have an influence on the bioavailability of essential feed constituents (Döll and Dänicke, 2004). Surprisingly, after 3 wk of feeding, no plasma levels above the LOQ were observed in any of the experimental groups. After 3 wk of feeding, the birds may have developed a kind of tolerance to the negative effect of deoxynivalenol. This resistance may be attributed to the age of the birds or to a kind of adaptation process through a variety of metabolic and hormonal compensatory mechanisms (Moon et al., 2008; Kobayashi-Hattori et al., 2011). Very low plasma concentrations of DON were measured during this trial, which correspond with the literature, where ^{14}C-labeled DON was described to be poorly absorbed from the gastrointestinal tract of hens given that peak plasma levels occurring 2 to 2.5 h postadministration accounted for only 0.64% of the administered dose (Prelusky et al., 1986). Our group also demonstrated an absolute oral bioavailability of only 19.3% for DON in broilers after a single oral bolus of DON (0.75 mg/kg of BW; A. Osselaere, unpublished data). Other researchers reported concentrations of DON and its de-epoxidized metabolite DOM-1 below the LOQ of 2 ng/mL in plasma after 5 wk of feeding DON in a concentration of 2.5 mg/kg (Dänicke et al., 2007). Because continuous feeding trials with plasma analysis do not seem to be promising to test detoxifiers for DON in poultry, bolus models to test the efficacy of detoxifying agents were developed at our department. Higher plasma concentrations were observed after a single oral administration of DON (0.75 mg/kg of BW), which was more appropriate to test the efficacy of the detoxifiers compared with continuous feeding trials (Devreese et al., 2012). In an effort to look for other possible end-points, the concentrations of DON and DOM-1 were determined in liver, kidneys, and bile fluid after 3 wk of feeding. Prelusky et al. (1986) described the important role of biliary excretion in the elimination of DON from the body. Again, no residues above the LOQ were detected in the collected organs. On the other hand, DON was detectable in the bile fluid, but only for these groups which received a diet with an amount of DON higher than 5 mg/kg of feed. Deepoxydeoxynivalenol was even found in the bile fluid of all the groups that received DON-contaminated feed. This is in contrast with the results reported by Dänicke et al. (2007), where no concentrations of DOM-1 were detected in the bile fluid (LOQ = 4 ng/mL) of chickens after 3 wk of feeding. Supplementation of the diets with an adsorbing or a biotransforming agent did not have significant effects on the concentration of DON and DOM-1 in the bile fluid.

The safety of mycotoxin-detoxifying agents is an important issue that did not receive much attention until now (Phillips et al., 2009). Detoxifying agents are able to modify the chemical structure of mycotoxins or to exert their activity by binding mycotoxins in the gastrointestinal tract. However, interactions with nutrients, feed additives, or veterinary drugs can possibly occur. Therefore, we also investigated the possible

interaction of detoxifying agents with the pharmacokinetic profiles and tissue residues of the antibiotics oxytetracycline and amoxicillin, which are frequently used in poultry practice (Abo El-Sooud et al., 2004; Ismail and El-Kattan, 2004). To our knowledge, only the recent EFSA scientific opinion (Anonymous, 2011) and a report of The Bureau of Veterinary Drugs of Canada (Anonymous, 1992) discuss a possible interaction between bentonite and tylosin or with coccidiostats. After 3 wk of feeding diets supplemented with a detoxifier, significantly different plasma profiles depending on the type of antibiotic were observed. For oxytetracycline, the birds receiving feed supplemented with a biotransforming agent had a significantly higher C_{max} and AUC than the other birds ($P < 0.05$). The results of the residue depletion study of oxytetracycline in tissues also showed higher concentrations of oxytetracycline in the kidneys of these birds compared with the control group. Kidneys play an important role in the elimination of oxytetracycline in poultry (Ismail and El-Kattan, 2004). In contrast, for amoxicillin, the group with the adsorbing agent reached significantly higher plasma concentrations and AUC, only when compared with the group with the biotransforming agent. For this antibiotic, no tissue residue levels above the limit of quantification were observed 12 h after the last administration, which can be explained by the rapid elimination of amoxicillin in poultry (Anadon et al., 1996). In this study, clay-based detoxifiers were used, which have a nonspecific binding capacity. Moshtaghian et al. (1991) noted that clays can absorb micronutrients and thus can have a negative impact on the bioavailability of minerals and trace elements. Clays consist of a porous structure with electric elementary charges which can trap particles with a particular size and electric charge (Jouany, 2007). Surprisingly, we observed an enhancement in bioavailability for these antibiotics rather than a reduction. Although the mechanism is still unclear, the possible consequences of these findings are very important. Adaptation of the dosage of the antibiotic can be necessary; otherwise, the higher plasma concentrations of the antibiotic can possibly lead to toxicity and to higher tissue residue levels. An advantage could be that the dosage of the antibiotic can be reduced and thus could lead to a reduced use of antibiotics. Influence of detoxifying agents on the pharmacokinetics of doxycycline has also been demonstrated in pigs by co-authors. Significantly higher plasma concentrations were observed in piglets that received T-2 toxin-contaminated feed supplemented with a mycotoxin-detoxifying agent (Goossens et al., 2012).

In conclusion, we have shown that the analysis of DON and its metabolite in vivo is not a relevant endpoint for the efficacy testing of mycotoxin detoxifying agents in plasma of broilers when fed in a continuous design. The safety testing of 2 commercially available detoxifiers revealed possible interactions with veterinary drugs, which can have important consequences and warrants further investigations to elucidate underlying mechanisms.

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