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Technical Literature

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Efficacy of TOXFIN® XL to prevent mycotoxin absorption by piglets – *In vivo* toxicokinetic study

Summary

The negative effects of mycotoxins on animal health and performance are widely known and mycotoxin-detoxifying agents such as mycotoxin binders are commonly added to animal feed to prevent these negative effects. Although previous *in vitro* and *in vivo* data already showed good effects of TOXFIN® XL on mycotoxin binding and mycotoxin excretion and a direct effect on reproductive organs in piglets, the European Food Safety Authority states that the efficacy of mycotoxin-detoxifying agents should be investigated based on toxicokinetic studies. Therefore, a toxicokinetic study using piglets was performed. In total 12 Belgium Landrace x Piétrain piglets were divided into 2 groups: a positive control group and a treatment group. All pigs were challenged with mycotoxins on day 8: 0.05 mg Ochratoxin A (OTA)/kg BW and 0.05 mg Deoxynivalenol (DON)/kg BW and at day 10: 3mg Zearalenone (ZEA)/kg BW, via an intragastric tube. The pigs in the treatment group received 150 mg of TOXFIN XL/kg BW on day 8 and day 10 via the same intragastric tube. Blood was collected at several time points and plasma levels of mycotoxins and their metabolites were quantified using LC-MS/MS or high-resolution mass spectrometry and toxicokinetic parameters were analysed. The addition of TOXFIN XL significantly reduced the relative oral bioavailability of the ZEA-GlcA (Glucuronidated Zearalenone, a phase II metabolite) biomarker to 37.6%, proving its ability to absorb ZEA. In addition, the absorption of OTA during the first 2 hours was significantly reduced by TOXFIN XL and the relative oral bioavailability of OTA was reduced to 72.6%, demonstrating the ability of TOXFIN XL to absorb OTA. Furthermore, the study showed the ability of TOXFIN XL to slow down and reduce the absorption of DON, although the effect was not significant. In conclusion, the present toxicokinetic trial shows the ability of TOXFIN XL to reduce the absorption of mycotoxins (ZEA, OTA and DON) by piglets.

KEYWORDS: *Zearalenone, Deoxynivalenol, Ochratoxin A, Piglets, Blood*

Introduction

Mycotoxins are secondary metabolites produced by different fungal species. It is a common practice to add mycotoxin-detoxifying agents, such as mycotoxin binders, to feed in order to reduce the bio-availability of the mycotoxins and prevent their negative effects on animal performance and health. The European Food Safety Authorization (EFSA), in its guideline states end-points for testing the efficacy of mycotoxin detoxifying agents (EFSA, 2010). Toxicokinetic studies, based on absorption, distribution, metabolization and excretion are accepted by EFSA as direct *in vivo* tests. In those tests, the plasma concentration of different mycotoxins or their metabolites is measured at different time points after oral bolus administration of the mycotoxins with or without the mycotoxin binder (Devreese *et al.*, 2012). The target mycotoxin of this toxicokinetic trial was Zearalenone (ZEA), but also the efficacy of TOXFIN XL to absorb the mycotoxins Ochratoxin A (OTA) and Deoxynivalenol (DON) was assessed in this trial.

Materials and Methods

Trial set-up

For this trial 12 healthy piglets (6 females and 6 males) from the same breed (Belgium Landrace x Piétrain) and with an average body weight of 19.5 kg were purchased from a local pig farm. Upon arrival in the research center, piglets were randomly assigned to two groups: a positive control group (challenged with mycotoxins) and a treatment group (challenged with mycotoxins and fed a mycotoxin binder). Each group was divided over 2 pens (3 animals/pen) of 4 m^2 each. All piglets from both groups followed an acclimatization period of 1 week during which they received a commercial feed, without mycotoxin challenges or any mycotoxin binder, and water *ad libitum*. The commercial feed was analyzed for the presence of main mycotoxins by a multi-mycotoxin LC-MS/MS method. Results showed a low contamination level only for DON and ZEA, with values significantly below the recommended level reported by the EU regulation 2006/576/EC.

At the end of the acclimatization period, the piglets were fasted for 12 hours (feed was withdrawn, water was still available), before the actual challenge. At day 8, the pigs of the treatment group received 150 mg of TOXFIN XL/kg BW via an intragastric tube. For easy administration, the mycotoxin binder was suspended in water. Immediately following the mycotoxin binder administration, the piglets were challenged with a combination of two mycotoxins, receiving 0.05 mg of DON/kg BW and 0.05 mg of OTA/kg BW, dissolved in ethanol/water (1/10, v/v) via the same intragastric tube. The piglets in the positive control group only received the mycotoxin challenge (same concentrations) via the intragastric tube.

On day 10, the piglets of the treatment group received again 150 mg of TOXFIN XL/kg BW, immediately followed by a challenge of 3 mg of ZEA/ kg BW (ZEA was dissolved in dimethylsulfoxide/water (1/10, v/v)). The piglets in the positive control group only received the ZEA challenge via the intragastric tube.

The challenge and treatment scheme is further elaborated in Table 1. After each challenge, the intragastric tube was rinsed with 50 ml of tap water to ensure that no mycotoxin nor mycotoxins binder remained in the tube.

Table 1. Mycotoxins challenges and treatment scheme.

Positive Control	mg/kg BW	Treatment	mg/kg BW
Day 8		*Day 8*	
DON	0.05	DON	0.05
OTA	0.05	OTA	0.05
TOXFIN XL	0	TOXFIN XL	150
Day 10		*Day 10*	
ZEA	3	DON	3
TOXFIN XL	0	TOXFIN XL	150

Blood samples of the 12 piglets were taken from the *vena jugularis externa*. The time points of blood sampling for the first challenge (day 8) were 0 h (just before administration), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48, 72 and 96 h (post administration).

The time points of blood sampling for the second challenge (day 10) were 0 h (just before administration) and 0.10, 0.20, 0.30, 0.45, 1, 1.5, 2, 3, 4, 6 and 8 h (post administration).

In both cases, all blood samples were centrifuged within 2 hours after collection. The plasma was stored at -20°C until analysis. DON, OTA and ZEA were determined according to the validated LC-MS/MS method described by Devreese *et al.* (2012b). The limit of quantification (LOQ) of DON, DOM-1, OTA, ZEA in pig plasma is 1, 2, 5 and 0.5 ng/mL, respectively. The extracted pig plasma samples were also analyzed by high resolution mass spectrometry (UHPLC-HR-

MS) analysis for semi-quantification of the phase II glucuronide conjugate of ZEA (ZEA-GlcA) based on Devreese *et al.* (2015).

Toxicokinetic analysis

Toxicokinetic modelling of the plasma concentration-time profiles of DON, OTA was done by non-compartmental analysis (WinNonlin 6.3, Pharsight Corporation, USA). Following parameters were calculated: area under the curve from time zero to time t ($AUC_{0\rightarrow t}$), area under the curve from time zero to infinite ($AUC_{0\rightarrow\infty}$), maximal plasma concentration (C_{max}) and time at maximal plasma concentration (T_{max}).

For ZEA, no toxicokinetic modelling could be performed since all plasma concentrations were below the LOQ of 0.5 ng/ml. For the ZEA-GlcA biomarker, the $AUC_{0\rightarrow 6h}$ of the instrument response ratio (chromatographic peak area of ZEA-GlcA divided by the peak area of the internal standard (IS)) –time profile was calculated, using Excel Pharmacokinetic Functions.

Effect of TOXFIN XL on oral absorption of ZEA, DON and OTA

The relative oral bioavailability ($F = \frac{AUC0\rightarrow\infty\ \text{mycotoxin+binder}}{AUC0\rightarrow\infty\ \text{mycotoxin}} x\ 100$ for DON and OTA, and $F = \frac{AUC0\rightarrow t\ \text{mycotoxin+binder}}{AUC0\rightarrow t\ \text{mycotoxin}} x\ 100$ for ZEN) was evaluated for each mycotoxin as a marker for efficacy of the mycotoxin binder in the study. The effect of the mycotoxin binder on the oral absorption of the mycotoxin was evaluated by comparing toxicokinetic parameters between the mycotoxin (Positive Control) and mycotoxin+binder (Treatment) pigs, with special emphasis on $AUC_{0\rightarrow\infty}$ and C_{max}. An independent sample t-test was performed on the PK parameter AUC with SPSS 24.0 (IBM, USA) to evaluate possible significant differences. The level of significance was set at 0.05. When the relative F value is below 80%, the treatments are considered as not bio-equivalent, indicating the efficacy of the binder to prevent the absorption of mycotoxins.

Results

The effect of administering TOXFIN XL to piglets challenged with ZEA is shown in Figure 1. As demonstrated in literature (Devreese *et al.* 2012, Osselaere *et al.* 2013, Devreese *et al.* 2015), ZEA level in plasma can be detected but not quantified as the concentration is below the LOQ (Limit of Quantification). For this reason, during toxicokinetic studies, ZEA metabolites are analyzed, with phase II metabolites like the ZEA-glucuronide being the main one. However, as reported by Devreese *et al.* (2015), there is no analytical standard of ZEA-GlcA commercially available making it impossible to run quantitative analysis. Only a semi-quantification was possible by comparing the area under the peak of ZEA-GlcA vs the area under the peak of Internal Standard (IS). Of course, as the IS was a constant, a higher ratio of ZEA-GlcA / IS means a higher level of ZEA-glucuronide in the blood, thus a higher absorption of ZEA by the animals.

Results show clearly that ZEA is immediately absorbed by piglets (Figure 1, max peak 10 min after ZEA administration). The administration of TOXFIN XL significantly prevents the absorption of ZEA by piglets. It is also important to notify that the SD of the Positive Control group is higher than that of the Treatment group, clearly proving that TOXFIN XL is not only reducing the average ZEA absorption, but it is working uniformly in all treated piglets. The relative oral bioavailability based on the ZEA-GlcA biomarker was 37.6% and it was statistically significant (P = 0.027). In a few words, the relative oral bioavailability represents the fraction of mycotoxins (or their metabolites) quantified in the plasma of piglets from the treatment group compared to the fraction of mycotoxins quantified in the plasma of piglets from the Positive Control group. A relative F value of 37.6% means that in the plasma of the Treatment group, 37.6% of the mycotoxins in the Positive Control group are found, or vice versa that TOXFIN XL prevents the absorption of 73.4% of mycotoxins.

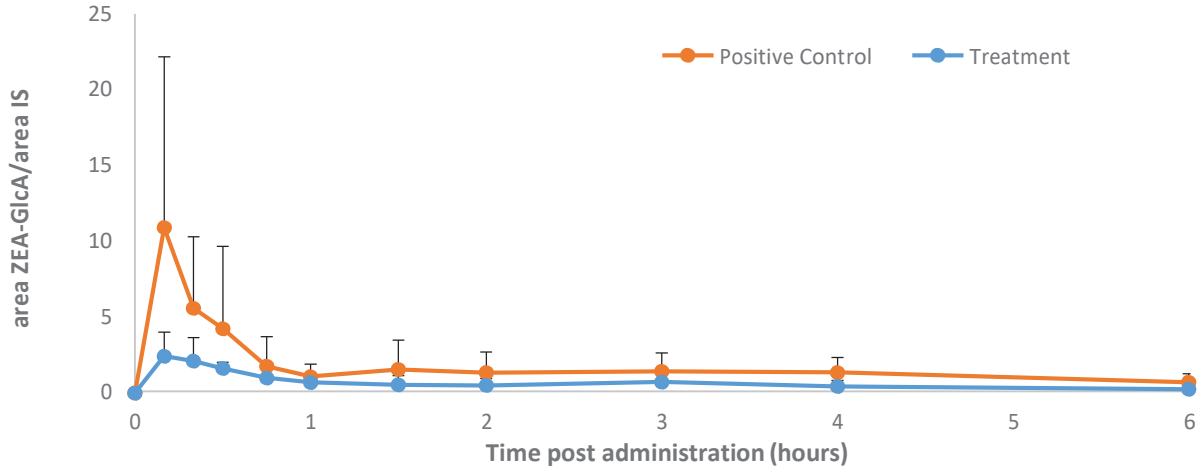

Figure 1. Ratio-time profile of area ZEA-GlcA / area IS and standard deviation (SD) in plasma from piglets challenged with 3 mg ZEA/kg BW (Positive Control) and piglets challenged with same level of ZEA plus administration of 150 mg of TOXFIN XL / kg BW (Treatment).

The effect of TOXFIN XL on the absorption of OTA is shown in Figure 2 and Table 2. TOXFIN XL can reduce (lower $AUC_{0\rightarrow\infty}$ and C_{max}) OTA absorption as well as slow it down (higher T_{max}). If we look at the absorption of OTA during the first two hours ($AUC_{0\rightarrow2h}$), a statistically significant effect of the treatment with TOXFIN XL can be observed ($P<0.05$). As a result, the relative oral bioavailability of OTA is reduced to 72.6% when feeding TOXFIN XL together with the mycotoxins. This result confirms the ability of TOXFIN XL to effectively prevent OTA absorption ($F < 80\%$).

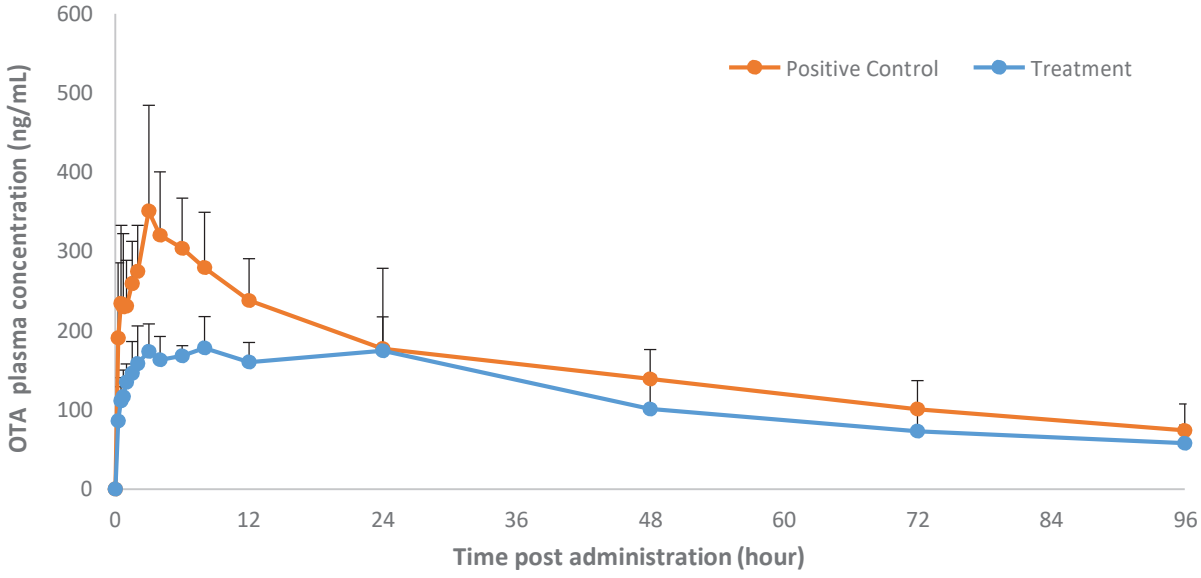

Figure 2. Mean (+ SD) plasma concentration-time profile of OTA from piglets challenged with 0.05 mg OTA/kg BW (Positive Control) and piglets challenged with same level of OTA plus administration of 150 mg of TOXFIN XL / kg BW (Treatment).

Table 2. Major toxicokinetic parameters registered after single OTA challenge.

Toxicokinetic parameters	Positive Control	Treatment	Treat. *vs* P.C. (%)
$AUC_{0\rightarrow 2h}$ (ng/ml)	480±80	320±110*	66.7
$AUC_{0\rightarrow \infty}$ (ng/ml)	21670±9250	15730±5740	72.6
C_{max} (ng/ml)	368.12±105.42	224.45±77.53	60.9
T_{max} (h)	4.33±2.88	10.41±10.77	240
Relative F (%)	100	72.6	

* $p < 0.05$

The effect of challenging piglets with DON, with or without the addition of TOXFIN XL is shown in Figure 3 and Table 3. The difficulty to prove the binding of DON by mycotoxins binders is well reported in literature (Avantaggiato *et al.*, 2005). However, we registered various field experiences; where using Kemin mycotoxin binders in feeds naturally contaminated with DON, resulted in a reduction of classic symptoms, including in very sensitive animals like swine. To give a scientific answer to what seen in the field, we decided to run also a toxicokinetic study for DON. Results of this study showed the ability of TOXFIN XL to slow down and to reduce the absorption of DON: lower $AUC_{0\rightarrow 8}$ and C_{max} and higher T_{max}. These changes in toxicokinetic parameters resulted in a reduction of the relative oral bioavailability to 84.97%, very close to the limit of 80% identified as a reference for proving the *in vivo* efficacy of a mycotoxin binder.

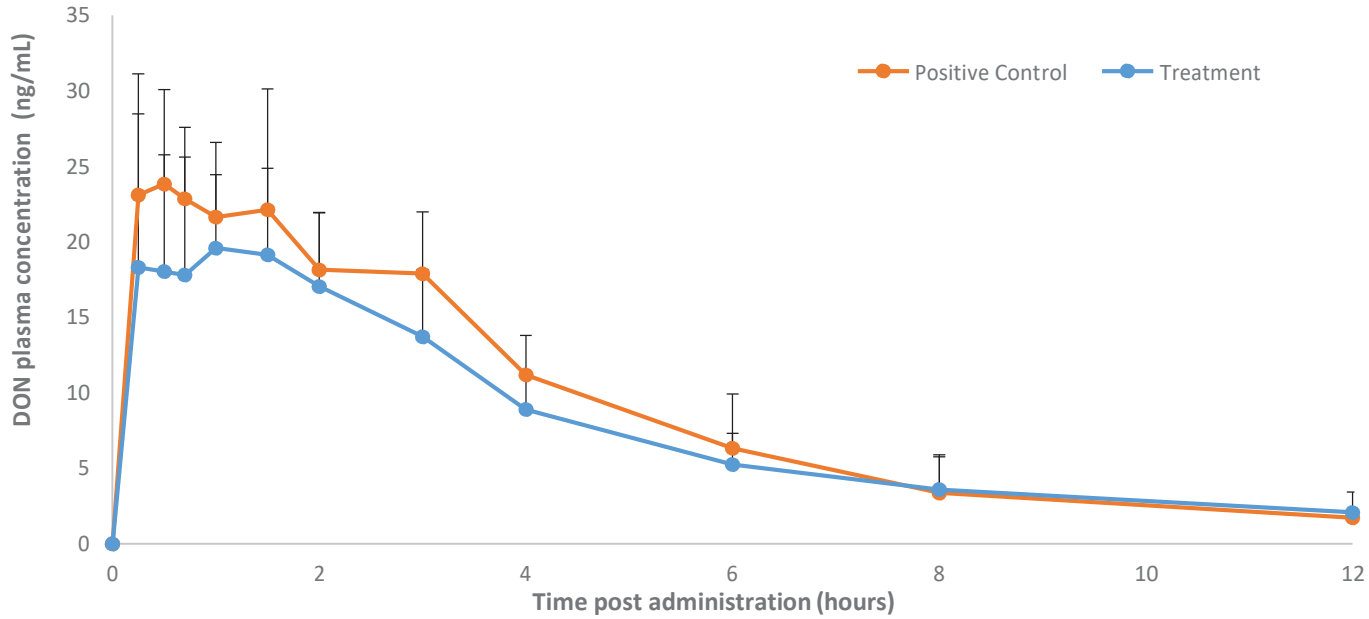

Figure 3. Mean (+ SD) plasma concentration-time profile of DON from piglets challenged with 0.05 mg DON/kg BW (Positive Control) and piglets challenged with same level of DON plus administration of 150 mg of TOXFIN XL / kg BW (Treatment).

Table 3. Major toxicokinetic parameters registered after single DON challenge.

Toxicokinetic parameters	Positive Control	Treatment	Treat. *vs* P.C. (%)
$AUC_{0\rightarrow 8}$ (ng/ml)	96.30±17.89	81.83±15.19	84.97
C_{max} (ng/ml)	29.40±5.71	22.97±7.72	78.13
T_{max} (h)	0.63±0.52	1.49±1.02	236.5
Relative F (%)	100	84.97	

Conclusions

The current *in vivo* trial once again confirmed the extraordinary efficacy of TOXFIN XL to prevent ZEA absorption in reared animals. This was demonstrated by a toxicokinetic trial. In this toxicokinetic trial, the relative oral bioavailability of ZEA (calculated by measuring the ZEA-GlcA metabolite) was significantly reduced (37.6%, p=0.027) when TOXFIN XL was administered to piglets immediately following a challenge with ZEA. The use of mycotoxin biomarkers (i.e ZEA-GlcA) is an approved methodology, recognized by EFSA, to determine mycotoxin binders' efficacy. In this case, the biomarker ZEA-GlcA was used as it was not possible to determine the direct/quantitative effect of ZEA, since the ZEA level in plasma was below the LOQ. The same toxicokinetic trial also demonstrated the efficacy of TOXFIN XL to reduce OTA absorption, showing a F value of 72.6% and with statistical differences within the first 2 hours after administration ($p<0.05$). This result is extremely important for a mycotoxin rapidly absorbed after ingestion.
Even though different *in vitro* tests confirm the difficulties of mycotoxin binders to effectively bind DON the results of this toxicokinetic trials showed a tendency of TOXFIN XL to reduce DON absorption. In fact, its inclusion reduced the relative oral bioavailability (84.97%, not statistically different, but very close to the referenced value of 80%), the maximum level of DON absorbed (C_{max} of 22.97±7.72 vs 29.40±5.71 µg/ml) and postponing the time required to register the high level (T_{max} of 1.49±1.22 vs 0.63±0.52 h).

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