SAFETY OF MYCOTOXIN BINDERS REGARDING THEIR USE WITH VETERINARY MEDICINAL PRODUCTS IN POULTRY AND PIGS:

AN *IN VITRO* AND PHARMACOKINETIC APPROACH

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in Veterinary Science

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“Inside of every problem lies an opportunity”

Robert Kiyosaki

CONTENTS

LIST OF ABBREVIATIONS

[*B*]: concentration of unoccupied binding sites on the binder in an *in vitro* binding experiment

[B_0]: initial concentration binding sites on the binder in an *in vitro* binding experiment

[*BD*]: concentration of occupied binding sites in an *in vitro* binding experiment

[*D*]: free drug concentration in an *in vitro* binding experiment

[D_0]: initial concentration of drug and in an *in vitro* binding experiment

AC: activated carbon

ACN: acetonitrile

AFB1: aflatoxin B1

AFM1: aflatoxin M1

ANOVA: analysis of variance

AUC: area under the curve

$AUC_{0\text{-}12h:}$ area under the plasma concentration-time curve from 0 h until 12 h time point

AUC_{0-24h}: AUC from time zero to the 24 h time point

AUC_{0-inf}: AUC from time zero to infinity

BC_{50}: amount of binder required to bind half of the drug in an *in vitro* binding experiment

BEMEFA: Belgian association for compound feed producers

BW: bodyweight

C_{ads}: adsorbed concentration in an *in vitro* binding experiment

C_{aq}: free concentration of drug in the medium in an *in vitro* binding experiment

CEC: cation exchange capacity

Cl/F: clearance scaled for absolute bioavailability

C_{max}: maximum plasma concentration

COLE: coefficient of linear extensibility

C_{ss}: plasma concentration at steady state

DDGS: distillers dried grain with solubles

DMCTC: demethylchlortetracycline

DON: deoxynivalenol

EDTA: ethylenediaminetetraacetic acid

EFSA: European Food Safety Agency

EMA: European Medicines Agency

EPT: 12,13 epoxytrichothec-9-ene

ESI+: electrospray ionization in the positive mode

F: bioavailability

FASCF: Federal Agency for the Safety of the Food Chain

FB1: fumonisin B1

g: goodness of fit

GAP: good agricultural practices

GIT: gastro-intestinal tract

HE: high exposure

HPLC: high-performance liquid chromatography

HSCAS: hydrated sodium calcium aluminosilicate

HT-2: HT-2 toxin

IARC: International Agency for Research on Cancer

ICP-AES: inductively coupled plasma-atomic emission spectrometry

IR: infra-red

IS: internal standard

K_{BD}: equilibrium constant in an *in vitro* binding experiment

K_d: distribution coefficient

k_{el}: elimination rate constant

K_f: Freundlich distribution coefficient

LAB: lactic acid bacteria

LC-MS/MS: liquid chromatography−tandem mass spectrometry

LD50: dose at which half of the population is expected to die

LE: low exposure

LOD: limit of detection

LOQ: limit of quantification

LSD: least significant difference

MeDIC: methyldiclazuril

MeOH: methanol

MF: mineral fraction

MIC: minimal inhibitory concentration

MIPs: molecularly imprinted polymers

MON: monensin

MOS: mannan oligo saccharides

MPN/MCN: mycotoxin induced porcine/chicken nephropathy

MRL: maximum residue limit

MS/MS: tandem mass spectrometry

NIG: nigericine

OTA: ochratoxin A

p.a.: post administration

p: significance level

PBPK: physiologically-based pharmacokinetic model

PBS: phosphate buffered saline

PD: pharmacodynamic

PK: pharmacokinetic

PPE: porcine pulmonary oedema

r: correlation coefficient

R: ideal gas constant

relative F: relative bioavailability

RH: relative humidity

RSD: relative standard deviation

Sa: sphinganine

SAL: salinomycin

SD: standard deviation

SHIME: simulator of the human intestinal microbial ecosystem

So: sphingosine

SRM: selected reaction monitoring

T: temperature

$T_{1/2,el}$: elimination half-life

T-2: T-2 toxin

tBME: tert-butyl methylether

TFA: trifluoroacetic acid

TIL: tilmicosin

T_{max}: time of maximum plasma concentration

TNO: Toegepast Natuurkundig Onderzoek, applied scientific research

TYL: tylosin

V_d/F: volume of distribution scaled for absolute bioavailability

VMP: veterinary medicinal product

XRD: X-ray diffraction

ZEN: zearalenone

ΔG^0: Gibbs free energy of the binding reaction

ΔH^0: bond-dissociation energy

ΔS^0: bond-entropy

GENERAL INTRODUCTION

1 MYCOTOXINS IN FEED

1.1 General

What are mycotoxins?

Etymologically the word mycotoxin stems from the Greek word *mykes* (mould) and the Latin word *toxicum* (poison). Mycotoxins are secondary metabolites that can be produced by various types of fungi. Some fungi develop mainly on the crop before harvest whereas other fungi usually emerge during storage. Mycotoxins are no primary metabolites for the fungi (Bennett, 1983), but they seem to have useful properties for the fungi that produce them. For example, the production of aflatoxins is closely related to the presence of reactive oxygen species (Reverberi et al., 2008). It is believed that the production of aflatoxins helps to manage oxidative stress inside the fungi (Narasaiah et al., 2006). The mycotoxins patulin and penicillic acid inhibit the communication between cells of *Pseudomonas aeruginosa*, a potentially dangerous bacterium (Rasmussen et al., 2005). Some parasitic and endophytic fungi produce mycotoxins to protect their host against predators such as insects or plant-eating animals (Vega, 2008). The phytotoxic effects of some mycotoxins, such as ochratoxin A (OTA), enables the fungi to invade a plant host by inhibition of plant immunity systems resulting in the induction of lesions (Peng et al., 2010) and interference with the hosts' metabolism (Paciolla et al., 2004). In general it is deemed that mycotoxin-producing fungi thrive better than fungi which do not (Fox and Howlett, 2008). Currently there are over 400 chemical entities classified as mycotoxins. The most important in the context of occurrence and toxicity, which are also the most relevant in the feed industry, include aflatoxins, ochratoxins, trichothecenes, fumonisins and zearalenone (Turner et al., 2009). Besides, modified forms produced by either fungi, plants or during processing also exist (Broekaert et al., 2015).

Fungi of the *Fusarium* genus are considered to be field fungi whereas fungi of the genera *Aspergillus* and *Penicillium* usually develop during storage, although they may also develop in earlier stages of the feed production chain. The growth of fungi is promoted in the presence of certain environmental factors. The main factors are temperature, humidity, presence of nitrogen and oxygen. Secondary factors include insects (control) and damage to the crops (Nelson, 1993).

Risk of mycotoxin contamination

The risk associated with mycotoxin contamination of crops is determined on the basis of the toxicity of the mycotoxin and the exposure. Figure 1 presents the key drivers for the risk and how they are related to each other. Toxicity is traditionally approached as an acute phenomenon, in which a single exposure to a high dose causes a set of symptoms. An important parameter to assess toxicity is the dose at which half of a population is expected to die, the lethal dose 50 or LD_{50}. Although LD_{50}-values are known for most toxins in rodents, this approach is not suitable to assess the risk of mycotoxins for animal production. Apart from the acute toxic effects, ingesting mycotoxins in low doses for a longer period can lead to a reduced feed intake, reduced immunity and impaired gut health (Osselaere et al., 2013b; Antonissen et al., 2015). Hence, they cause mainly economic damage by reducing the zootechnical performance of food producing animals. They also increase the susceptibility to infections (Vandenbroucke et al., 2011; Antonissen et al., 2014). The exposure depends on the consumption of contaminated feed and the level of contamination. Important for the consumption driver is the product mix that is consumed. Both the amount and type of consumption (product mix), is subject to the laws of supply and demand for feed, which might

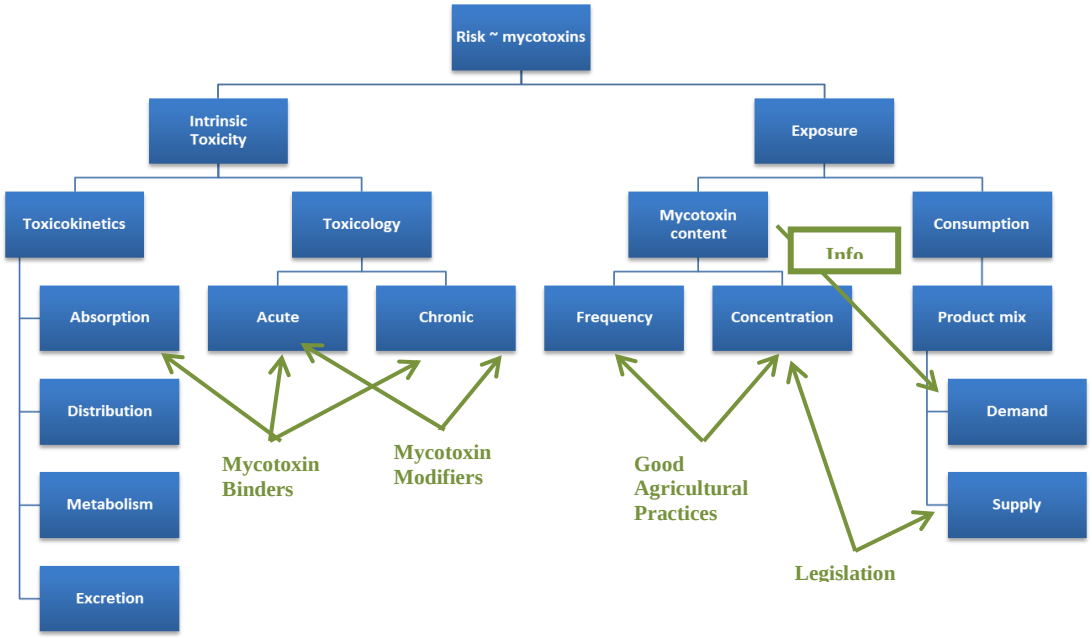

Figure 1: Key drivers of the risk associated with mycotoxin contamination of crops. The green arrows connect factors that can reduce the risk of the applicable driver. Adapted from (Tirado et al., 2010).

be contaminated by mycotoxins. Information about the mycotoxin content can influence the demand for certain types of feed and thereby shift the product mix towards a less mycotoxin-containing mix. On the other hand, legally enforced maximum limits or guidance levels for maximum mycotoxin contamination will prevent contaminated batches from entering the market. This can be achieved by reducing the supply of heavily contaminated crops and limit the concentration in batches that will be consumed by food producing animals.

1.2 Toxicology of the most important mycotoxins

Aflatoxins

Aflatoxins are produced by *Aspergillus* fungi, differentiation between the toxicologically most relevant types of aflatoxins is based on their fluorescence, namely those reflecting blue light are labelled B1 and B2, those reflecting green light are labelled G1 and G2. Aflatoxin B1 (AFB1) is the most important of the family, it is classified as a class I carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 2002) indicating the carcinogenic effects are proven for humans. Its structure is presented in Figure 2. AFB1 has a difuranocoumarin structure. Sterigmatocystine, also an *Aspergillus* mycotoxin, is an intermediate in the biosynthesis of AFB1.

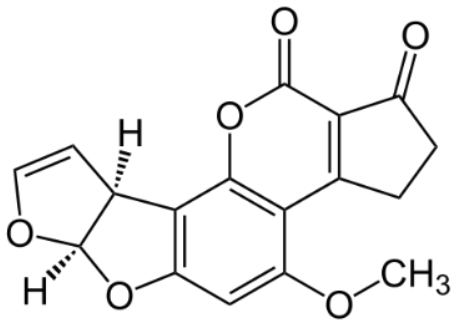

Figure 2: Skeletal formula of aflatoxin B1 (AFB1).

The absorption is rapid and complete in most species (Yunus et al., 2011), the main metabolic pathway is the phase I bio-activation to the genotoxic AFB1-epoxide and primarily occurs in the liver which is the target organ for toxicity. Metabolism to other metabolites can take place, including aflatoxicol and aflatoxin M1 (AFM1). Excretion is slower than most other toxins, after 7 days about 71% could be recovered (Sawhney et al., 1972). At levels <0.3 mg/kg feed for pigs and 1 mg/kg for chickens, weight reduction is the most obvious symptom (Dersjant-Li et al., 2003). Aflatoxin M1 is categorized as class 2B carcinogen (probably carcinogenic to humans) (IARC, 2015).

Ochratoxins

Ochratoxins are mainly produced by *Aspergillus* and *Penicillum* fungi. Ochratoxin A (OTA) is the most important mycotoxin in this group. In pigs, OTA (Figure 3) affects primarily the liver and kidneys. Ingestions of concentrations up to 1 mg/kg feed for prolonged periods of time may cause nephropathy (Krogh et al., 1974). In chickens, OTA also primarily affects the kidneys and results in weight loss (Golinski et al., 1983). Higher concentrations of OTA (> 2 mg/kg) were able to decrease bone density, lower the absorption of carotenoids, cause glycogen accumulation in the breast muscle, and increase the number of intestinal ruptures (Huff et al., 1983). Synergistic interaction between OTA and other mycotoxins are believed to be the main cause of mycotoxin induced porcine/chick nephropathy (MPN/MCN) (Stoev and Denev, 2013). The IARC classifies OTA in category 2B (IARC, 2015).

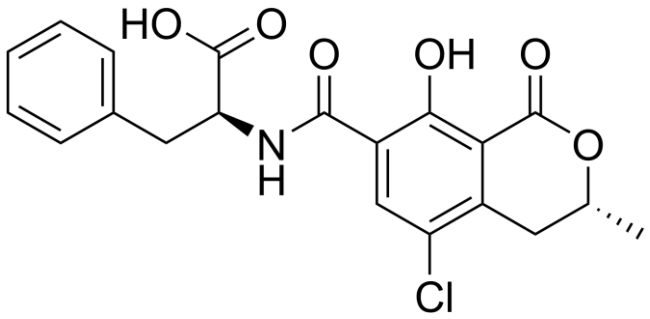

Figure 3: Skeletal formula of ochratoxin A (OTA).

Trichothecenes

Trichothecenes are produced by *Fusarium* fungi and have a sesquiterpenoid structure (Figure 4) with an epoxide ring which is responsible for their toxicity. Trichothecenes are powerful inhibitors of the protein synthesis through interaction with ribosomes. Therefore, tissues with a high regeneration rate are most sensitive. Four types (A, B, C and D) are distinguished by substitutions of the 12,13-epoxytrichothec-9-ene (EPT) core structure, which is composed of four rings (McCormick et al., 2011). Type A is in general the most toxic group, however type B trichothecenes are most common in Europe, therefore, these groups are considered the most important classes. Type A trichothecenes include T-2 toxin (T-2), its metabolite HT-2 and diacetoxyscirpenol; type B trichothecenes include nivalenol, deoxynivalenol (DON) and fusarenon-X. High doses of T-2 cause oral lesions and weight loss in broilers, in lower doses systemic effects lead to a reduced bodyweight (BW) gain (Wyatt et al., 1973). Pigs also show lesions of the mucosa which come into contact with T-2. It has a pronounced effect on the

immune system, thereby affected animals are more sensitive to secondary or opportunistic infections (Rafai et al., 1995a; Rafai et al., 1995b). DON is the most abundant trichothecene. It differs from nivalenol by the absence of a hydroxyl group at position 4 of the ring structure. Chickens are relatively resilient to the exposure to DON because of 1) a low oral bioavailability (Dänicke and Brezina, 2013; Osselaere et al., 2013a) and 2) the extensive phase II biotransformation capacity to non-toxic sulfate-conjugates (Devreese et al., 2015). Pigs on the other hand are very sensitive to DON, it elicits emesis at high concentration levels and DON is therefore sometimes named vomitoxin. Feed refusal is the most obvious symptom of DON, even at lower contamination levels. The IARC has classified DON and T-2 toxin as a class 3 carcinogenic substance (not classifiable as to its carcinogenicity to humans) (IARC, 2015).

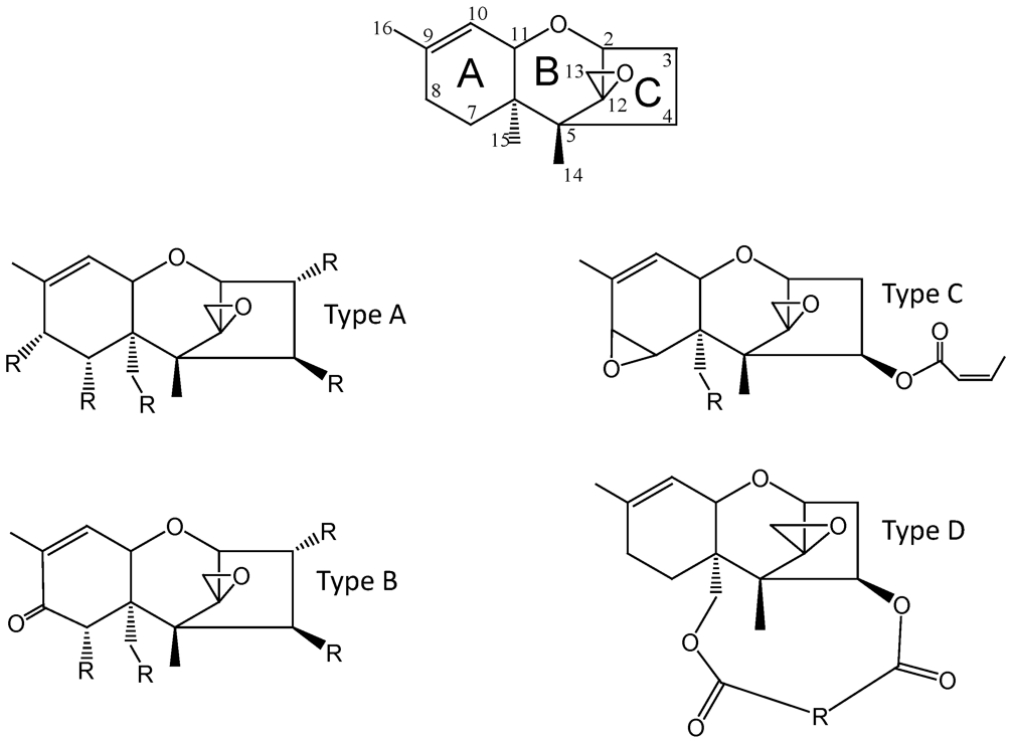

Figure 4: Classification of trichothecene structures: EPT (12,13-epoxytrichothec-9-ene); R groups may be H, OH, OAcyl, or variations in the macrolide chain (McCormick et al., 2011).

Fumonisins

Fumonisins are mainly produced by *Fusarium* fungi, they have a central carbon chain, two tricarballylic acid groups are esterified to this central carbon chain and they also contain several hydroxyl groups. Fumonisin B1 (FB1, Figure 5) is the most frequently occurring mycotoxin, it differs from B2 and B3 in the position of the hydroxyl groups. Fumonisins

interfere with the cell membrane synthesis by inhibiting the ceramide synthase. This enzyme plays an important role in the formation of sphingolipids, important components of the cell membrane. Because of the inhibition of the ceramide synthase, accumulation of sphinganine (Sa) and sphingosine (So) is observed, also the Sa:So ratio will increase and is considered an appropriate biomarker of exposure to fumonisins (Riley et al., 1994). Chickens are relatively resistant to the effects of fumonisins, yet caution is advised. Elevated liver markers (aspartate amino transferase, alkaline phosphatase and lactate dehydrogenase) have been described. Also the weight of the liver, gizzard and proventriculus was increased. Finally, the total weight of the day-old chicks decreased after administration of 100 mg FB1/kg feed (Ledoux et al., 1992). Pigs are more susceptible and effects on the respiratory system, liver and cardiovascular system are most pronounced. Contaminations of 12 mg/kg feed may cause fatal pulmonary oedema (porcine pulmonary oedema, PPE) due to myocard insufficiency (Haschek et al., 2001). The IARC categories FB1 as a class 2B toxin (IARC, 2015).

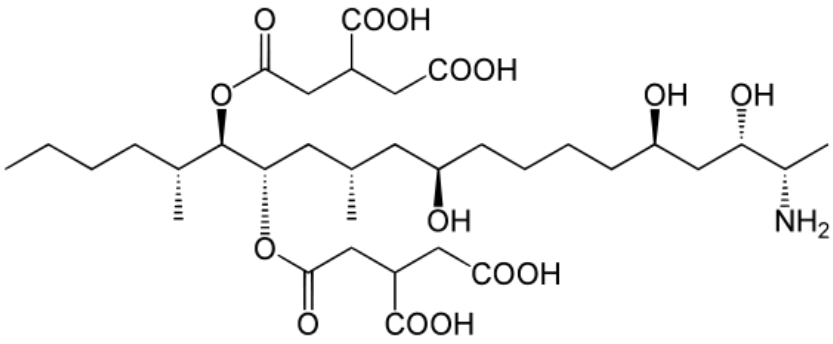

Figure 5: Skeletal formula of fumonisin B1 (FB1).

Zearalenone

Zearalenone (ZEN, Figure 6) is also produced by *Fusarium* fungi. It is a small lipophilic molecule which is extensively metabolised (phase I biotransformation) in liver to α- or β-zearalenol. Both are agonists for the estradiol receptor, ZEN is therefore called a myco-estrogen. However, α-zearalenol has a 92-times higher affinity for the estradiol receptor whereas the affinity of β-zearalenol is 2.5-times lower compared to ZEN (Malekinejad et al., 2006). Biotransformation to α-zearalenol can thus be considered as activation of ZEN, biotransformation to β-zearalenol as deactivation. In chickens, the effects of ZEN are limited because of the extensive biotransformation to β-zearalenol. Broiler chickens can tolerate feed contamination levels up to 800 mg/kg feed (Allen et al., 1981). Pigs however, are the most

sensitive species because they mainly form α-zearalenol, the reproductive organs being the primarily affected tissues, especially in females. The main symptoms are related to hyperestrogenism and include turgid sex organs (uterotropism), prolapse of the vulva, stillbirth, nymphomania, etc. (Kuiper-Goodman et al., 1987). The IARC categorizes ZEN as a class 3 toxin (IARC, 2015).

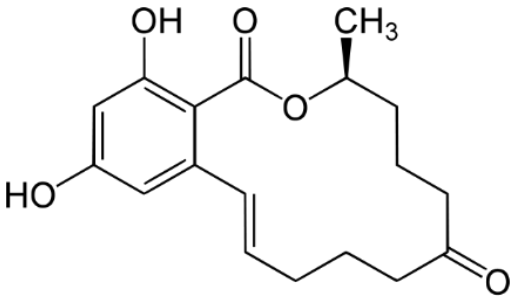

Figure 6: Skeletal formula of zearalenone (ZEN).

1.3 Prevalence

Both humans and animals are exposed to mycotoxins through contamination of food and feed (Jard et al., 2011). In 2013, a large scale survey was reported in which more than sixty thousand feed samples were analysed for the presence of aflatoxins, ZEN, DON, fumonisins and ochratoxins (Schatzmayr and Streit, 2013; Streit et al., 2013). Figure 7 and Table 1 show the percentage of samples that were positive for each of these mycotoxins.

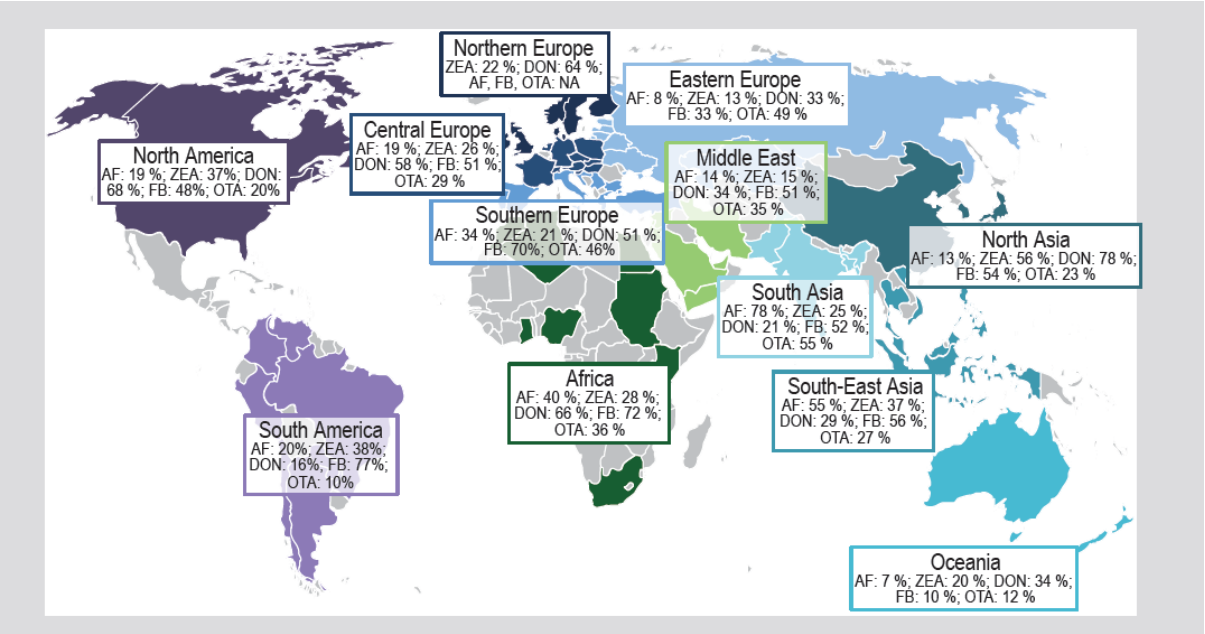

Figure 7: Global mycotoxin prevalence in surveyed regions. Finished feed and maize accounted for 27% of the samples each. The pool of samples further comprised wheat and wheat bran (9%), barley (8%), silage (8%), soybean meal (4%), distillers dried grain with solubles (DDGS; 2%), corn gluten meal (1%), rice and rice bran (1%), straw (1%) and other feed ingredients (e.g. cotton seed, sorghum, cassava, peanut, copra, etc.; 12%). Number of samples analysed for aflatoxins (AF), zearalenone (ZEA), deoxynivalenol (DON), fumonisins (FB), ochratoxin A (OTA), respectively: North America: 812; 832; 844; 820; 265; South America: 1,521; 784; 768; 1,544; 360; Northern Europe (ZEA; DON): 596; 789; others not analysed (NA); Central Europe: 241; 3,632; 5,521; 206; 235; Southern Europe: 299; 381; 463; 233; 242; Eastern Europe: 59; 106; 111; 70; 86; Africa: 302; 227; 286; 271; 47; 70; 86; Middle East: 167; 172; 170; 156; 69; South Asia: 495; 489; 478; 486; 433; South-East Asia: 2,383; 2,350; 2,237; 2,357; 1,623; Oceania: 859; 873; 873; 842; 681; North Asia: 4,723; 4,799; 4,855; 4,365; 3,352. Adopted from (Schatzmayr and Streit, 2013).

Table 1: Global mycotoxin prevalence in surveyed regions. Finished feed and maize accounted for 27% of the samples each. The pool of samples further comprised wheat and wheat bran (9%), barley (8%), silage (8%), soybean meal (4%), distillers dried grain with solubles (DDGS; 2%), corn gluten meal (1%), rice and rice bran (1%), straw (1%) and other feed ingredients (e.g. cotton seed, sorghum, cassava, peanut, copra, etc.; 12%). For the number of samples analysed per region, see Figure 7 caption. Adopted from (Schatzmayr and Streit, 2013).

	Aflatoxins[1]	Zearalenone	Deoxynivalenol	Fumonisins[1]	Ochratoxins
Number of samples	11,967	15,533	17,732	11,439	7,495
Number of positive samples	3,142	5,797	9,960	6,204	1,902
% positive samples	26	37	56	54	25
Average of the positive samples (µg/kg)	57	286	1,009	1,647	14
Median of the positive samples (µg/kg)	11	85	453	750	2.6
1th quartile of the positive samples (µg/kg)	3	43	234	332	1.1
3rd quartile of the positive samples (µg/kg)	40	225	972	1,780	6.2
Maximum of positive samples (µg/kg)	6,323	26,728	50,289	77,502	1,589
Origin sample with highest measured concentration	Myanmar	Australia	Central Europe	China	China
Sample type (year) with highest measured concentration	Other feed (2012)	Silage feed (2007)	Wheat (2007)	Compound feed (2011)	Compound feed (2011)

Results of the analysis of 19,757 samples of feed and feed raw materials sourced globally, specifying the number of samples analysed for each of the mycotoxins/mycotoxin groups, the number and percentage of samples testing positive for the respective mycotoxin as well as the average, median, maximum, first quartile and third quartile of the concentrations detected in positive samples (in µg/kg); regarding maximum values, the type and origin of the sample and the year of analysis are given.
[1]Aflatoxins: sum of aflatoxin B1, B2, G1 and G2; Fumonisins: sum of fumonisin B1, B2 and B3.

The levels are considered relatively low by the authors, the average values are shown in the Table 1 accompanying Figure 7. Only 17% of the samples did not meet the European legislation for AFB1. It should be noted that levels lower than the permissible values already can cause damage, especially if the contaminated feed is fed to the animals for longer periods. The simultaneous presence of different mycotoxins can have an additive or synergistic effect which can cause significant damage even at low concentrations (Grenier and Oswald, 2011). This was the case in 39% of the samples and in 59% of the finished feed samples.

1.4 Legislation

Mandatory limits for maximum mycotoxin content in feed in Europe are limited to levels for AFB1. There are however also guidelines for DON, ZEN, OTA and fumonisins. For T2 and HT2, action levels are provided. Action levels are concentrations, which, once surpassed, require further investigation. Investigation is required regarding the sources of the samples and source of contamination. Guidelines and recommendations for complete compound feed for pigs and poultry are summarized in Table 2.

Table 2: Maximum levels, guidance values and indicative levels for mycotoxins in complete feed (mg/kg). Values for young animals, if applicable, are presented between brackets.

Mycotoxin	Chicken	Pig	Legislative text
AFB1	0.02 (0.01)	0.02 (0.01)	Directive 2002/32/EC
DON	5	0.9	Recommendation 2006/576/EC
ZEN	2	0.25 (0.1)	Recommendation 2006/576/EC
OTA	0.1	0.05	Recommendation 2006/576/EC
T2+HT2	0.25[1]	0.25[1]	Recommendation 2013/165/EC
FB1+FB2	20	5	Recommendation 2006/576/EC

AFB1: aflatoxin B1; DON: deoxynivalenol; ZEN: zearalenone; OTA: ochratoxin A; T2: T2-toxin; HT2: HT2-toxin; FB1: fumonisin B1; FB2: fumonisin B2; [1]action level: above these concentrations further investigation is needed for the sources of the contamination

In Belgium, the enforcement of these laws is the responsibility of the Federal Agency for the Safety of the Food Chain (FASCF). The controls ought to be compliant with regulation 882/2004/EC (European Commission, 2004). This document states that the authorities, for Belgium represented by the FASFC, are obliged to control the feed and feedstuffs. Control should be done at regular time points, at all stages of the production and distribution, including export and import. The frequency should be appropriate to the risk associated with the type of feed, animal species, production process, past record of the compliance, reliability of the procedures, etc. In addition, *ad hoc* controls should be carried out if any information might indicate non-compliance.

1.5 Risk management

There are several methods for the prevention of mycotoxins in feed which can be applied before and/or after harvest (respectively pre- and/or post-harvest). Although difficult and not always sufficient, the best pre-harvest procedure is to minimize the production of mycotoxins in the field by applying Good Agricultural Practices (GAP) which comprise cultivar/variety choice, crop rotation, crop residue management, fungicide usage, minimizing insect and mechanical damage and optimal irrigation (Kabak et al., 2006; Jouany, 2007). These measures reduce the development of fungi on the crops.
To reduce susceptibility of the crop to storage fungi, the method of harvesting can have an important impact. As an example, damage to the crops may lead to kernels for fungal development.

After harvesting, crops can also become contaminated with mycotoxins. Either through new mycotoxin-producing fungi and/or the fungi already present on crops can continue to produce mycotoxins. Finally, fungi already present on the corps may start producing mycotoxins triggered by a change in conditions. GAP-guidelines for minimizing mycotoxin contamination post harvest include appropriate storage and transport conditions and measures (temperature, humidity and pest control) to prevent damage to crops. Furthermore, there are several chemical and physical methods available to tackle the mycotoxin problem. The chemical methods are based on the principle of transformation of the mycotoxin into less toxic products. The efficacy of various chemicals, including acids, bases, oxidizing and reducing agents has been described (Kabak et al., 2006; Jard et al., 2011). Only a few are indeed effective against mycotoxins, such as ammonia, hydrogen peroxide and hydrochloric acid. However, these methods are often time consuming and expensive, they also have a negative impact on the nutritional value and organoleptic quality (Kabak et al., 2006), and are therefore not allowed in Europe. Examples of physical methods are washing, grinding and heat treatment. These are also time-consuming, expensive and their efficiency depends on the degree and type of contamination (Kolosova and Stroka, 2011).
These pre- and post-harvest strategies can prevent many problems, however, they cannot always deliver the hoped-for outcome, alternatives are being applied. Nowadays, the mixing of mycotoxin detoxifiers in the feed is a frequently used practice (Jard et al., 2011).

2 MYCOTOXIN DETOXIFIERS

2.1 Concept and legislation

Mycotoxin detoxifiers are additives mainly added to compound animal feed. They aim to reduce the effects of mycotoxins on the animal, or to prevent damage by lowering the activity of mycotoxins in the gastro-intestinal tract (GIT) (Jard et al., 2011; Devreese et al., 2013a). The legislation regarding mycotoxin detoxifiers originates from the European Commission, and is the jurisdiction of the Directorate General of Agriculture and Rural Development and the Directorate General of Health and Food Safety. Policies of both Agriculture and Rural Development, and Health and Food Safety, are completely regulated on an European level. Mycotoxin detoxifiers belong to the category of technological feed additives and are defined by Regulation 386/2009/EC as: 'substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action' (European Commission, 2009a). This class of feed additives can be divided into two groups: mycotoxin binders and modifiers. The mycotoxin binders are non-resorbable materials which aim to adsorb mycotoxins in the GIT. Ideally, the non-resorbable complex is then excreted along with the faeces. Mycotoxin binders are usually clays, or derivatives of yeast and are discussed in detail in section 2.3. Mycotoxin modifiers are enzymes or micro-organisms which are able to transform, or to degrade the mycotoxins into less toxic derivatives (Kolosova and Stroka, 2011). To date there are mycotoxin modifiers registered for FB1 in pigs (EFSA FEEDAP Panel, 2014) and for DON in ruminants, poultry and pigs (EFSA FEEDAP Panel, 2013b; European Commission, 2013a).

Yeast derived mycotoxin binders are usually registered as feed material. The only registered mycotoxin binder is bentonite, a clay of the smectite-type for protection against the effects of AFB1 in pigs, poultry and ruminants, this was registered by implementing Regulation 1060/2013 (European Commission, 2013a). Nevertheless there are many more substances on the market that make the claim of mycotoxin binder. They are registered as a different type of technological feed additives, namely as an anti-caking agent, or to improve the hardness of the pellets in pelleted feed. These products include clays such as zeolite, clinoptilolite, kaolin, vermiculite, etc. The feed additives are described in the Regulation 1831/2003/EC (European Commission, 2003). In Annex I of this Regulation, a list can be found on the authorized additive groups for use in animal nutrition (European Commission, 2015). A summary of the legislation is presented below.

Definitions

Feed additives are not feed materials, processing aids or veterinary drugs. Feed materials are defined as products of vegetable or animal origin with the main purpose to meet the animals nutritional requirements and are regulated by EU Regulation 767/2009 (European Commission, 2009b). Processing aids are substances not consumed as a feedstuff by itself but intentionally used in the production or processing of feed materials in order to fulfil a technological purpose. This may result in the unintentional presence of residues of the substance in the final product, but provision is made that these residues do not have an adverse effect on animal health, human health or the environment and do not have any technological effects on the finished feed. An example of a processing aid are extraction solvents.

If single or compounded substances are presented as possessing curative or prophylactic properties with respect to a certain disease or condition, e.g. mycotoxicosis; or the substance which can be administered to animals to restore, improve or alter physiological functions by exerting a pharmacological, immunological or metabolic effect or to make a diagnosis, they are considered as a veterinary drug and are subject to the legislation of veterinary drugs, i.e. Directive 2001/82/EC (European Commission, 2001).

Feed additives are substances which are intentionally added to the feed to:

- improve one or more characteristics of the feed
- improve the animal production, performance or welfare and the characteristics of animal products
- favourably affect the colours of ornamental fish and birds
- satisfy the nutritional requirements of the animals
- favourably affect the environmental impact of animal production
- have a coccidiostatic or histomonostatic action

Classification of feed additives

Feed additives can be classified into five different categories according to their function: technological-, sensory-, nutritional-, zootechnical additives and hygienic condition enhancers.

The category 'technological additives', which contains most mycotoxin binders, comprises the following functional groups. The categories marked with an * are those for which bentonite can be registered.

a) preservatives: substances or micro-organisms which protect feed against deterioration caused by micro-organisms or their metabolites
b) antioxidants: substances prolonging the storage life of feedingstuffs and feed materials by protecting them against deterioration caused by oxidation
c) emulsifiers: substances that make it possible to form or maintain a homogeneous mixture of two or more immiscible phases in feedingstuffs
d) stabilisers: substances which make it possible to maintain the physico-chemical state of feedingstuffs
e) thickeners*: substances which increase the viscosity of feedingstuffs
f) gelling agents: substances which give a feedingstuff texture through the formation of a gel
g) binders*: substances which increase the tendency of particles of feedingstuffs to adhere
h) substances for control of radionucleide contamination*: substances that suppress absorption of radionucleides or promote their excretion
i) anticaking agents*: substances that reduce the tendency of individual particles of a feedingstuff to adhere
j) acidity regulators: substances which adjust the pH of feedingstuffs
k) silage additives: substances, including enzymes or micro-organisms, intended to be incorporated into feed to improve the production of silage
l) denaturants: substances which, when used for the manufacture of processed feedingstuffs, allow the identification of the origin of specific food or feed materials
m) substances to reduce the contamination of feed by mycotoxins*: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins, or modify their mode of action

*application for bentonite

Bentonite is registered as a mycotoxin binder for AFB1 for ruminants, poultry and pigs since 2013 (European Commission, 2013a). However, there are a number of conditions attached to the use of bentonite as a mycotoxin binder. A minimum content of smectites (determined by

X-ray diffraction (XRD)-analysis) of 70% is required. Furthermore, the levels of feldspar and opal should not exceed 10%, and the levels of calcite and quartz cannot exceed 4%. Finally, the candidate mycotoxin binder has to be able to bind AFB1 in a well described *in vitro* setup, discussed in section 2.3. The registration is not brand-specific, any substance which fulfils the above conditions and the general safety rules for feed additives can make the claim of mycotoxin binder for AFB1. Confusion may arise because the same bentonite is also used as a thickener (category e), binding agent (g), for control of radionucleide contamination (h), or anticaking agent (i). The requirements for this application are less strict than those for mycotoxin binders. A content of smectite minerals of ≥50% is required and there are no specifications for other minerals.

The total content of bentonite, regardless of the application for which it is used, can be up to 20 g/kg of complete feed (2%) (European Commission, 2013a). Most manufacturers of mycotoxin binders, however, recommend a lower content, typically between 0.1 and 0.25% (m:m) and in some cases 0.5%. Since these clays can be used for different applications, the final content can be higher than the amount intended as mycotoxin binder. For example, a bentonite can be used in the mixing of different raw materials in order to improve the flowing properties, next a different bentonite can be used as a mycotoxin binder, and finally, a third bentonite can be used as the pelletizing agent to improve the hardness of the pellets. As the various stages can be executed by different parties, the probability that the total content of the bentonite approximates to 2% exists, although should not exceed 2%.

In order to assess the mycotoxin binder producers, an important distinction needs to be made for the mycotoxin detoxifier market according to the type of detoxifier, namely mycotoxin modifiers and -binders. For mycotoxin modifiers, the value chain up to the wholesale level, consists currently of only a few products sold by one company. For the mycotoxin binders, the producers of the raw materials include operators of clay mines, especially bentonite mines, and producers of yeast (by-products). About 15 producers are members of the association of European bentonite producers (Industrial Mineral Association, 2014). On a global level, more than 54 countries produce over 20 million ton of bentonite each year (British Geological Survey, 2013). Through wholesalers or brokers, the raw materials are provided to the manufacturers of feed additives. In Europe, there are about 100 registered members in the European Association of Specialty Feed Ingredients and their Mixtures (FEFANA) (FEFANA, 2015). The finished additives are sold to compound feed producers, premixers or

companies which market the additive as different products, whether or not supplemented with other ingredients.

2.2 Chemical structure of mycotoxin binders

In order to understand the mechanism of action of the mycotoxin binders, a good understanding of the structure of these compounds is necessary. Therefore, an overview will be presented here of the molecular structure of clays and mycotoxin binders derived from yeasts.

Clays

Clays are finely grinded minerals (≤ 2 µm), which consist mainly of silicate minerals, and optionally other materials, so-called associated phases (Guggenheim et al., 2006). The only registered clay for mycotoxin binding so far is bentonite which is defined as predominantly (≥ 70%) composed of montmorillonite (EFSA FEEDAP Panel, 2015). The silicates can be subdivided into tectosilicates and phyllosilicates. Zeolites belong to the tectosilicates family whereas phyllosilicates include, among other clays, sepiolites, smectites and kaolinites. The smectites includes montmorillonite, which can be found in bentonite. Bentonite is defined in the applicable legislation as containing at least 70% montmorillonite. An overview of the minerals used in the additives can be found in Figure 8.

The silicates all have in common that their base unit is a tetrahedron composed of a central silicon atom (Si^{4+}) surrounded by four oxygen atoms, usually in the fully oxydized form O^{2-}. The residual charge of such a tetrahedron is negative and may be neutralized in various ways. In tectosilicates the charge is neutralized by sharing the oxygen atoms of the different tetrahedrons, in this way the ratio of oxygen atoms and silicon atoms is reduced, as well as the residual charge. The sharing of oxygen atoms results in a porous structure of tetrahedrons which are connected to each other at their corner points as shown in Figure 9.

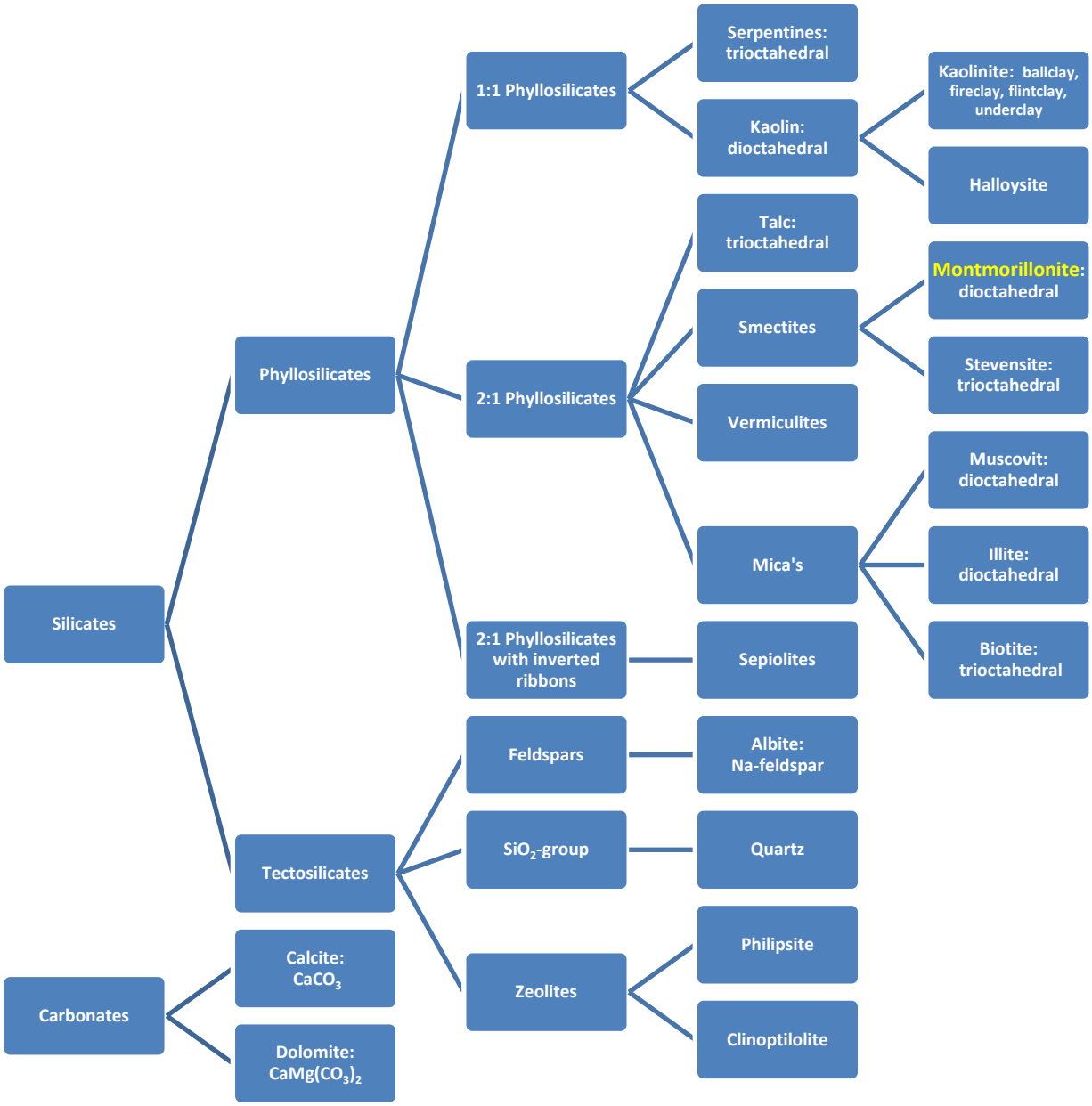

Figure 8: Overview of the nomenclature of clays and other minerals used as a feed additive, adapted from (Bailey, 1980). 1:1 phyllosilicate sheets are composed of one octahedra layer aligned by one tetrahedra layer; 2:1 phyllosilicate sheets are composed of one octahedra layer aligned by a tetrahedra layer on each side.

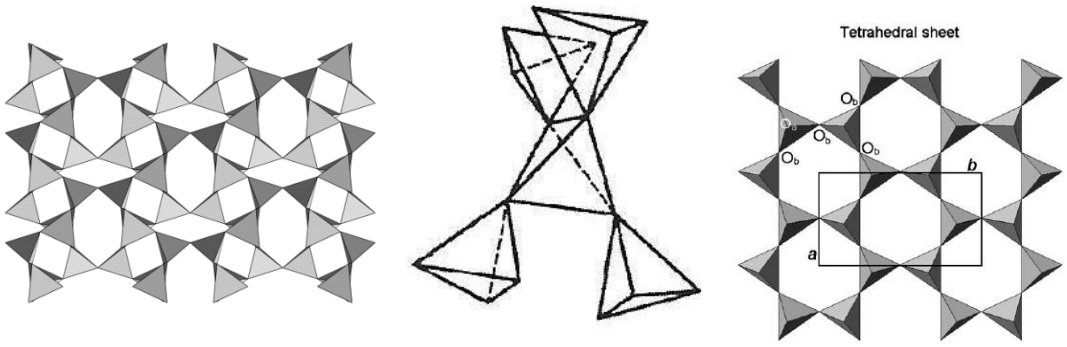

Figure 9: Left and centre: Three-dimensional structure of a tectosilicate (Colville and Ribbe, 1968), (Van Ranst, 2013). The tetrahedra represent Si^{4+}, surrounded by four O^{2-} ions (corners of the tetrahedra). The residual negative charge is reduced by sharing O^{2-} ions and thereby reducing the O^{2-}/Si^{4+} ratio. Right: Sheet configuration of tetrahedra (Brigatti et al., 2006).

The phyllosilicates (Figure 10) also share oxygen atoms in order to neutralize the residual charge, but do so by forming a sheet structure. Typically for the phyllosilicates is the formation of octahedra adjacent to the SiO_4 tetrahedra. The SiO_4 tetrahedra are arranged so that three O^{2-} ions of each tetrahedron are shared with three other tetrahedra. The remaining oxygen atoms at the top of the tetrahedral sheets are linked to the octahedra sheets.

Octahedra occur when six oxygen atoms are arranged around a central atom. In octahedral sheets, the oxygen atom usually appears as the reduced hydroxide OH^-, except where they are shared with apical oxygens of the tetrahedral sheets. Stereochemical, the OH^- form has very little consequences because the hydrogen occupies only a small volume compared to the oxygen atom. The central void is smaller as in the tetrahedra, therefore the ions that may occupy are not the same than in the tetrahedral configuration. In practice, the central void of octahedra is mostly occupied by Mg^{2+} of Al^{3+}, whereas the configuration around the Si^{4+} is always tetrahedral. Substitution of the central octahedral atom might occur, these substitutions contribute to the diversity between similar clays. They also have an important impact on the residual charge on the phyllosilicate layers, which is also an important feature in differentiating different clays.

The octahedra can be arranged in two different manners, namely dioctahedral and trioctahedral, see Figure 10. A trivalent central atom, such as Al^{3+} give rise to a trioctahedral configuration. Each oxygen is shared between three octahedra resulting in a ratio of oxygens and Al^{3+} of 3 to 1. In case the oxygens are in the reduced OH^- form, this configuration as such is stable. A divalent central atom, such as Mg^{2+}, gives rise to a dioctahedral configuration. In

this configuration, each oxygen in the sheet is shared by two octahedra, resulting in a ratio of 2 oxygens for each Mg^{2+} which is electrochemically stable in case all oxygen atoms are in the reduced form.

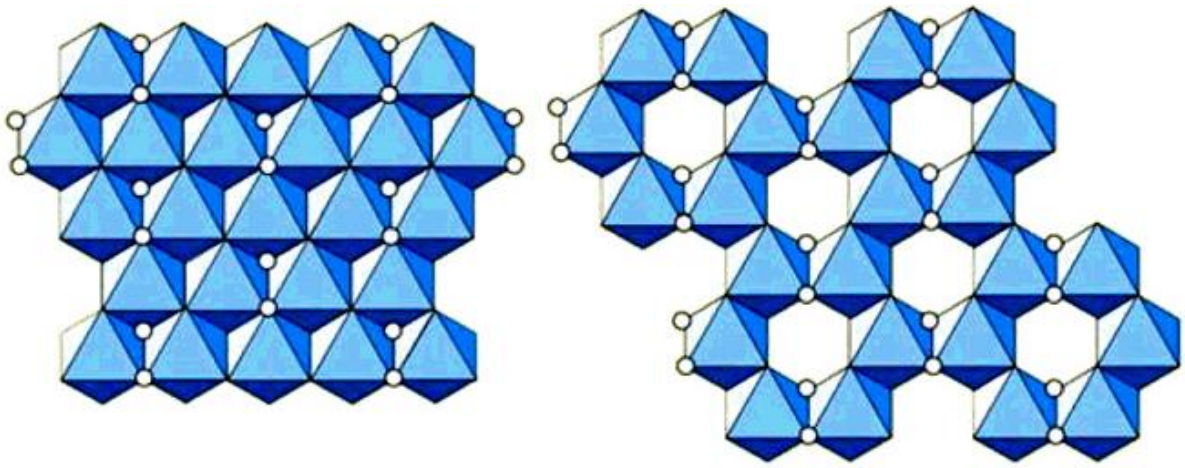

Figure 10: Left: Trioctahedral configuration. Right: Dioctahedral configuration (Wittke and Bunch, 2014).

Depending on whether on one side or on both sides the octahedral sheets are linked with tetrahedra planes, 1:1 and 2:1 phyllosilicates are formed, respectively. The apical oxygens of the tetrahedral sheet are shared with oxygens of the octahedral sheet. The oxygen atoms of the octahedral sheet that are not shared, i.e. fall in the cavities formed by the tetrahedral sheet, remain in the reduced form OH^-. By forming a layered sheet structure, not all of the residual charges are neutralized. The oxygen atoms at the ends of the sheet structure can be neutralized by free hydrogen atoms and thus form pH-sensitive -OH groups. The non-neutralized charges inside the sheet structure are neutralized by the so-called exchangeable cations. These atoms have a positive charge, they are usually Na^+, K^+, Ca^{2+}, Mg^{2+} or NH_4^+ and are located between the layers, but are not included in the crystal structure of the phyllosilicate. In an aqueous environment, these ions attract water because of their high osmotic value, and are responsible for the swelling of the clay. In a hydrated clay these ions can be exchanged. When exchanging these ions, other molecules may be placed between the sheets but only if they have the appropriate stereochemical properties. This has been described for AFB1 (Phillips et al., 2006; Deng et al., 2010), and is shown graphically in Figure 11. Distinction between the clays is based on their molecular and supramolecular structure, dioctahedral or trioctahedral octahedron configuration of the sheet, residual charge between the different layers, specific

substitutions of the silicon, aluminum or magnesium atoms and type of exchangeable cations. Furthermore, different crystal structures may occur within the same crystal, these are called 'mixed-layer' clays.

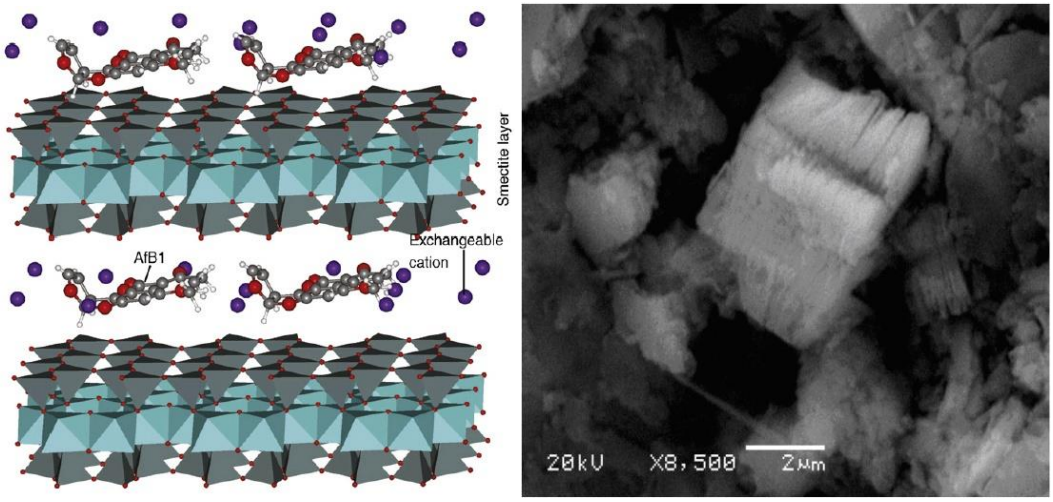

Figure 11: Left: Model of aflatoxin B1 held between smectite layers. The water molecules are not shown in the intermediate layer (Deng et al., 2010). Right: Scanning Electron Microscopic image of a phyllosilicate clay (Al-ani and Sarapaa, 2008).

Yeasts

Dead yeast cells (*Saccharomyces cerevisiae*) are readily available as a by-product of the bakery and brewery industry. The different parts, including specific proteins and certain fractions, up to complete yeast cells are used as a mycotoxin binder. The best known are glucomannans and mannan-oligosaccharides (MOS). The yeast cell wall is shown schematically in Figure 12. These products are usually registered as feed material. Cell walls – or cell wall fractions – offer many different, easily accessible, adsorption sites such as polysaccharides, proteins and lipids. Due to the different properties of these adsorption sites, adsorption may occur through multiple, sometimes simultaneous, mechanisms such as hydrogen bonding, ionic or hydrophobic interactions. These are relatively weak bonds compared to covalent or ionic bonds, however, when large in number, these interactions can play an important role. Hydrophobic interactions become more important with increasing pH (Picollo, 1999; Huwig et al., 2001).

Of the yeast-derived products used as mycotoxin binders, the MOS products have been best studied. These products interact with intestinal flora by binding to the lectin receptor of

Salmonella and *Escherichia* bacteria, a second important effect of MOS is the increase in the Ig's A and B in the intestine (Newman, 1994). Further, an increased villus length was observed in pigs (Goossens et al., 2012). Other effects such as an altered pH of the ceca, altered intestinal microflora (other than *Salmonella* and *Escherichia* spp.) and moisture content or a modified BW sometimes contradict each other.

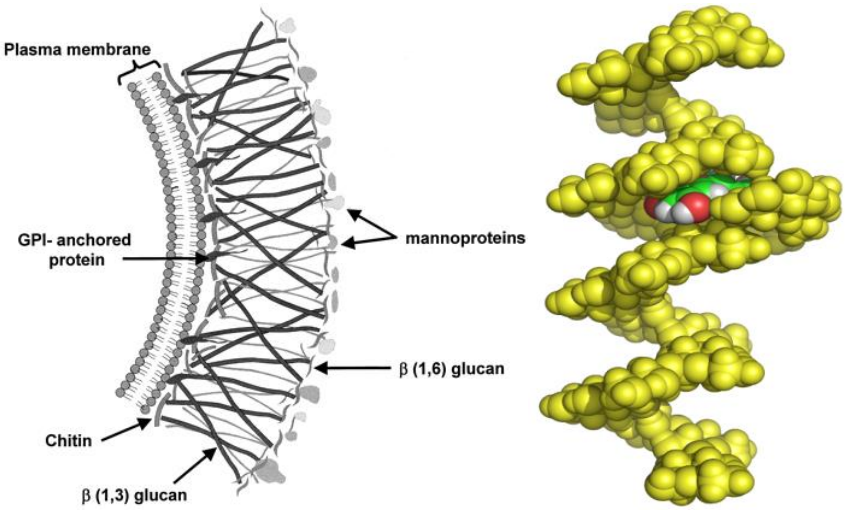

Figure 12: Left: Schematic representation of a yeast cell wall (Selitrennikoff, 2001). Right: *In silico* simulation of the adsorption of zearalenone (green and red structure) by β-D-glucans (yellow structure) of the *Saccharomyces cerevisiae* cell wall (Yiannikouris et al., 2004).

Other mycotoxin binders

In addition to the clay minerals and yeast-derivatives, a number of other substances are also used to adsorb mycotoxins. These mycotoxin binders, with the exception of Lactic Acid Bacteria (LAB), are not used in feed but only for research purposes. Some examples are: LAB (Lahtinen et al., 2004), molecularly imprinted polymers (MIPs) (Lenain et al., 2012), cholestyramine and polyvinylpyrrolidone (Kolosova and Stroka, 2011).

2.3 Adsorption kinetics

Adsorption processes are interactions between the surface of a non-dissolved solid (= adsorbent, e.g. mycotoxin binder), and a solute molecule (= adsorbate, e.g. veterinary drug). The forces that are responsible for the adsorption are non-covalent forces, and can be classified into hydrophobic interactions (e.g. Van der Waals), hydrogen bonds and electrostatic interactions. Multiple bond mechanisms can simultaneously play a role in the

adsorption reaction, the number of interactions and stereochemistry are important for bond strength. To form a bond between adsorbent and adsorbate, a bond has to be broken in between the adsorbent and solvent. Therefore, the matrix in which the adsorption takes place will play an important role. Because of the complexity of binding interactions, they are often pragmatically approached. For most applications, quantitative information is sufficient instead of elucidating the binding mechanism.

Consider the equilibrium reaction between adsorbent [*B*] and adsorbate [*D*]:

$$[B] + [D] \leftrightarrow [BD]$$

Wherein [*D*] is the free drug concentration (mol/L), [*B*] is the concentration of unoccupied binding sites on the binder (mol/L) and [*BD*] is the concentration of occupied binding sites (mol/L). The latter is equal to the concentration of adsorbed drug. Following statements apply to the adsorption reaction:

$$K_{BD} = \frac{[BD]}{[B][D]}$$

$$[D_0] = [D] + [BD]$$

$$[B_0] = [B] + [BD]$$

K_{BD} with the equilibrium constant, B_0 and D_0 are the initial concentration of the drug and binding sites on the adsorbent, respectively. In binding experiments [D_0] is *a priori* known.
In single concentration adsorption experiments, a certain quantity of binder, and a known amount of adsorbate in a known volume of a known matrix are exposed to each other. The results are expressed as % adsorption or as the distribution coefficient K_d. The K_d value is calculated as follows:

$$K_d = \frac{C_{ads}}{C_{aq}} = \frac{1}{BC_{50}}$$

With C_{ads} the adsorbed concentration (amount of drug absorbed per unit weight of *B*), and C_{aq} the free concentration of *D* in the medium. This parameter can be interpreted as the equilibrium constant in case that the ratio between *D* and *B* is very small, in other words, when there is an excess of binding sites available relative to the amount of *D*. If C_{ads} is expressed in mg substance adsorbed per gram of adsorbent, K_d indicates how much drug can be adsorbed by 1 g of binder if in equilibrium with a 1 µg/mL solution. The BC_{50} is, in this

respect, a somewhat more intuitive parameter which indicates the concentration of binder required to bind half of the drug. It can be derived that BC_{50} is equal to the reciprocal of K_d.

When a higher concentration of adsorbate is exposed to the binder, it is possible that the values obtained for K_d are lower than expected. This is because, at first, the most optimal binding sites are occupied followed by the more inferior binding sites. This can be visualized by plotting C_{ads} against C_{aq}, the slope of the adsorption isotherm curve decreases with increasing concentration of the drug (Figure 13). To correct for this deflection, a curve described by the Freundlich equation is fitted (Freundlich, 1906):

$$C_{ads} = K_f * \sqrt[n]{C_{aq}}$$

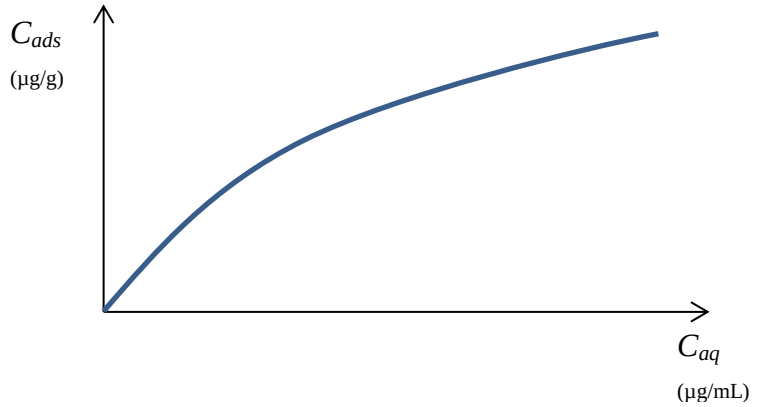

Figure 13: Adsorption isotherm which exhibits non-linear kinetics. In the ordinate, the adsorbed amount of the adsorbate C_{ads} (µg/g adsorbent) is shown, and in the abscissa, the dissolved concentration of the adsorbate C_{aq} (µg/mL).

The Freundlich distribution coefficient K_f has similarities with the distribution coefficient K_d, but the C_{aq} value is adjusted with the power 1/n.

If the adsorbate concentration increases even more, the capacity of the adsorbent will be reached and saturation occurs (Figure 14). The Freundlich equation is no longer suitable for this situation, the Langmuir equation is used instead (Langmuir, 1918) which takes into account the saturation concentration C_{sat}:

$$C_{ads} = K_l * C_{sat} * \frac{C_{aq}}{1 + K_l * C_{aq}}$$

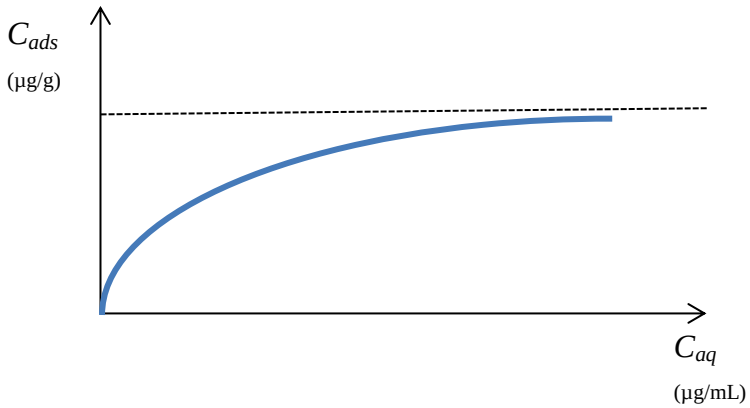

Figure 14: Adsorption isotherm with saturation of the binding sites. In the ordinate, the adsorbed amount of the adsorbate C_{ads} (µg/g adsorbent) is shown, and in the abscissa, the dissolved concentration of the adsorbate C_{aq} (µg/mL).

The distribution coefficient, Freundlich and Langmuir isotherms give an idea about the quantitative aspects of the adsorption process. Variations on these equations are made and may provide a better description of the binding isotherms (Skopp, 2009). It should also be mentioned that the equations described above are purely empirical, they provide only limited information regarding the structure of the adsorbent and/or the mechanism of the adsorption process (Giles et al., 1974). The qualitative part, namely how strong the bond is, can be determined through the Van 't Hoff equation (Van 't Hoff, 1884):

$$\ln K_{d,T} = -\frac{\Delta H^0}{R}\frac{1}{T} + \frac{\Delta S^0}{R}$$

Where R is the ideal gas constant, ΔH^0 the bond-dissociation energy and ΔS^0 the bond-entropy. The latter is under laboratory conditions usually negligible. The bond-dissociation energy is a measure of the strength of the bond. In order to derive the bond-dissociation energy, the distribution coefficient K_d is determined at different temperatures: $K_{d,40}$, $K_{d,30}$, $K_{d,20}$, etc. Next, the logarithm is plotted against the reciprocal of the temperature. The slope of the curve is a measure of the bond-dissociation energy (Figure 15).

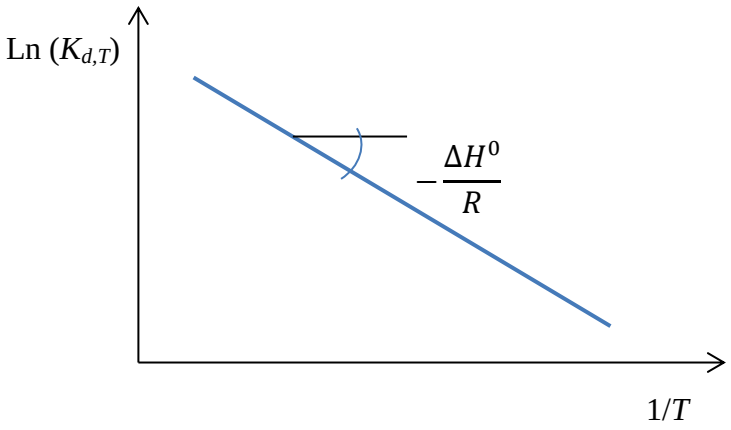

Figure 15: Van 't Hoff isotherm to calculate the bond-dissociation energy. In the ordinate, $K_{d,T}$: the distribution coefficient at a given temperature, shown on a logarithmic scale. In the abscissa, the reciprocal of the temperature T. ΔH^0: bond-dissociation energy; R: ideal gas constant.

2.4 Efficacy testing

In vitro

In vitro models for assessing adsorption properties of mycotoxins in the context of efficacy studies of mycotoxin binders include static and dynamic models. Dynamic models such as the TNO-gastro-Intestinal Model (TIM), developed by the Dutch organisation for applied scientific research (Toegepast Natuurwetenschappelijk Onderzoek or TNO) (Minekus et al., 1995; Avantaggiato et al., 2003; Blanquet et al., 2004) or Simulator of the Human Intestinal Microbial Ecosystem (SHIME) (Molly et al., 1993; Molly et al., 1994), could be representative for the *in vivo* situation. However, they are time consuming and require many resources, hence, they are not best suited for high throughput screening experiments. Static models are often cheap and quick and thereby suitable for rapid screening experiments, however, they are less representative for the GIT and the results should be interpreted carefully. Static models include adsorption-isotherm studies (Ramos and Hernandez, 1996b) or single concentration studies (Sabater-Vilar et al., 2007; Vekiru et al., 2007; Devreese et al., 2013b). For the authorisation of a bentonite for AFB1 binding, efficacy testing needs to be carried out using a static adsorption test in a buffered matrix of pH 5.0 with a concentration of 4 mg AFB1/L buffer and 200 mg feed additive/L buffer (European Commission, 2013a).

In vivo

For the assessment of the *in vivo* efficacy of mycotoxin binders, several approaches are being used. The criteria animal performance and incidence of mycotoxin related pathologies, or the prevalence of mycotoxins in products intended for human consumption (e.g. AFB1 in milk), are used as clinical endpoint. However, the performance and incidence of pathologies related to mycotoxins as criteria are non-specific, even controlled clinical studies which assess these endpoints can be subject to many confounding factors such as influence on GI microbiota, GI morphology, nutritional value of the mycotoxin binder (e.g. containing micro-nutrients), etc. Study of the toxicokinetics and residues in (edible) tissues can avoid some of the problems described above. This is sometimes difficult to accomplish because the concentrations in which the mycotoxins appear in edible tissues can be very low; the analytical performance is key for the success of the experiment. In addition, the relation between concentrations in the biological matrices and the endpoint should be well understood to allow interpretation of the results and the establishment of maximum levels of the mycotoxins in the specified matrix.
In some cases, surrogate endpoints such as the use of biomarkers, or examination of target organs can circumvent some of the problems described with assessing the toxicokinetics or residues of mycotoxins. These surrogate endpoints are suitable provided that their relation to the clinical endpoints is completely elucidated. Table 3 presents the most relevant endpoints for exposure to selected mycotoxins.

Table 3: Most relevant *in vivo* endpoints for evaluating the efficacy of mycotoxin detoxifiers for selected mycotoxins (EFSA FEEDAP Panel, 2010).

Mycotoxin	Endpoint
Aflatoxin B1	Aflatoxin M1 in milk/egg yolk
Deoxynivalenol	Deoxynivalenol or its metabolites in blood serum
Zearalenone	Zearalenone + α- and β-zearalenol in plasma Excretion of zearalenone/metabolites
Ochratoxin A	Ochratoxin A in kidney or blood serum
Fumonisins B1 + B2	Sphinganine/Sphingosine ratio in blood, plasma or tissues

In 2001, Huwig et al. summarized the results of the research carried out so far (Huwig et al., 2001), while in 2011 Kolosova and Stroka presented a more recent review (Kolosova and Stroka, 2011). The most extensively studied is the binding of aflatoxins by Hydrated Sodium Calcium Aluminosilicate (HSCAS), which is marketed as Novasil® (Phillips et al., 1988; Kubena et al., 1991; Edrington et al., 1996; Bailey et al., 1998; Ledoux et al., 1999). HSCAS is a broad term for aluminium silicate minerals, containing sodium and calcium, hence, almost the entire family of silicates can be labelled as HSCAS. After the introduction of Novasil®, other products entered the market labelled containing HSCAS as active ingredient, for example Milbond-TX®, Ethecal®, etc. The inclusion rate used in the studies mentioned is 0.5 g/kg feed or 1.0 g/kg feed. The species in which most *in vivo* tests are executed are rats, (broiler) chickens, turkeys, lambs, pigs, mink, trout and cows. A reduced growth rate due to the administration of aflatoxins in the feed was the most obvious symptom in the test groups, in most cases this could be prevented, totally or partially, by the addition of HSCAS. The concentration of the biomarker AFM1 in milk was, when included in the study, also significantly lower. Similar results were obtained with the use of various types of bentonite (Pappas et al., 2014).

For other mycotoxins, the evidence is limited. If positive effects are observed, they are usually non-specific such as effects on inflammatory biomarkers or BW loss due to mycotoxin administration (Kolosova and Stroka, 2011). It can be concluded that the efficacy for the binding of AFB1 is proven for a number of montmorillonite clays, for other toxins no effective mycotoxin binders have been identified yet in scientific literature.

2.5 Risks of mycotoxin binders

Binding to mycotoxin binders is deemed to be non-specific. These non-specific interactions with other feed ingredients or additives are not explicitly mentioned in the legislation of mycotoxin binders, although concerns are communicated regarding this subject (EFSA FEEDAP Panel, 2010). The non-specific interactions of interest are interactions with nutritional compounds in the feed and interactions with veterinary medicinal products which are mixed into the feed or drinking water.

Interactions with nutritional compounds

Reports on the interaction with nutritional compounds are limited to a few studies in which the uptake of vitamins was investigated in animals or humans which received a clay-based additive (Papaioannou et al., 2002; Pimpukdee et al., 2004; Afriyie-Gyawu et al., 2008). In

general, the risk for binding vitamins, or other micronutrients, when administering clay-based mycotoxin binders is considered limited (EFSA, 2009).

Interactions with orally administered veterinary medicinal products

Previous studies reported possible interactions of mycotoxin binders and veterinary medicinal products which are also mixed in the feed or drinking water. The first report of the potential interactions dates from 1992 when the Canadian Bureau of Veterinary Drugs reported a case in which lack of efficacy of tylosin (TYL) was seen in cattle fed a bentonite supplemented feed (Canadian Bureau of Veterinary Drugs, 1992). In 1994, Shryock et al. investigated the efficacy of tilmicosin (TIL) for preventing airsacculitis caused by *Mycoplasma gallisepticum* in broiler chickens. A decrease in the effectiveness of TIL was seen as from an inclusion rate of bentonite of 2% in the feed (Shryock et al., 1994). In 1998, the effect of sodium bentonite on the performance of chickens whose feed was supplemented with the coccidiostats monensin (MON) or salinomycin (SAL) was studied. A reduced growth-promoting effect could be seen in the group which also received bentonite together with the coccidiostats, but only when the inclusion rate of the coccidiostats was below the recommended dose (Gray et al., 1998). In a trial in which chickens were challenged with an *Eimeria* infection, more severe clinical symptoms were seen in the groups which received clinoptilolite – a tectosilicate – together with SAL compared to the groups only receiving SAL (Nesic et al., 2003). An interaction between lincomycin and a non-specified anti-mycotoxin agent was also observed in broilers (Amer, 2005).

In 2010, the European Food Safety Authority (EFSA) stated that the safety of mycotoxin binders with respect to non-specific binding of drugs for veterinary use must be examined (EFSA FEEDAP Panel, 2010). Devreese et al. studied the pharmacokinetic (PK) properties of TYL in fasted broiler chickens, using an oral bolus model in which TYL or TYL + bentonite was administered. The daily dose of bentonite corresponding to an inclusion rate of 1 g/kg feed, was administered to fasted broilers before administration of a dose of TYL, which also corresponded to the recommended daily dose. The oral bioavailability of TYL was significantly reduced in the test group receiving TYL + bentonite, as demonstrated by the plasma concentration-time curves in Figure 16 (Devreese et al., 2012).

To date, the concomitant use of bentonite and macrolide antibiotics is prohibited for all species (European Commission, 2013a). For poultry, the use of bentonite with the coccidiostat

robenidine should be avoided, for other coccidiostats, the maximum inclusion rate for poultry feed is set at 5 g/kg (European Commission, 2013a).

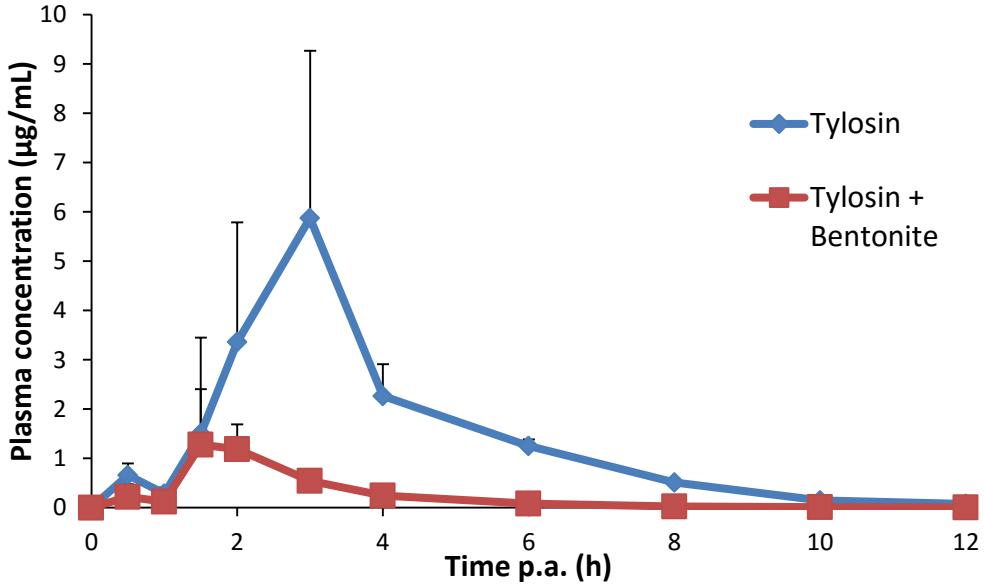

Figure 16: Plasma concentration–time profile of tylosin after a single oral bolus administration of tylosin (24 mg/kg) with or without bentonite (1 mg/kg) to broiler chickens. Values are presented as means + standard deviation (SD, n = 8) (Devreese et al., 2012).

Veterinary medicinal products and pharmacokinetics

Over 200 veterinary drug formulations are available on the Belgian market to apply orally to pigs and chickens by mixing in the feed or drinking water. Oral administration is applied in 91.5% of the treatments with antimicrobials in food producing animals in Europe (% of sales, expressed as mg/produced kg meat). For poultry and pig rearing, usually multiple animals are treated together by medicating the drinking water or feed, mainly because of practical reasons. The categories of antimicrobials that are mostly used for food producing animals are tetracyclines (37%), β-lactams (22%), sulfonamides (10%) and macrolides (8%). The antimicrobials in these categories are mostly (> 90%) applied orally (European Medicines Agency, 2013). An overview ot the registered products is presented in table 4.

Table 4: Overview of the chemical entities registered as antimicrobial drugs or coccidiostats for pigs and poultry. For the antimicrobial drugs, only following categories are included: penicillins, tetracyclines, sulfonamides+trimethoprim and macrolides (Belgisch Centrum voor Farmacotherapeutische Informatie; http://www.bcfi-vet.be, 2016)

	Antimicrobials of the categories penicillins, tetracyclines, potentiated sulfonamides and macrolides registered for use in pigs or poultry	**Registered coccidiostats (feed additive) for use in pigs and poultry**
Pigs	benzylpenicillin, ampicillin, amoxycillin, doxycycline, oxytetracycline, sulfonamides (sulfadiazine/ sulfamethoxazole/ sulfadoxine/ sulfachlorpyridazine) + trimethoprim, gamithromycin, tilmicosin, tildipirosin, tulathromycin, tylosin, tylvalosin	
Poultry	fenoxymethylpenicillin, amoxycillin, doxycycline, sulfachlorpyridazine/sulfadiazine + trimethoprim, tilmycosin, tylosin, tylvalosin	monensin, decoquinate, robenidine, lasalocid, halofuginone, narasin, salinomycin, maduramicin, diclazuril, semduramicin, nicarbazin

The antimicrobials are absorbed by the animal in the GIT and distributed to the site of the infection, where they hopefully reach concentrations that are sufficiently high, to kill or inhibit growth of the target pathogen. This concentration is defined as the Minimal Inhibitory Concentration (MIC) of a specific pathogen (Andrews, 2001). The different classes of antimicrobials differ with regard to the time and degree their concentration should surpass the MIC in order to be effective. This is defined as the pharmacokinetic/pharmacodynamic (PK/PD)-indices for antimicrobials, and different classes can be considered: 1) time-dependent antimicrobials: the period their concentration surpasses the MIC is decisive for their efficacy (time/MIC or T/MIC), examples are macrolides and β-lactams; 2) concentration-dependent antimicrobials: the efficacy depends upon the extent the maximal plasma concentration surpasses the MIC (C_{max}/MIC), examples are fluoroquinolones and aminoglycosides; and 3) co-dependent antimicrobials: their efficacy is related to both duration of exposure and maintained concentration: the PK/PD index is area under the plasma concentration-time curve (AUC)/MIC, examples are tetracyclines and azalides (Mouton et al., 2012).

In most cases the drug is indeed intended to exhibit a systemic effect, and the availability at the site of action is in most cases related to the blood (plasma) concentration. Therefore, the bioavailability of the drug is defined as the rate and extent to which the parent drug substance becomes available in the systemic circulation (Rang et al., 2007). Consequently, the bioavailability is based on the AUC after extravascular administration divided by the AUC after IV administration, and correcting for the dose. The AUC can be measured until the last blood sampling time point (AUC_{last}) or extrapolated until infinity (AUC_{inf}). AUC_{inf} is preferred to calculate the (oral) bioavailability. Oral bioavailability is applicable when the drug is administered orally, and it is the result of surpassing biological membranes and possible first-pass degradation and/or biotransformation processes. The extent to which orally administered drugs express a high bioavailability depends on a number of physicochemical and biological factors. Most important physicochemical factors include pKa and lipophilicity of the drug, GIT stability, solubility, dissolution rate of the active ingredient from its pharmaceutical formulation and composition/pH of GIT juices. Important biological factors are the physiology of the GIT and the presystemic biotransformation of gut and liver (Hu and Li, 2011).

For oral medicinal products with a systemic mode of action, a lower oral bioavailability may lead to inefficient therapy, meaning that the peak concentration is not high enough or drug concentration cannot remain above the MIC long enough to be efficient. An increase in oral bioavailability on the other hand, may lead to residues above Maximum Residue Limit (MRL) in animal products intended for human consumption. An increase of oxytetracycline plasma concentrations was seen in broilers after three weeks exposure to a clay-based mycotoxin binder (Osselaere et al., 2012). In pigs, the plasma concentrations of doxycycline and paromomycin were higher after exposure of seven days to a yeast derived mycotoxin binder in combination with T-2 or DON (Goossens et al., 2012).

Coccidiostats are feed additives which inhibit the development of coccidia (e.g. *Eimeria spp.*) and histomona (e.g. *Histomonas meleagridis*) in the GIT. Although they are registered as feed additives, they have many properties in common with medicinal products. They claim prevention of coccidiosis, have a well described pharmacological mode of action and their registration procedure is quite similar to those of medicinal products. These are described in Directive 2001/82 and Regulation 429/2008 (European Commission, 2001, 2008).

Many mycotoxin binders are available on the European market and many new products are entering the market (EFSA, 2009). Given the multitude of veterinary drugs that can be mixed in the feed and drinking water, and coccidiostats that are readily mixed in the feed, the number of possible combinations with mycotoxin binder is very high and still increasing. Current studies on the possible interactions between mycotoxin binders and medicinal products cover only a small fraction of the possible combinations, moreover, these studies are limited to date and predominantly apply models that are not specific and sensitive for detecting interactions.

2.6 Safety testing

Safety of mycotoxin binders comprises testing of the direct and indirect risks. Tests and regulation regarding the direct risks are provided in the European Directive 429/2008 for registration of (technological) feed additives (European Commission, 2008). For indirect risks, such as the adsorption of beneficial chemical entities in the feed, e.g. veterinary medicinal products, no specific tests are provided.

The static, single concentration *in vitro* models required for the registration of a bentonite are designed to assess the efficacy, as described above. When similar models are applied for safety testing, they should be adapted to maximize sensitivity. This can be done by reducing the total volume or using concentrations of mycotoxin binder and adsorbate that maximise the probability of interactions, i.e. mimicking worst case scenarios. These adaptations usually mean a decrease in specificity, which could lead to prohibition of suitable therapies. Hence, *in vitro* interactions should be further evaluated *in vivo*.

To date, no *in vitro* models are available that are designed for screening the safety (interactions with veterinary medicinal products) rather than efficacy. Sensitive and specific *in vitro* models which allow a quick screening for interactions are urgently needed (EFSA, 2009).

For *in vivo* safety studies which investigate the interactions of mycotoxin binders with oral veterinary medicinal products, the endpoints are the clinical outcome of the pharmacological treatment and whether or not the beneficial effects of the medicinal product are diminished by the concomitant use of mycotoxin binders. Studies which assess the endpoints face the same problems as for the efficacy studies. A kinetic approach, i.e. effects of the mycotoxin binders on the PK of the veterinary medicinal products, is an approach of interest because it avoids confounding factors. Moreover, for most medicinal products, plasma (or tissue)

concentrations after administration of the recommended dose are high enough to allow accurate quantification, and the relation of plasma or tissue concentrations with the clinical outcome is usually well known. Therefore more studies should be executed using the PK approach, this was also recommended by the European authorities (EFSA, 2011).

SCIENTIFIC AIMS

Mycotoxin binders are mixed in the feed to counter the deleterious effects of mycotoxins. Veterinary medicinal products, such as antimicrobials and coccidiostats, are usually administered in the feed of poultry and pigs. Antimicrobials are also administered by mixing in the drinking water, hence these veterinary medicinal products can come into contact with mycotoxin binders in the gastrointestinal tract. This may compromise their pharmacological effect and/or oral bioavailability. There is limited information on the possible interactions between mycotoxin binders and oral veterinary medicinal products. The European Food Safety Authority (EFSA) stated that the non-specific binding needs to be investigated, using unambiguous methods such as pharmacokinetic studies. The **general aim** of this thesis is to investigate the safety of mycotoxin binders regarding the possibility for non-specific interactions with oral veterinary medicinal products. Knowledge on the effects of mycotoxin binders on the pharmacokinetic properties of these products is needed to safeguard their optimal pharmacotherapy. Therefore, the results described in this thesis can support competent authorities in the safety assessment of these feed additives.

The goal of the **first chapter** of this doctoral thesis is to get a better understanding of the composition of mycotoxin binders available on the European market. Characterization experiments in chapter 1 aim to map the physicochemical properties of the mycotoxin binders and to assess a link with the binding potential. The binding of zearalenone, a *Fusarium* mycotoxin, is chosen as model. These characteristics are also used to select a number of binders which are subject to further *in vitro* and *in vivo* experiments.

In the **second chapter** of this thesis, the binding potential of a number of representative mycotoxin binders is studied for the antimicrobial doxycycline using an *in vitro* model. The techniques applied are set up as a screening test using single concentration of drug and binder. The *in vitro* results are compared with the results of an *in vivo* pharmacokinetic experiment, which resembles a worst-case scenario in broiler chickens.

The **third** and **fourth chapter** of this thesis focuses on interactions between selected mycotoxin binders and veterinary drugs from a pharmacokinetic perspective. These experiments apply models which are closer related to the field situation than the worst-case setup used in chapter 2. Several *in vivo* studies are executed in broiler chickens (chapter 3) and pigs (chapter 4). The veterinary medicinal products that are studied are selected antimicrobials and coccidiostats.

In the **fifth chapter**, a sensitive and specific *in vitro* model is presented for screening for interactions. Feed is incorporated in the design and this approaches the *in vivo* situation better than the model presented in chapter 1. Furthermore the influence of different inclusion rates are tested. Finally, the results are compared with the results of *in vivo* experiments, presented in this thesis and published elsewhere.

EXPERIMENTAL STUDIES

Chapter 1: Characterization of 27 mycotoxin binders and the relation with *in vitro* zearalenone adsorption at a single concentration

Adapted from:

Thomas De Mil, Mathias Devreese, Siegrid De Baere, Eric Van Ranst, Mia Eeckhout, Patrick De Backer and Siska Croubels (2015b). Characterization of 27 Mycotoxin Binders and the Relation with *in vitro* Zearalenone Adsorption at a Single Concentration. Toxins, 7, 21-33; doi:10.3390/toxins7010021

Abstract

The aim of this study was to characterize 27 feed additives marketed as mycotoxin binders and to screen them for their *in vitro* zearalenone (ZEN) adsorption. Firstly, 27 mycotoxin binders, commercially available in Belgium and The Netherlands, were selected and characterized. Characterization was comprised of X-ray diffraction (XRD) profiling of the mineral content and *d*-spacing, determination of the cation exchange capacity (CEC) and the exchangeable base cations, acidity, mineral fraction, relative humidity (RH) and swelling volume. Secondly, an *in vitro* screening experiment was performed to evaluate the adsorption of a single concentration of ZEN in a ZEN:binder ratio of 1:20,000. The free concentration of ZEN was measured after 4 h of incubation with each of the 27 mycotoxin binders at a pH of 2.5, 6.5 and 8.0. A significant correlation between the free concentration of ZEN and both the *d*-spacing and mineral fraction of the mycotoxin binders was seen at the three pH levels. A low free concentration of ZEN was demonstrated using binders containing mixed-layered smectites and binders containing humic acids.

Keywords: mycotoxin; binders; characterization; zearalenone; adsorption screening

1 Introduction

The contamination of feed with mycotoxins is a continuing feed safety issue, leading to economic losses in animal production (Wu, 2007). Consequently, a variety of methods for the decontamination of feed has been developed, but the addition of mycotoxin detoxifiers to the feed is the most commonly-used method (Jard et al., 2011; Kolosova and Stroka, 2011). The additives used for this purpose can be divided into two groups: binders and modifiers. Mycotoxin binders aim to prevent the absorption of the mycotoxins from the intestinal tract of the animal by adsorbing the toxins to their surface. Mycotoxin binders are generally clay- (inorganic) or yeast-derived (organic) products (Kolosova and Stroka, 2011). Mycotoxin modifiers, on the other hand, aim to alter the chemical structure of the mycotoxins and, consequently, reduce their toxicity. Mycotoxin modifiers are usually of microbiological origin comprised of whole cultures of bacteria or yeasts, as well as specifically extracted components, such as enzymes (Kabak and Dobson, 2009).

The extensive use of specialized additives to diminish the effects of mycotoxins has led to the establishment of a new group of feed additives in Regulation 386/2009: "substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action" (European Commission, 2009a). However, most of the mycotoxin detoxifiers are registered as technical additives, feedstuff or digestibility enhancers, as those are more easily being registered in comparison to the claim of a mycotoxin detoxifier. At the moment, only two products are registered in annex I of Regulation 1831/2003 as being a mycotoxin detoxifier (European Commission, 2015), whereas a wide variety of products indirectly claiming mycotoxin binding or modifying abilities is available. In addition, European legislation does not require full transparency with regard to the content of these technical additives.

Although many different types of ingredients are known to be used in additives marketed as mycotoxin binders (in brief, binders), no studies are available that provide a comprehensive overview of their exact composition. In most reports, the description of the products is limited to the product name and an entry of a generic name, such as hydrated sodium calcium aluminosilicate (HSCAS) or bentonite (Kolosova and Stroka, 2011). Despite this generic nomenclature of commercially-available binders, several physicochemical properties have been identified as having a possible association with adsorption of mycotoxins and might therefore be used to categorize the different available products. These characteristics originate from soil science and comprise cation exchange capacity (CEC), exchangeable K^+, Na^+, Mg^{++} and Ca^{++}, acidity, linear swelling, mineral fraction and relative humidity (Burt, 2011).

Exchangeable cations neutralize the interlayer charges in phyllosilicates and are involved in the binding mechanism of aflatoxin B1 (Phillips et al., 2008; Deng et al., 2010). The CEC is a measure of the amount of exchangeable cations, whereas the different types of exchangeable base cations (K^+, Na^+, Mg^{++} and Ca^{++}) have different properties in terms of their affinity for the clay and osmolarity (Fletcher and Sposito, 1989). Although a correlation between the binding properties of mycotoxins and CEC values is not documented in the literature, this parameter is cited by manufacturers when discussing the binding properties of inorganic mycotoxin binders.

The pH of the binder can provide insight into the saturation of a clay with exchangeable base cations, which results in a pH of seven or higher. An increase in pH can be due to the solvation of the exchangeable base cations or the presence of carbonates. A low pH is indicative for exchangeable Al^{3+} or the presence of acidic functional groups, e.g., humic acids.

Adsorption to clays is not limited to the surface of the clay particles, but extends also to the interlayer space of the clay. This interlayer space, characterized by the *d*-spacing, can be determined with X-ray diffraction (XRD) and is restrictive for the formation of one or more adsorbent layers. This space can increase if the clay swells, thereby increasing the number of binding sites (Chang et al., 2009). Hydration of the minerals plays an important role in this process, as well, since it is related to the osmotic power of the mineral (Vidal and Dubacq, 2009; Morrow et al., 2013) and, hence, the ability to hydrate the interlayer space.

Non-enzymatic organic compounds used as additive in feeds are mostly products derived from yeast cell walls or organic mineraloids, such as leonardite and lignite, which are a rich source of humic and fulvic acids. Adsorption to these compounds can occur through hydrophobic interactions (Picollo, 1999). Such interactions were proposed for the binding of the antibiotic, oxytetracycline, to montmorillonites in the presence of dissolved organic matter (Kulshrestha et al., 2004). To determine the mineral fraction of a sample, the organic compounds are discarded by dry combustion.

With regard to the adsorption of mycotoxins, zearalenone (ZEN) is a secondary metabolite produced by several fungi of the *Fusarium* genus. It has lipophilic properties and exerts its effects on the reproductive system of animals (Kanora and Maes, 2009; Cozzini and Dellafiora, 2012). Sabater-Vilar *et al.* described the ZEN-adsorption of three smectite-based minerals, six humic substances, four yeast-derived detoxifiers and six commercial products, which include, according to the commercial brochures, two yeast products, three mineral binders and a mixture of clay and yeast products. A large variation in the adsorption of ZEN is seen in all of the types of binders (Sabater-Vilar et al., 2007). Yiannikouris *et al.* compared the ZEN binding properties of a yeast cell extract and a mineral binder and concluded that the yeast-based product had better adsorption properties than the mineral in the higher concentration range (Yiannikouris et al., 2013). Avantaggiato *et al.* studied 19 binders and also

found a large variation in ZEN adsorption (Avantaggiato et al., 2005). These results indicate that ZEN can be adsorbed, but only by a limited number of binders, and there is a large variation in binding percentage. Therefore, ZEN binding can be used as model to evaluate which physicochemical properties are related to the binding of rather lipophilic mycotoxins. All of the studies cited above used activated carbon or charcoal as the positive control and found binding percentages of over 90%.

The first aim of this study was to identify the qualitative composition of 27 commercially-available feed additives marketed as mycotoxin binders by XRD analysis and to determine the following physicochemical properties: CEC, exchangeable K^{+}, Na^{+}, Mg^{++} and Ca^{++}, acidity, swelling, mineral fraction, presence of carbonates (HCl effervescence test) and relative humidity.

The second aim was to discuss the relation between the observed free concentration of ZEN after incubation with the mycotoxin binders and the physicochemical properties of these binders.

2 Experimental Section

2.1 Mycotoxin binders, chemical products and reagents

Feed additives marketed as mycotoxin binders (n = 27) were collected after a market study to identify the most relevant products. The suppliers include the international companies, Poortershaven, Sanluc, Kemin, Biomin, Alltech, Agrimex, Cenzone tech, Tesgo international, Selko, Clariant, Tolsa, BASF, Miavit, Special Nutrients and American Colloid. Acid-washed sea sand, HCl, $CaCl_2 \cdot 2H_2O$, NaCl and MgO were supplied by VWR (Leuven, Belgium). Technical ethanol was provided by Fiers (Kuurne, Belgium). The ammonium acetate, boric acid, H_3PO_4, Na_2HPO_4 and Neβler reagent were provided by Merck (Darmstadt, Germany). Sigma Aldrich (Bornem, Belgium) supplied KCl, $MgCl_2 \cdot 6H_2O$ and glycol. Acros Organics (Geel, Belgium) supplied methyl red, bromocresol green and tert-butyl methyl ether (tBME). Water and acetonitrile (ACN) used for the HPLC analysis were of MS-grade and provided by Fisher Scientific (Wijnegem, Belgium). ZEN and $^{13}C_{18}$-ZEN were purchased from Fermentek (Jerusalem, Israel) and Romerlabs (Tulln, Austria), respectively.

2.2 CEC and exchangeable base cations

A glass burette with a porous bottom was filled with, respectively, 10 g of acid-washed sand, 25 g of acid-washed sand that was thoroughly shaken on a horizontal shaker for 30 min with 0.5 g of binder and 5 g of acid-washed sand to avoid splattering. After 20 min of equilibration, 150 mL of technical ethanol was percolated over the burette for two hours. Next, 150 mL of a 1 mol/L aqueous ammonium acetate solution was percolated in the same manner for a total time of 4 h. The ammonium acetate percolate was analyzed with inductive coupled plasma-atomic emission spectrometry (ICP-AES), as described by Burt *et al.* (Burt, 2011). The device used was an IRIS Interpid II XSP (Thermofisher, Waltham, UK). The characteristic wavelengths used were 317.9 nm for Ca^{2+}, 766.4 nm for K^+, 285.2 nm for Mg^{2+} and 589.5 nm for Na^+.

After the ammonium acetate percolation, the column was rinsed with 150 mL of technical ethanol to remove ammonium that was not adsorbed by the sample. This washing step was performed over a period of 2 h, respecting 20 min of equilibration. The percolate was tested for the presence of ammonia with the Neβler reagent. In case the test was positive, an extra 100 mL of ethanol was used to remove all ammonia. Next, 500 mL of KCl 1 mol/L were percolated over 4 h, again respecting 20 min of equilibration. Fifty milliliters of the KCl percolate were transferred to a Buchi-tube (Buchi labortechnik AG, Flawill, Switzerland), together with about 5 g of MgO. Ammonia was captured in a boric acid-containing solution (20 mL, 0.3 M). The boric acid solution was supplemented with indicators methyl red and bromocresol green. The formed tetrahydroxyborate was titrated back to boric

acid with 0.01 mol/L of HCl, and the titration was considered complete when the red color reappeared. The reactions involved and formulas to calculate the CEC value are presented below:

$$NH_4^+ \xrightarrow{T\uparrow;MgO} NH_3(\uparrow) \quad (1)$$

$$NH_3 + B(OH)_3 + H_2O \rightarrow NH_4^+ + B(OH)_4^- \quad (2)$$

$$B(OH)_4^- + H_3O^+ \leftrightharpoons B(OH)_3 + 2H_2O \quad (3)$$

$$CEC_{pH7} = \frac{(V_2 - V_0) \times T \times V \times 100}{V_1 \times G} \quad (4)$$

with (V_2−V_0) representing the volume of HCl used, T the titer of HCl (= 0.01 mol/L), V the volume of KCl percolate (= 500 mL), V_1 the volume of KCl percolate sample (= 50 mL) and G the mass of the binder (= 0.5 g). A KCl solution was used as a blank sample for the titration; a pure sand sample was included for the percolation.

2.3 Other characterization tests

To measure the acidity of the samples, a 1:10 binder:water suspension was shaken for 2 h and was left to sediment for another 2 h under closed lid. The pH of the supernatant was measured using a glass-calomel electrode (Inolab WTW, Weilheim, Germany).

The presence of carbonates in the samples was tested with a HCl effervescence test: a small amount of binder was mixed with a few droplets of concentrated HCl on a glass dish. The reaction in the first 10 seconds was monitored and scored as follows: −, no reaction; +, moderate reaction; ++, strong reaction.

To determine the relative humidity and the mineral fraction, 10 g of binder were dried in an oven (Memmert, Swabach, Germany) at 110 °C overnight. The sample was weighed before and after drying, and the moisture content was calculated based on the weight reduction. The mineral fraction was assessed by the dry combustion method by heating in a Muffle® furnace (Nabertherm, Lilienthal, Germany) to 400 °C for 16 h and then cooled in a desiccator (Burt, 2011).

The swelling volume was assessed by using an adaptation of the coefficient of linear extensibility (COLE) (Marroquin-Cardona et al., 2009; Burt, 2011). An aliquot of the binder (2.5 mL tapped bulk volume) was mixed with 15 or 50 mL of water, depending on the extent of swelling. The mixture was thoroughly vortexed (15 s) in the volumetric tube, incubated (4 h) and centrifuged (1070× *g*, 10 min, 4 °C) before measuring the volume of the sediment.

XRD patterns, including *d*-spacing, were obtained with a Philips X'PERT SYSTEM (Phillips, Eindhoven, The Netherlands), the diffractometer (type: PW 3710) was equipped with a copper tube

anode, a secondary graphite beam monochromator, a proportional xenon filled detector and a 35-position multiple sample changer. The incident beam was automatically aligned, and the irradiated wavelength was 12 mm. The secondary beam side surpassed a 0.1-mm receiving slit, a Soller slit and a 1° anti-scanner slit. The tube was operated at 40 kV and 30 mA. XRD data were collected in a theta, 2-theta geometry from 3.00' onwards at a step of 0.020° 2-theta and a counting time of 1 s per step. XRD patterns of powder samples, oriented samples and glycol-saturated oriented powder samples were recorded.

2.4 Zearalenone adsorption screening

A saline solution was made by adding 24.0 g of NaCl, 0.3 g of $MgCl_2 \cdot 6H_2O$, 0.6 g of KCl and 0.4 g of $CaCl_2 \cdot 2H_2O$ to 3L HPLC-grade water. Next, a phosphate buffer system was added to 1 L of saline solution to obtain phosphate buffered saline (PBS). The buffer system consisted of H_3PO_4 and KH_2PO_4 for the acidic (pH 2.5) buffer and of KH_2PO_4 and Na_2HPO_4 for the buffers of pH 6.5 and 8.0. Total buffer concentration was calculated with the Henderson–Hasselbalch equation and the constraint to obtain a total osmolarity of 9.6 mmol/L in each buffer. The pH was measured and adjusted with H_3PO_4 or Na_2HPO_4 to obtain buffers of pH 2.5, 6.5 or 8.0. A 60-mL flask was filled with 20 mg of each of the binders and 5 mL of PBS; this was done for each pH, in triplicate. ZEN was added to a final concentration of 200 ng/mL. The flask was then shaken for 4 h at 37 °C in an incubator (New Brunswick Scientific, Rotselaar, Belgium). Next, samples were centrifuged (10 min, 1070× *g*, 25°C), and 2 mL of the supernatant were transferred to a test tube. Next, 25 µL of the internal standard (IS, $^{13}C_{18}$-ZEN, 1 µg/mL) were added and vortexed, followed by 4 mL of tBME. The tube was swirled on a roller bench (Stuart Scientific, Surrey, UK) for 20 min and centrifuged (10 min, 2,851× *g*, 4 °C). The supernatant was evaporated to dryness under a gentle nitrogen stream (40 ± 5 °C). The dry residue was reconstituted in 200 µL of ACN and transferred to a glass vial for LC-MS/MS analysis.

The HPLC system consisted of a Waters 2690 pump and autosampler system with a Zorbax Eclipse C-18 HPLC column (3 mm × 100 mm; i.d. 3.5 µm) and a pre-column of the same type (Agilent, Diegem, Belgium). The injection volume was 10 µL. The mobile phases were ACN (A) and HPLC-grade water supplemented with 0.3% ammonia (B). The gradient elution program was as follows: 0–0.5 min, 50% A/50% B; 0.5–1 min, linear gradient to 70% A/30% B; 1–4.5 min, 70% A/30% B; 4.5–5.5 min, linear gradient to 50% A/50% B; 5.5–8 min, 50% A/50% B. The flow rate was set at 0.6 mL/min. The MS/MS detection system was a Micromass Quattro Ultima (Micromass, Manchester, UK) operated in the ESI-negative mode. The *m*/*z* transitions for quantification were 335 > 140 ($^{13}C_{18}$-ZEN) and 317 > 131 (ZEN). The capillary and cone voltages were −3.47 kV and 60 V, respectively, and source

temperature was set at 120 °C and desolvation temperature at 200 °C. The cone gas flow and desolvation gas flow were set at 848 L/h and 60 L/h, and the optimized collision energy was 30 eV.

The analytical method was validated for the three pHs independently according to European guidelines (2002/657/EC, 2002) and was adapted from the method by De Baere *et al.* (2012) (De Baere et al., 2012). The validation included evaluation of linearity, within- and between-run accuracy and precision, limit of detection (LOD), limit of quantification (LOQ), specificity and carry-over. The correlation coefficients (*r*) and goodness-of-fit coefficients (*g*) of the 7-point calibration curves were calculated and fell within the limits of specification, ≥0.99 and ≤10%, respectively. For the precision, the relative standard deviation (RSD, %) fell within 2/3 of the values calculated according to the Horwitz equation,$RSD_{max} = 2^{(1-0.5\log Conc)} \times 2/3$, for within-run precision, with a minimum of 10%, and within the values calculated according to the Horwitz equation for between-run precision, $RSD_{max} = 2^{(1-0.5\log Conc)}$. The LOQ was determined by analyzing six samples spiked at 3.13 ng/mL, on the same day. Detection limits for pH 2.5, 6.5 and 8.0 were respectively 0.70, 1.07 and 0.66 ng/mL.

2.5 Statistical analysis

Analysis of variance (ANOVA) was used to compare the free concentration of ZEN for the different binders. The free ZEN concentration was correlated with the continuous explanatory variables. *p*-values below 0.05 were considered statistically significant. All analyses were conducted using SPSS 22 (IBM, Chicago, IL, USA), and graphs were obtained with GraphPad Prism® version 5 (La Jolla, CA, USA).

3 Results and discussion

3.1 Physicochemical characterization

The physicochemical properties of the 27 binders are presented in Table 1. These samples represent the vast majority of additives marketed as mycotoxin binders in Belgium and The Netherlands and are available in most European countries. All binders contain one or more mineral constituent, and some products contain organic compounds. Most binders are mixtures of different mineral constituents, and most prevalent compounds are smectites, such as montmorillonite. The ratio of exchangeable base cations varies widely, even among products with similar compounds. The non-mineral content of a binder with a low mineral fraction (*i.e.*, Sample Numbers 5, 12, 15 and 16) was confirmed by information provided by the manufacturer of the binder, who labelled these products as containing humic acids, leonardite or yeast-derived binders.

3.2 Zearalenone adsorption screening and correlation with physicochemical characteristics

The *in vitro* ZEN adsorption is assessed using a high throughput screening model applied at different pHs, which are representative for the gastro-intestinal tract of most monogastric animals. Similar models were successfully applied in previous *in vitro* experiments (Ramos and Hernandez, 1996b; Avantaggiato et al., 2005; Sabater-Vilar et al., 2007; Marroquin-Cardona et al., 2009; Joannis-Cassan et al., 2011; Yiannikouris et al., 2013; Sasaki et al., 2014). Major differences include the use of other buffer systems or media and the construction of adsorption isotherms. The use of other buffers or media may influence chemical equilibria, whereas adsorption isotherms may reveal information on the binding mechanism, affinity and capacity. This study focused on the determination of the free concentration of ZEN in phosphate buffered saline (PBS) after incubation with each of the 27 binders. The amount of ZEN and mycotoxin binder used for incubation is in accordance with the ZEN-binder ratio of 1:20,000, which is based on the maximum guidance level for ZEN in European piglet feed of 0.1 mg/kg described in Recommendation 2006/576 (European Commission, 2006) and the conventional binder inclusion level of 2 g/kg feed. The individual results of three replicates for the different pHs are presented in a ranked manner (Figure 1) to facilitate comparison between the binders. One-way analysis of variance (ANOVA) for the different binders indicates significant differences in free ZEN concentration ($p < 0.05$). Next, the free ZEN concentration was correlated with the physicochemical characteristics. The correlation matrix of free ZEN concentration and the physicochemical properties is presented in Table 2.

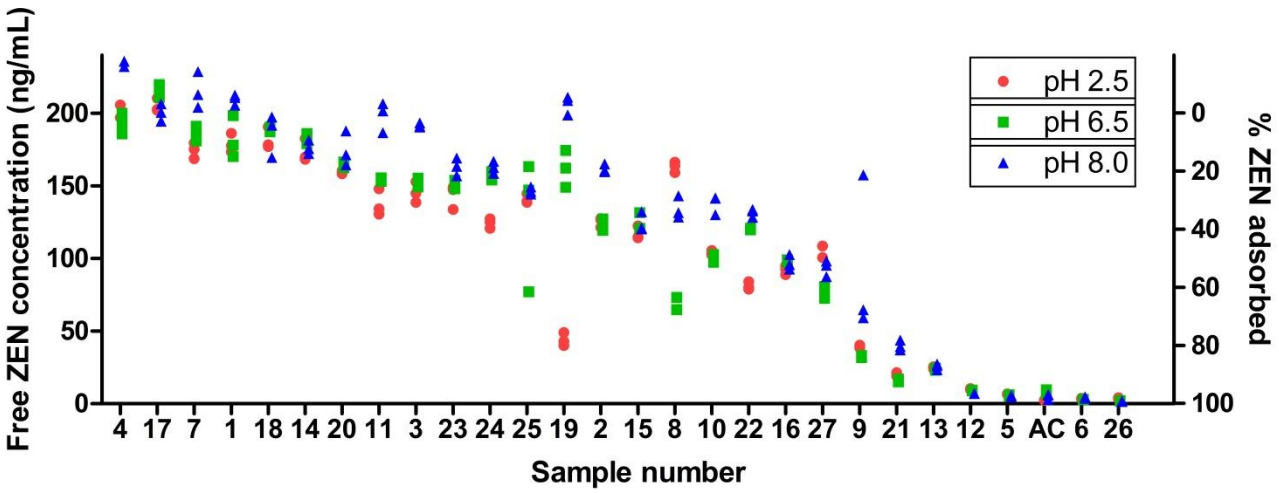

Figure 1: Free zearalenone (ZEN) concentration after incubation of ZEN with 27 mycotoxin binders at three different pHs (Sample Numbers 1–27). Individual results of three replicates are shown. AC represents activated carbon, which is included as the positive control.

Table 1: Physicochemical characteristics of 27 additives marketed as mycotoxin binders and available in Belgium and The Netherlands. Mean values of triplicate analyses are presented.

Sample Number	XRD Result	HCl	*d*-spacing (10^{-10} m)	CEC ($cmol_c\ kg^{-1}$)	pH	Ca^{2+} ($cmol_c\ kg^{-1}$)	K^{+} ($cmol_c\ kg^{-1}$)	Mg^{2+} ($cmol_c\ kg^{-1}$)	Na^{+} ($cmol_c\ kg^{-1}$)	Swelling (mL)	MF (%)	RH (%)
1	Zeolite	+	9.5	172.9	8.3	16.8	102.4	0.8	24.6	2.1	94.4	4.6
2	Sepiolite, smectite	+	12.4	31.9	7.7	7.3	1.5	9.8	1.3	7.7	96.0	8.7
3	Clinoptilolite	−	10.2	120.3	7.7	8.4	58.7	1.2	10.2	2.5	97.7	4.9
4	Zeolite	−	12.5	413.5	10.3	n.d.	35.4	0.1	363.3	0.0	93.8	7.1
5	Humic substance, quartz	−	26.2	185.9	4.2	7.2	1.5	3.4	19.2	2.5	15.8	10.6
6	Mixed layer montmorillonite, quartz	−	19.1	51.0	7.7	10.0	10.7	3.8	21.8	2.7	78.6	3.4
7	Montmorillonite	++	12.8	82.9	9.8	12.5	2.8	4.0	63.8	43.7	97.1	10.1
8	Montmorillonite	−	15.5	100.5	3.7	19.2	1.8	3.0	0.8	2.2	95.9	13.3
9	Sepiolite, montmorillonite, quartz (t), dolomite (t), albite (t)	+	12.1	39.3	8.2	8.2	0.6	10.2	0.6	7.9	96.3	5.4
10	Montmorillonite, sepiolite, quartz (t), calcite (t)	++	12.4	56.7	8.5	16.9	0.6	8.0	26.9	9.1	96.9	9.1
11	Montmorillonite, quartz (t), calcite (t), feldspars (t)	++	12.6	64.1	9.3	19.6	3.0	6.7	54.3	31.8	98.3	11.9
12	Humic substance, quartz	−	25.9	166.4	4.4	1.3	11.5	0.9	18.4	2.5	6.0	12.4
13	Sepiolite, montmorillonite, calcite (t), quartz (t)	+	12.2	22.1	7.1	17.7	2.2	9.3	4.4	5.9	80.3	6.7
14	Montmorillonite	−	9.2	109.4	5.6	21.7	17.2	1.9	4.2	2.9	92.8	7.2
15	Calcite, dolomite, organic material	++	6.9	12.6	5.7	35.5	19.1	4.2	26.0	7.5	38.9	5.1
16	Thenardite, montmorillonite, quartz, organic material		14.8	7.8	4.1	2.3	26.0	7.0	131.8	4.0	27.3	6.4
17	Montmorillonite	−	12.6	71.8	8.0	9.5	4.0	2.7	49.5	7.6	90.2	9.8
18	Clinoptilolite	−	10.2	176.6	7.4	15.2	44.7	2.0	6.0	2.5	96.3	4.7
19	Quartz, mica, montmorillonite, kaolin	−	14.7	59.7	7.9	18.1	1.9	9.0	0.3	4.3	95.4	7.9
20	Mica, kaolin, quartz, montmorillonite	+	14.7	59.6	7.9	14.4	2.5	8.7	0.6	3.5	97.0	9.0
21	Mixed layered smectite	+	12.4	23.7	9.9	13.3	0.7	19.2	47.7	24.2	97.5	7.5
22	Mica, calcite, smectite	+	15.5	77.9	8.0	33.9	1.8	4.1	0.9	4.3	88.6	11.4
23	Montmorillonite, sepiolite, calcite (t)	++	12.4	46.5	7.9	24.2	1.4	4.7	55.2	8.6	92.7	7.3
24	Montmorillonite, mica, feldspars	−	12.3	7.0	6.2	8.1	12.9	3.3	4.9	3.8	94.8	5.2
25	Calcite, montmorillonite (t)	++	13.1	26.1	6.6	55.8	10.7	2.4	11.6	3.7	97.0	3.0
26	Mixed layered montmorillonite, quartz, feldspars	−	21.5	27.9	7.7	9.3	1.4	2.6	4.9	2.5	98.0	2.0
27	Montmorillonite	−	12.7	111.7	9.5	8.7	1.3	4.0	69.5	5.7	86.8	13.2

−, + and ++ indicate minor, moderate and strong reaction in the HCl-effervescence test; n.d., not detectable; CEC, cation exchange capacity; MF, mineral fraction; RH, relative humidity; (t) indicates trace amounts.

Table 2: Correlation matrix of the free zearalenone (ZEN) concentration and the physicochemical properties of the 27 mycotoxin binders.

Parameters		Free ZEN concentration pH 2.5	Free ZEN concentration pH 6.5	Free ZEN concentration pH 8.0	Average free ZEN concentration
Free ZEN concentration pH 2.5	R	1	0.887 **	0.874 **	0.948 **
	Sig.	-	0.000	0.000	0.000
Free ZEN concentration pH 6.5	R	0.887 **	1	0.955 **	0.979 **
	Sig.	0.000	-	0.000	0.000
Free ZEN concentration pH 8.0	R	0.874 **	0.955 **	1	0.976 **
	Sig.	0.000	0.000	-	0.000
Average free ZEN concentration	R	0.948 **	0.979 **	0.976 **	1
	Sig.	0.000	0.000	0.000	-
d-spacing	R	−0.631 **	−0.632 **	−0.659 **	−0.662 **
	Sig.	0.000	0.000	0.000	0.000
Swelling	R	0.090	0.122	0.182	0.137
	Sig.	0.654	0.545	0.364	0.495
CEC	R	0.319	0.237	0.266	0.282
	Sig.	0.104	0.234	0.179	0.153
pH	R	0.192	0.285	0.357	0.290
	Sig.	0.339	0.149	0.067	0.142
Ca^{2+}	R	0.257	0.258	0.256	0.266
	Sig.	0.205	0.204	0.207	0.189
K^{+}	R	0.394 *	0.379	0.360	0.389 *
	Sig.	0.042	0.051	0.065	0.045
Mg^{2+}	R	−0.399 *	−0.316	−0.227	−0.321
	Sig.	0.039	0.108	0.254	0.102
Na^{+}	R	0.302	0.240	0.267	0.278
	Sig.	0.125	0.227	0.178	0.160
RH	R	0.082	−0.006	0.055	0.045
	Sig.	0.684	0.977	0.785	0.824
MF	R	0.421 *	0.419 *	0.525 **	0.472 *
	Sig.	0.029	0.030	0.005	0.013

R: Pearson correlation coefficient; Sig.: significance level; * significant at the 0.05 level (two-tailed); ** significant at the 0.01 level (two-tailed); CEC, cation exchange capacity; pH, acidity of the samples; Ca^{2+}, K^{+}, Mg^{2+}, Na^{+}, exchangeable base cations; RH, relative humidity; MF, mineral fraction.

A large variability in free ZEN concentration was observed, ranging from 200 ng/mL, which is indicative for no adsorption, to the limit of quantification, which corresponds with 100% adsorption under the given conditions. This is in accordance with previous binding experiments, where a large variability was also observed (Avantaggiato et al., 2005; Sabater-Vilar et al., 2007; Yiannikouris et al., 2013). A significant correlation could be demonstrated

between the free ZEN concentration and both the *d*-spacing and mineral fraction (MF). Figure 2 presents the two biplots of these parameters with the free ZEN concentration. In the low pH range (pH 2.5), exchangeable K^+ and Mg^{2+} were also significantly correlated. The pH may influence the phenolic hydroxyl group of ZEN or the ionization-state of the functional groups of the mycotoxin binders and thereby alter the chemical sorption due to ionic interactions. A low pH can facilitate degradation of the minerals, but this effect is mostly seen over a longer period. Deng *et al.* (2009) described the binding mechanism for aflatoxin B1 (AFB1) to montmorillonite clays, a mechanism involving the exchangeable cations and water (Deng et al., 2010). The correlation between the *d*-spacing and the free ZEN concentration suggests a cut off-value between 16 and 19 × 10^{-10} m, as can be seen in the left plot in Figure 2. From this cut off-value, a similar mechanism might apply for ZEN as for AFB1, explaining the low free ZEN concentration in binders expressing a large *d*-spacing. However, some aspects need to be considered: AFB1 has a rather planar structure, which facilitates interlayer adsorption, whereas ZEN has a more spherical molecular geometry. Furthermore, AFB1 is more hydrophilic than ZEN (estimated log $P_{\text{Aflatoxin B1}}$ = 1.58 *vs.* estimated log P_{ZEN} = ca. 4.37 (Chemaxon, 2013)). This is important, since the interlayer space is hydrophilic (Sposito et al., 1999).

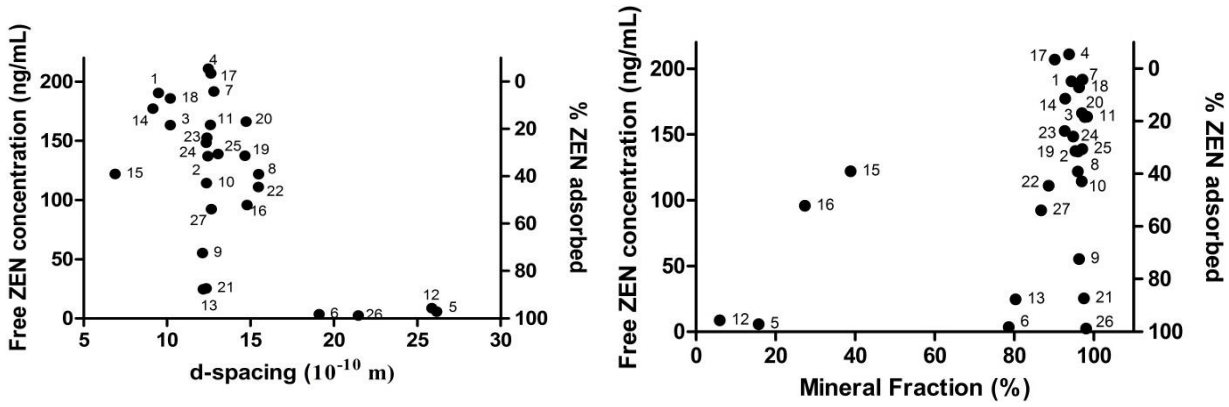

Figure 2: Biplots of the average free concentration of zearalenone (ZEN) with the *d*-spacing (left) and the mineral fraction (right) of the 27 mycotoxin binders (Numbers 1–27).

A low free ZEN concentration over the complete pH range was seen with the mixed-layer smectites (Sample Numbers 6, 21 and 26), which was also reported by (Avantaggiato et al., 2005). The exact mechanism for this remains to be elucidated. XRD and infra-red (IR) spectroscopy of the binding complex can be used to study the role of the *d*-spacing and may unravel the binding mechanism.

The humic acid-containing binders (Sample Numbers 5, 12 and 13) also presented a low free ZEN concentration. Similar results were observed in three out of five humic substance samples examined by Sabater-Vilar *et al.* (Sabater-Vilar et al., 2007). Yeast cell wall-derived products also expressed a low free ZEN concentration, which was also observed by (Sabater-Vilar et al., 2007) and (Yiannikouris et al., 2013), but not by (Avantaggiato et al., 2005). A high affinity of organic substances for oxytetracycline and AFB1 was described by (Diaz et al., 2003; Kulshrestha et al., 2004). The low free ZEN concentration when incubated with organic substances can be explained by the additional binding possibilities that these substances offer. The extra binding possibilities are hydrophobic in nature and comprise van der Waals, π–π and CH-π bonds (Picollo, 1999). Hydrophobic interactions were also suggested for the binding of ZEN to modified Japanese acid clay (Sasaki et al., 2014). In addition, hydrated humic substances are more flexible than the ridged minerals; this flexibility enables a larger interaction surface with the humic substances. These binding possibilities are independent of possible interlayer adsorptions and might be a parallel mechanism for toxin binding, as can be seen in the right plot of Figure 2. The zeolites and sepiolites expressed a rather high free ZEN concentration and are probably not fit for ZEN adsorption. Zearalenone was effectively adsorbed by active carbon, and this was also the case in previously published studies (Avantaggiato et al., 2005; Sabater-Vilar et al., 2007; Yiannikouris et al., 2013).

4 Conclusions

Twenty-seven frequently-used feed additives and marketed as mycotoxin binders were characterized. A single concentration *in vitro* adsorption screening of ZEN was executed in three different PBS-buffers (pH 2.5, 6.5 and 8.0). A significant correlation between free ZEN concentration and both the *d*-spacing and mineral fraction could be demonstrated. In the low pH range (pH 2.5), an additional correlation between the exchangeable K^+ and Mg^{2+} could be demonstrated. Humic acid-containing binders and mixed-layered smectite-containing binders achieved the lowest free ZEN concentration.

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Chapter 2: *In vitro* adsorption and *in vivo* pharmacokinetic interaction between doxycycline and frequently used mycotoxin binders in broiler chickens

Adapted from

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Abstract

Mycotoxin binders are readily mixed in the feed to prevent uptake of mycotoxins by the animal. Concerns were raised for non-specific binding with orally administered veterinary drugs by the European Food Safety Authority in 2010. This paper describes the screening for *in vitro* adsorption of doxycycline - a broad spectrum tetracycline antibiotic - to six different binders that were able to bind more than 75% of the doxycycline. Next, an *in vivo* pharmacokinetic interaction study of doxycycline with two of the binders, which demonstrated significant *in vitro* binding, was performed in broiler chickens using an oral bolus model. It was shown that two montmorillonite-based binders were able to lower the area under the plasma concentration-time curve of doxycycline with more than 60% compared to the control group. These results may indicate a possible risk for reduced efficacy of doxycycline when used concomitantly with montmorillonite-based mycotoxin binders.

Keywords: Doxycycline, mycotoxin binder, montmorillonite, *in vitro*, *in vivo*, pharmacokinetic, broiler chickens

1 Introduction

Mycotoxins are secondary fungal metabolites and are potentially harmful for animals after ingestion. They are often detected in feed (Binder, 2007; Streit et al., 2013) and can be responsible for economic losses even at subclinical levels (Binder, 2007). Measures such as crop rotation, application of fungicides, heat- or chemical treatment and optimal storage are often not sufficient to eliminate the production of and the damage caused by mycotoxins, hence other methods are used to counteract the effects of mycotoxins (Jard et al., 2011). Mixing specialized additives, i.e. mycotoxin detoxifiers, in the feed is nowadays the most commonly used method (Kolosova and Stroka, 2011). The mycotoxin detoxifiers can be divided in two groups: mycotoxin modifiers and -binders. The modifiers are of microbiological origin and aim to transform the chemical structure of mycotoxins into less- or non-toxic compounds. Mycotoxin binders aim to adsorb the toxin to their surface in the gastro-intestinal tract of the animal, thereby preventing the systemic uptake of the mycotoxin (Devreese et al., 2013a). Compounds used as mycotoxin binders are most of all clays, but also yeast cell walls and organic humic and fulvic acids, such as leonardite, are used. In a previous study (De Mil et al., 2015b), 27 mycotoxin binders commercially available in Belgium and The Netherlands were collected and characterized. The clays were mostly smectite clays, e.g. montmorillonites/bentonite, but some also contained sepiolites, zeolites, feldspars and kaolins. Indeed, 19 of the 27 samples contained montmorillonites, sepiolites or leonardites (Kolosova and Stroka, 2011; De Mil et al., 2015b). Besides, montmorillonites are also used because of their pellet binding, anti-caking or coagulant properties (EFSA, 2011; Kolosova and Stroka, 2011). It has been demonstrated that montmorillonite can effectively adsorb the mycotoxin aflatoxin B1 (AFB1) both *in vitro* and *in vivo,* which prevents the uptake of this mycotoxin in the animal (Desheng et al., 2005). The adsorption mechanism is by means of hydrogen bonds with exchangeable cations of the clay (Phillips et al., 2006; Deng et al., 2010). These mechanisms are deemed to be non-specific and in 2010 the European Food Safety Authority (EFSA) stated that next to efficacy testing of mycotoxin binders, also their safety should be investigated (EFSA FEEDAP Panel, 2010). Safety concerns the non-specific adsorption of vitamins, nutrients and veterinary medicinal products to these clays. Only few literature reports have investigated the adsorption of veterinary medicinal products to clays. Interactions were reported for the macrolide antibiotics tilmicosin (TIL) (Shryock et al., 1994) and tylosin (TYL) (Canadian Bureau of Veterinary Drugs, 1992; Devreese et al., 2012), and for the coccidiostats monensin (MON) and salinomycin (SAL) (Gray et al., 1998).

Schryock et al. (1994) (Shryock et al., 1994) studied the effectiveness of TIL for prevention of airsacculitis in broiler chickens infected with *Mycoplasma gallisepticum*. A decrease of the protective effect of TIL was seen from an inclusion rate of bentonite of 2% onwards. Furthermore, the Canadian Bureau of Veterinary Drugs (1992) (Canadian Bureau of Veterinary Drugs, 1992) reported a case of lack of efficacy of TYL when concurrently administered with bentonite in cattle. Therefore, the EFSA (2012) discourages the simultaneous use of bentonite clay with macrolides, coccidiostats and other medicinal products (EFSA, 2011). In 2012, Devreese et al. (Devreese et al., 2012) also described the interaction between TYL and bentonite in broiler chickens, using a pharmacokinetic (PK) approach with single oral bolus administration of TYL whether or not combined with bentonite clay. A significant decrease of the area under the plasma concentration-time curve (AUC), maximum plasma concentrations (C_{max}) and time when C_{max} occurs (T_{max}) of TYL were observed when combined with an inclusion rate of bentonite of 1 g/kg feed. Consequently, a relative oral bioavailability (F) of only 23.3% could be calculated for the birds receiving TYL+bentonite, compared to 100% in the TYL group alone.

Next, Gray et al. (1998) (Gray et al., 1998) studied the efficacy of MON and SAL in the presence of sodium bentonite. The authors concluded that sodium bentonite could reduce the efficacy of MON and SAL but only at levels below the recommended dosages.

In vitro or *in vivo* literature data for other clays and/or other mycotoxin binders and for other veterinary medicinal products are not available despite the possible risk of binder-drug interactions and consequently the reduced efficacy of the drug. In case of antibiotics, not only the lack of efficacy is of concern but also the possible increase of antimicrobial resistance due to exposure to sub-therapeutic concentrations (Kobland et al., 1987; Levy, 2002; Phillips et al., 2004). These concerns were also incorporated in the EFSA recommendations for this class of additives (Wache et al., 2009). Besides macrolides, tetracycline antibiotics are frequently used in veterinary medicine, more specifically in feed or drinking water medication in pig and poultry farming. Doxycycline (DOX) is a broad spectrum, bacteriostatic tetracycline of the second generation. It is mainly used in broiler chickens to treat respiratory and systemic infections caused by *Mycoplasmata, Ornithobacterium rhinotracheale, Avibacterium paragallinarum, Pasteurella multocida* and *Chlamydia spp.* (Butaye et al., 1997; Avrain et al., 2003; Johansson et al., 2004; Cauwerts et al., 2007) and in pigs for respiratory infections caused by *Actinobacillus pleuropneumoniae, Pasteurella multocida,, Bordetella bronchiseptica, Mycoplasma hyorhinis and Streptococcus suis* (Pijpers et al., 1989).

Therefore, the aim of present study was to evaluate the *in vitro* adsorption of DOX to four montmorillonite and one sepiolite clay, one leonardite-based binder, and including activated carbon (AC) as positive control. Next, to confirm and validate the *in vitro* model, an *in vivo* pharmacokinetic study was performed using oral bolus dosing of DOX and two of the *in vitro* studied mycotoxin binders in broiler chickens.

2 Materials and methods

2.1 Chemicals, solutions and mycotoxin binders

Sigma-Aldrich (Bornem, Belgium) supplied doxycycline (DOX) hyclate, demethylchlortetracycline (DMCTC, used as internal standard), potassium chloride (KCl) and phosphoric acid (H_3PO_4). Water (H_2O), methanol (MeOH) and acetonitrile (ACN) used for the high performance liquid chromatography (HPLC) analysis and preparation of stock solutions were of HPLC-grade and provided by Fisher Scientific (Wijnegem, Belgium). Stock solutions of 10 mg/mL were made for DOX and DMCTC in respectively H_2O and MeOH/H_2O (50/50; v/v). Sodium chloride (NaCl), magnesium chloride ($MgCl_2 \cdot 6H_2O$) and calcium chloride ($CaCl_2 \cdot 2H_2O$) were supplied by VWR (Leuven, Belgium). Potassium dihydrogen phosphate (KH_2PO_4), disodiumhydrogenphosphate (Na_2HPO_4), formic acid (HCOOH), trifluoroacetic acid (TFA) and sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany). Six mycotoxin binders were evaluated and obtained from Sigma-Aldrich, Kemin (Herentals, Belgium) and Poortershaven (Rotterdam, The Netherlands) (De Mil et al., 2015b), and were identified as 'montmorillonite number 1, 2, 3 and 4', 'leonardite' and 'sepiolite'. Regarding the composition of the evaluated binders, X-Ray Diffraction spectrometry (XRD) analysis of montmorillonite 1 indicated montmorillonite as the most prevalent mineral, but also the presence of mica, quartz and kaolin was detected, the mineral fraction was 97.0%. XRD analysis of montmorillonite 2 indicated mixed layered montmorillonite as the most prevalent mineral, but also trace amounts of quartz were detected, the mineral fraction was 78.6%. XRD analysis of montmorillonite 3 indicated pure montmorillonite as the only mineral, the mineral fraction was 86.8%. Montmorillonite 4 contained montmorillonite, sepiolite and trace amount of calcite, the mineral fraction was 92.7%. XRD analysis of the leonardite indicated the presence of humic substance and small amounts of quartz. The mineral fraction was less than 16%. The sepiolite contained small amounts of a smectite clay, mineral fraction was 96% (De Mil et al., 2015b). AC (Norit Carbomix®), used as positive control, was obtained from Kela (Hoogstraten, Belgium) and was of pharmaceutical grade and applied in a granulated form. Doxycycline hyclate (Soludox 15%®) used for the *in vivo* study was supplied by Dechravet (Heusden-Zolder, Belgium).

2.2 *In vitro* binding assay

The protocol used for the *in vitro* binding assay was adapted from Sabater-Vilar et al. (Sabater-Vilar et al., 2007) The main adaptations were the use of Phosphate Buffered Saline

(PBS) buffers of two different pH values instead of adjusting the pH during the conduct of the experiment. Also the sample preparation, i.e. centrifugation instead of filtering, and chromatographic sample analysis differed. PBS was prepared by adding first 24.0 g NaCl, 0.3 g $MgCl_2.6H_2O$, 0.6 g KCl and 0.4 g $CaCl_2.2H_2O$ to 3 L of HPLC-grade water. Next, a buffer system consisting of H_3PO_4 and KH_2PO_4, or KH_2PO_4 and Na_2HPO_4 was added to 1 L of salt solution to obtain a buffer solution of ± 9.6 mM (Sabater-Vilar et al., 2007). The pH was adjusted with H_3PO_4 or NaOH to obtain buffers of pH 2.5 and 6.5, respectively. Five mL of the respective PBS at pH 2.5 or 6.5 were added to a 60 mL flask containing 20 mg of the respective binder or AC. Finally, DOX hyclate was added to a concentration of 100 µg DOX/mL. All samples were prepared and analyzed in triplicate. The ratio DOX/binder of 1/40 (w/w) corresponds with a dose of 20 mg DOX/kg BW and a feed intake of 80 g/kg BW with inclusion of 1% binder.

The flask was shaken on a lateral shaker (IKA, Staufen, Germany) for 4 h at 37 °C, 180 rpm (New Brunswick Scientific, Rotselaar, Belgium). Next, samples were transferred to a 15 mL tube and centrifuged (10 min, 524 x *g*, 4 °C), 100 µL of the supernatant was transferred to an Eppendorf cup, supplemented with 50 µL of a 50 µg/mL aqueous solution of DMCTC and diluted with HPLC-grade H_2O to a volume of 1 mL. After thorough vortex mixing, 100 µL was transferred into an autosampler vial and diluted with ACN:H_2O 80:20 (v:v) to a final volume of 1 mL for further liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis. Negative control samples were included at 10 µg/mL and 100 µg/mL DOX and were submitted to the same treatment as the other samples, but without addition of binder.

2.3 *In vivo* oral bolus pharmacokinetic study

Montmorillonite 2 and 3 were selected for further research since montmorillonites are the most frequently used mycotoxin binder and because of the high purity of these two montmorillonites. Furthermore, montmorillonite 2 differs from montmorillonite 3 in terms of Cation Exchange capacity CEC (51.0 $cmol_c/kg$ vs. 111.7 $cmol_c/kg$) (De Mil et al., 2015b), type of montmorillonite - i.e. mixed layered and normal layered - and mineral fraction. Thirty-two 14-day-old broiler chickens (Ross 308, as hatched) were randomly divided in four groups of eight birds, namely DOX, DOX+AC, DOX+montmorillonite 2 and DOX+montmorillonite 3. The animals were housed and treated according to European guidelines for animal experiments (Council of Europe, 2009). Each group was housed in floor pens of 2 m² covered with wood shavings and equipped with a heating lamp. The animals had *ad libitum* access to

feed and drinking water. Temperature and relative humidity were controlled between 15-25 °C and 40-80% respectively. After 1 week acclimatization, the animals were weighed and fasted 12 h before the onset of the experiment. According to their group, the birds received an oral intra-crop bolus (2 mL) of tap water for the animals of the negative control group (DOX), AC dispersed in 2 mL tap water for the positive control group (DOX+AC) and montmorillonite 2 or montmorillonite 3 also dispersed in 2 mL of tap water for the other two groups (DOX+montmorillonite 2 and 3, respectively). The montmorillonite and AC dose was equivalent with the expected daily intake when 1% (w/w) is included in the feed. Immediately after this bolus administration, an oral Soludox 15%® bolus (1 mL) containing 20 mg DOX/kg BW in tap water was administered, followed by 1 mL of tap water to flush the tube. The ratio DOX/binder was in respect with the *in vitro* trial, namely 1/40 (w/w). Next, blood samples were taken at 0 min (just before administration), 15, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 hours post-administration (p.a.) of the binder and DOX. Blood samples were collected in heparinized tubes, centrifuged (524 x *g*, 10 min, 4 °C) and plasma was stored at ≤ -15 °C until analysis. This experiment was approved by the local Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University (case number EC 2014/08).

2.4 Quantification of doxycycline in buffer (*in vitro*) and in plasma (*in vivo*)

The sample preparation for the *in vitro* buffer samples is presented in the section 2.2 on the *in vitro* binding assay. Sample preparation for plasma samples was as follows: 100 µL of plasma was supplemented with 15 µL of TFA, 50 µL of a 10 µg/mL aqueous DMCTC solution and 50 µL of HPLC water. The sample was vortex mixed for 30 seconds before centrifuging at 10800 x *g* for 10 min at room temperature. The supernatant was diluted 5-fold in HPLC water before analysis. The HPLC system for all samples consisted of a Waters 2690 pump and autosampler (Waters, Milford, USA). Chromatographic separation was achieved on a Hypersil Gold column (100 mm x 2.1 mm, particle size 5 µm) and corresponding guard column (Thermo Scientific, Erembodegem, Belgium). The mobile phases were A: 0.1% HCOOH in H_2O; B: 0.1% HCOOH in ACN. Following gradient elution program for the samples derived from the *in vitro* study was applied: 0-2 min: 98% A, 2% B; 2-5.5 min: 40% A, 60% B; 5.5-12 min: 98% A, 2% B. The flow rate was set at 0.30 mL/min, 5 µL of sample was injected. For the analysis of plasma samples, following gradient was applied: 0-0.3 min: 95% A, 5% B, 0.3-3.5 min: 10% A, 90% B, 3.5-8.5 min: 70% A, 30% B, 8.5-15 min: 95% A,

5% B. The flow rate was set at 0.45 mL/min, a 5 µL aliquot of the sample extract was injected.

The HPLC effluent was interfaced to a Quattro Ultima tandem mass spectrometer (Micromass, Manchester, UK). The capillary- and cone voltage were 3.0 kV and 40 V, respectively, the source temperature was set at 120 °C and desolvation temperature at 250 °C. Cone gas flow and desolvation gas were respectively 101 L/h and 898 L/h. The positive electrospray ionization (ESI+) mode was applied, collision energy was optimized to 25 eV for both DOX and DMCTC in plasma samples, and 25 and 20 eV for DOX and DMCTC in the samples from the *in vitro* study, respectively. Following selected reaction monitoring (SRM) transitions were monitored and used for quantification: *m/z* 445.0>428.2 for DOX and 465.0>448.1 for DMCTC.

The method was validated for DOX in plasma as well as samples from the *in vitro* study according to a validation protocol previously described (De Baere et al., 2011), using matrix matched calibration curves. All parameters met the requirements and were in compliance with the recommendations and guidelines defined in Directive 2002/32 and Decision 2002/657 (European Commission, 2002b, a) and with international criteria described in the literature (Knecht and Stork, 1974; Heitzman, 1994; U.S. Department of Health and Human Services, 2015), except for carry-over of DOX on the instrument. A small interfering peak of 0.4% - 1% area relative to the area of the highest calibrator was detected when injecting a solvent sample directly after the highest calibrator, no peaks were detected when injecting solvents after the other calibrators. Since the majority of the samples fell below the concentration range in which carry-over was expected, no overestimation was present. The limit of quantification (LOQ) was 1 µg/mL for PBS at pH 6.5 and 2.5, and 50 ng/mL for plasma. Between-run recovery ± standard deviation (SD, n=6) of the quality control samples prepared in PBS at pH 2.5 were 10.9 ± 0.5 µg/mL and 94.2 ± 2.2 µg/mL for blank samples spiked at 10 and 100 µg/mL, respectively. For pH 6.5 the recoveries were 10.4 ± 0.5 µg/mL and 101.3 ± 3.9 µg/mL. Within-run recovery (n=6) in PBS at pH 2.5 was 10.7 ± 0.2 µg/mL and 95.3 ± 0.2 µg/mL, whereas in PBS at pH 6.5 the recoveries were 10.1 ± 0.2 µg/mL and 99.8 ± 1.8 µg/mL. Validation of the analytical method for the plasma samples included blank plasma samples spiked at 250 ng/mL (n=6) and 2500 ng/mL (n=6, both within- and between-run), between-run recovery was 242.2 ± 4.6 ng/mL and 2518.2 ± 59.8 ng/mL, respectively. For the within-run recovery, values of 243.8 ± 5.6 ng/mL and 2460.4 ± 68.1 ng/mL were obtained.

2.5 Pharmacokinetic analysis

A non-compartmental analysis of the DOX plasma concentration-time data was performed to determine the pharmacokinetic (PK) characteristics of DOX using WinNonlin 6.3 (Phoenix Pharsight, St. Louis, USA). Following main PK variables were determined for each animal: C_{max}, T_{max}, area under the plasma concentration-time curve from time 0 to infinite and 24 h ($AUC_{0\text{-}inf}$ and $AUC_{0\text{-}24h}$), elimination rate constant (k_{el}), elimination half-life ($T_{1/2el}$), volume of distribution relative to the absolute oral bioavailability (Vd/F) and clearance relative to the absolute oral bioavailability (Cl/F). The relative oral bioavailability (relative F) was calculated according to following formula:

$$relative\ F\ = \frac{AUC_{0-inf.,DOX+tested\ binder}}{AUC_{0-inf.,DOX\ negative\ control}} * 100$$

2.6 Statistical analysis

The free DOX concentrations in the *in vitro* trial were analyzed using a one-way analysis of variance (ANOVA), and executed using the statistical package SPSS for Windows (IBM, Brussels, Belgium). The PK values for each group in the *in vivo* study were compared using a one-way ANOVA, p-values below 0.05 were considered as statistical significant.

3 Results

Results of the *in vitro* binding assay are presented in Figure 1. The concentration of DOX in the negative control samples at pH 2.5 were 10.6 ± 0.3 and 91.0 ± 1.8 µg/mL for the samples spiked at 10 and 100 µg/mL, respectively. For the samples at pH 6.5 the concentration of DOX was 11.0 ± 6.8 and 100.3 ± 4.7 µg/mL, respectively. Low free concentrations of DOX after incubation are indicative for high adsorption and are seen in all of the tested binders. No significant differences were observed between the binders and AC, however, all of the tested binders differed significantly ($p<0.001$) from the negative control samples.

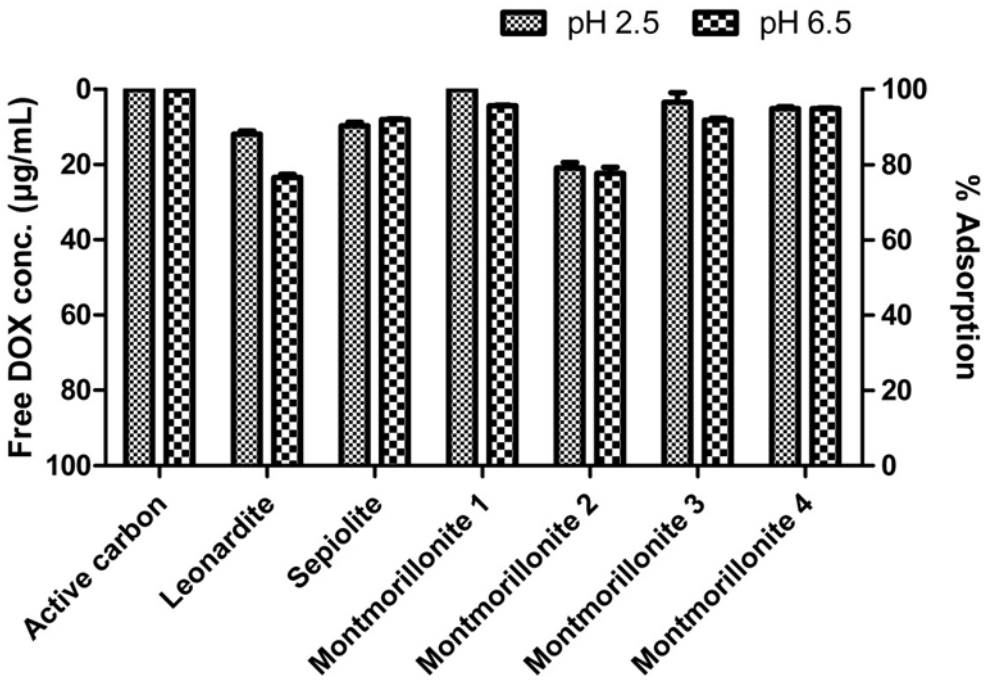

Figure 1: Doxycycline (DOX, 100 µg/mL) was incubated for 4 h at 37 °C with 20 mg of one of following binders in 5 mL PBS buffer at pH 2.5 and pH 6.5: activated carbon (positive control), leonardite, sepiolite and 4 different montmorrilonites. The free DOX concentration (µg/mL) is presented on the left y-axis as average of 3 independent measurements + standard deviation (SD). The % adsorption is presented on the right y-axis. No significant differences were observed between the binders and activated carbon, however, all of the tested binders differed significantly ($p<0.001$) from the negative control samples.

The plasma concentration-time profiles of DOX after single oral bolus administration of the tetracycline whether or not combined with one of the binders are presented in Figure 2. Mean plasma concentration of DOX in the negative control group reached a first maximum at about 2.5 h and a second at 6 h. The area under the plasma concentration-time curves of the test

groups are clearly lower compared to the control group, and the curves display only one maximum at the same time of the second maximum of the control group (at about 6 h). The PK parameters of DOX are presented in Table 1. Significant differences between the negative control group and test groups can be seen for the $AUC_{0\text{-}inf}$, $AUC_{0\text{-}24h}$ and C_{max} ($p<0.001$). The absorption rate constant (k_a) was calculated using the curve stripping method as described by Gabrielsson and Weiner (Gabrielsson and Weiner, 2000). However, variance on the calculated k_a values did not allow meaningful comparison, therefore, this parameter was omitted from Table 1.

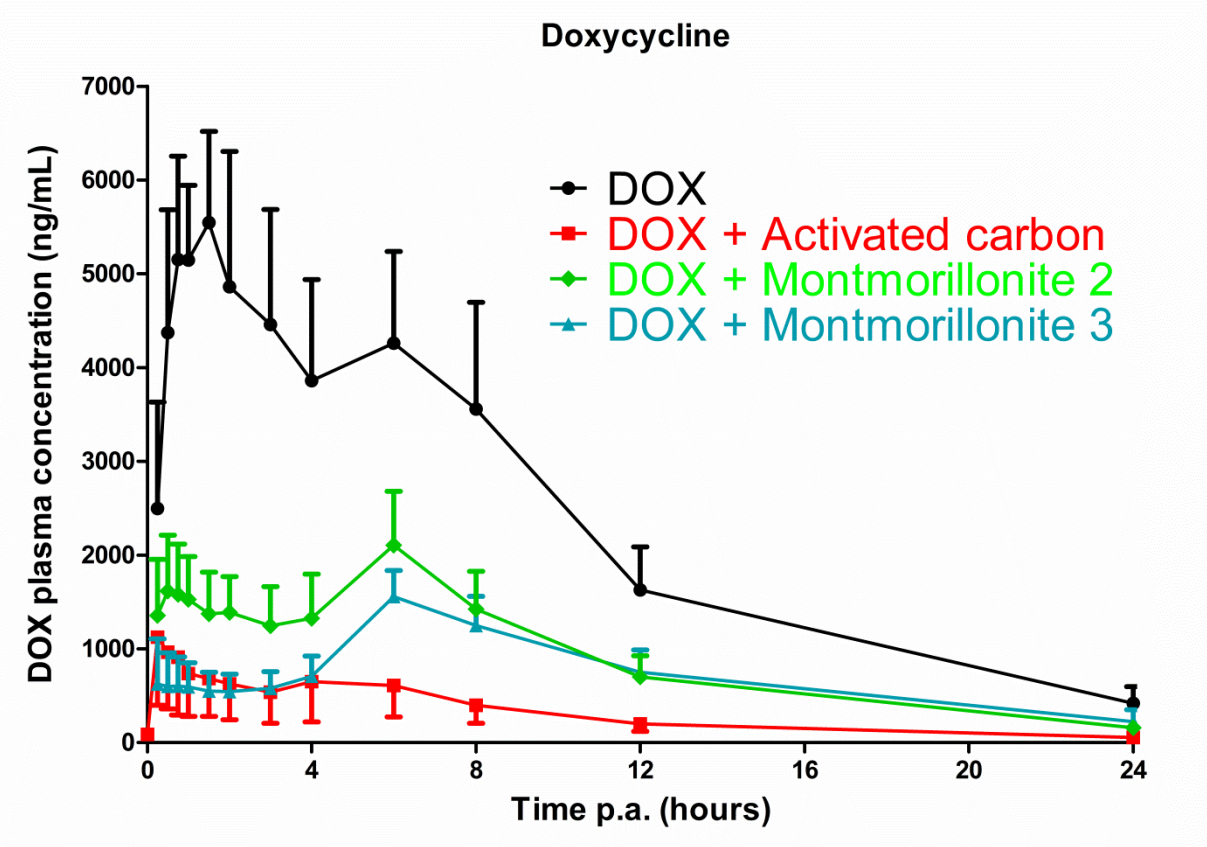

Figure 2: Four groups of eight broiler chickens were given an oral bolus containing water (negative control), activated carbon (positive control), montmorillonite 2 or montmorillonite 3 directly followed by an oral bolus containing doxycycline (DOX) at 20 mg/kg bodyweight. Plasma concentration-time profiles of DOX are presented as means of 8 observations + standard deviation.

Table 1: Four Groups of Eight Broiler Chickens Were Given an Oral Bolus Containing Water (Negative Control), Activated Carbon (Positive Control), Montmorillonite 2 or Montmorillonite 3 Directly Followed by an Oral Bolus of Doxycycline (DOX) at 20 mg/kg Bodyweight. The Pharmacokinetic Parameters of DOX for the Different Test Groups Are Presented as Mean of the Group ± Standard Deviation.

Parameter	Negative control	Activated carbon	Mont. 2	Mont. 3
$AUC^{b}_{0\text{-inf}}$ (h·µg/mL)	57.58±16.37	7.91±4.86 ‡ [a]	23.01±5.82 ‡ [a]	20.46±6.22 ‡ [a]
$AUC^{b}_{0\text{-24h}}$ (h·µg/mL)	54.70±13.96	6.99±4.77 ‡ [a]	21.73±5.70 ‡ [a]	16.98±4.35 ‡ [a]
Relative F (%)	100.00±28.42	13.74±8.43 ‡ [a]	40.0±10.10 ‡ [a]	35.52±10.80 ‡ [a]
$T_{max}{}^{c}$ (h)	2.84±2.73	0.88±1.28	4.63±2.55	6.00±0.00 * [a]
$C_{max}{}^{d}$ (µg/mL)	5.55±1.40	1.16±0.86 ‡ [a]	2.14±0.53 ‡ [a]	1.56±0.33 ‡ [a]
$k_{el}{}^{e}$ (1/h)	0.13±0.02	0.14±0.05	0.13±0.02	0.11±0.03
$T_{1/2el}{}^{f}$ (h)	8.22±1.51	7.97±2.64	7.82±1.24	9.82±3.20
Vd/F^{g} (L/kg)	3.05±0.99	30.50±24.21 * [a]	7.20±2.15	10.01±3.06
Cl/F^{h} (L/h/kg)	0.38±0.12	3.73±2.48 ‡ [a]	0.91±0.20	1.09±0.47
$MRT^{i}_{0\text{-inf}}$ (h)	8.87±1.16	8.49±2.17	9.02±1.27	12.35±2.62 * [a]

[a] *, † and ‡ indicate significant difference compared to the control at the 0.05, 0.01 and 0.001 level respectively; [b] AUC: Area Under the Curve from time 0 to infinity or 24 h; [c] T_{max}: time of maximum concentration; [d] C_{max}: maximum concentration; [e] k_{el}: elimination rate constant; [f] $T_{1/2el}$: terminal elimination half-life; [g] Vd/F: distribution volume relative to the absolute oral bioavailability; [h] Cl/F: clearance relative to the absolute oral bioavailability; [i] Mean Residence Time

4 Discussion

The aim of this study was to investigate the interactions between DOX and different feed additives used as mycotoxin binder. Firstly an *in vitro* adsorption screening study was performed. Therefore an *in vitro* model as reported by De Mil et al. (2015b) (De Mil et al., 2015b) and based on the model used by Sabater-Vilar et al. (2007) (Sabater-Vilar et al., 2007), was applied. This model uses PBS at pH's 2.5 and 6.5, which represent respectively the gastric and duodenal pH conditions of monogastric animals. Less than 25% of the initial concentration could be detected as free DOX after 4 h of incubation at 37 °C in all groups, indicating that the majority of DOX is adsorbed by the additives. Deng et al. (2010) (Deng et al., 2010) described the binding mechanism between AFB1 and montmorillonite clays, the adsorption is facilitated by the exchangeable cations which form hydrogen bonds with AFB1. Along with the exchangeable cations, AFB1 can then be 'trapped' between the silicate sheets of the montmorillonite and effectively bound. A similar mechanism might apply here since DOX has many sites suitable for hydrogen bonding.

When used as a mycotoxin binder, most recommendations mention an inclusion rate in the feed of 0.1 to 0.5% (w/w), however, when used as pelletizing - or binding agent, inclusion rates up to 2% are recommended. The ratio DOX/binder of 1/40 (w/w) is representative for the *in vivo* application of both substances when considering a clay inclusion rate of 1% (w/w), this ratio was the same in both the *in vitro* and the *in vivo* trial.

The *in vitro* models used for the assessment of the efficacy include static (Vekiru et al., 2007; Devreese et al., 2013b) and dynamic (Ramos and Hernandez, 1996a; Avantaggiato et al., 2003) gastro-intestinal (GI)-models, construction of adsorption isotherms (Ramos and Hernandez, 1996b) and single concentration studies (Sabater-Vilar et al., 2007). Although the simple setup in this study cannot provide the same information as the complex dynamic- and isotherm models, by using representative buffers and binder-drug ratio's, this setup still can provide relevant information regarding the safety of the feed additives which can be directly extrapolated to the *in vivo* situation. To verify the latter, *in vivo* experiments have to be carried out. An *in vivo* oral bolus model was applied to study the interactions from a PK perspective (Devreese et al., 2012), this is in accordance with EFSA guidelines for the safety testing of these group of additives (EFSA FEEDAP Panel, 2010). Concerning the DOX PK parameters in the negative control group, the reported $T_{1/2,el}$ are between 5.69 h (El-Gendi et al., 2010) and 7.93 h (Ismail and El-Kattan, 2004) in broiler chickens, whereas this study found a $T_{1/2,el}$ of 8.22 h which can be considered to be in the same range. The reported T_{max},

C_{max} and $AUC_{0\text{-}inf}$ values for most references are also in the same range as found for the control group in this study, namely between 1.73 h (Laczay et al., 2001) and 2.9 h (Gutierrez et al., 2012) for T_{max}, between 3.18 µg/mL (El-Gendi et al., 2010) and 7.84 µg/mL (Ismail and El-Kattan, 2004) for C_{max} and between 39.84 µg·h/mL (El-Gendi et al., 2010) and 97.6 µg·h/mL (Ismail and El-Kattan, 2004) for $AUC_{0\text{-}inf}$. The appearance of a second maximum in the plasma concentration-time profile is likely to be due to an enterohepatic recycling, this means excretion of DOX from the blood to the gastro-intestinal lumen by both hepatic/biliary or direct passive unionized diffusion from blood to gut, followed by reabsorption. This phenomenon, seen for most tetracyclines, has been described for many species such as pigs (Riond and Riviere, 1990), humans (Gibaldi, 1967) and sheep (Castro et al., 2009). Pharmacokinetic analysis of DOX of the test groups clearly indicates that $AUC_{0\text{-}inf}$ and C_{max} are decreased when AC or the tested montmorillonites are used simultaneously with DOX. Table 1 shows a somewhat delayed T_{max} of DOX in the montmorillonite test groups. However, this T_{max} coincides with the second absorption maximum of DOX in the control group (Figure 2), which indicates that this shift is rather due to the absence of the first maximum than a delayed absorption. Differences in Vd/F and Cl/F can be explained by a difference in bioavailability although the latter cannot be calculated with the given data. As expected, no differences in elimination kinetics were seen, since k_{el} and $T_{1/2el}$ were similar in both control and test groups. The 60% decrease in $AUC_{0\text{-}24h}$ in the test groups results in a lower AUC/Minimal Inhibitory Concentration (MIC) ratio, which is of major importance for the clinical outcome (Hesje et al., 2007). Therefore, a decrease in treatment efficacy can be expected since a decrease in oral bioavailability F is linearly correlated to a decrease in AUC (Gabrielsson and Weiner, 2006) and therefore also to the AUC/MIC ratio. Antimicrobials for which the efficacy depends on the time the plasma concentration is above the MIC, such as TYL, might even be more sensitive to a decrease in oral bioavailability since the time above MIC decreases in a logaritmic manner in function of F. Moreover, sub-therapeutic concentrations of tetracyclines can induce microbial resistance (Phillips et al., 2004). This interaction is in line with previously reported interactions of montmorillonite-based feed additives with the macrolides TIL (Shryock et al., 1994) and TYL (Devreese et al., 2012) in broiler chickens.

Based on the *in vitro* adsorption and *in vivo* pharmacokinetic results, it can be concluded that AC and the tested montmorillonite clays can substantially diminish the oral absorption of DOX in broiler chickens and this is most likely due to the adsorption of DOX to the clay

additives. Therefore, in case DOX is orally administered to chickens that are also fed montmorillonite-based feed additives, a possible interaction needs to be carefully considered. Further research is needed to investigate the safety of other additives when used simultaneously with other veterinary medicinal products.

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Chapter 3: Influence of mycotoxin binders on the oral absorption of tylosin, doxycycline, diclazuril and salinomycin in fed broiler chickens

Adapted from:

Thomas De Mil, Mathias Devreese, An Maes, Sarah De Saeger, Patrick De Backer and Siska Croubels (2016c). Influence of mycotoxin binders on the oral bioavailability of tylosin, doxycycline, diclazuril and salinomycin in fed broiler chickens. Submitted to Poultry Science.

Abstract

The presence of mycotoxins in broiler feed can have deleterious effects on the wellbeing of the animals and their performance. Mycotoxin binders are feed additives which aim to adsorb mycotoxins in the intestinal tract and thereby preventing the oral absorption of the mycotoxin. Coccidiostats are also feed additives frequently administered to poultry and antimicrobials are given as oral mass medication in the poultry industry as well. If the binding of mycotoxin binders is non-specific, the simultaneous administration of coccidiostats and/or antimicrobials with mycotoxin binders can lead to a reduced oral bioavailability of these veterinary medicinal products. This paper describes the influence of four mycotoxin binders on the oral bioavailability and pharmacokinetic parameters of the antimicrobials doxycycline and tylosin, and the coccidiostats diclazuril and salinomycin. A feeding study was performed which evaluates the long-term effects of feeding 2 g mycotoxin binder/kg feed on the possible interactions and can therefore be considered as an approximation of the field situation. No interactions were observed between any of the mycotoxin binders and the coccidiostats, whereas a trend but no significant interactions could be noticed between some mycotoxin binders and the antimicrobials doxycycline and tylosin.

Keywords: Mycotoxin binders, antimicrobials, coccidiostats, broiler chickens, pharmacokinetics, oral bioavailability

1 Introduction

Mycotoxins are secondary fungal metabolites frequently contaminating feed which can be acute toxic at high doses, whereas chronic exposure to lower contamination levels can result in poor animal performance and consequent economical damage (Binder, 2007). It is estimated that in the USA *Fusarium* mycotoxins may cause damage equivalent to 20 million US dollars each year (Wu, 2007). Because of a high prevalence of *Fusarium* mycotoxins in cereal based feed, pigs and poultry are frequently exposed to these toxins (Binder et al., 2007; Streit et al., 2013). Special feed additives, called mycotoxin detoxifiers, are frequently used in the pig and poultry industry to counter the deleterious effects of mycotoxins (EFSA FEEDAP Panel, 2010). Based on their mode of action, two categories can be distinguished, namely mycotoxin modifiers and mycotoxin binders (Jard et al., 2011). Mycotoxin modifiers aim to alter the chemical structure of the mycotoxin into chemical entities that are less or non-toxic. To date, all the modifiers available on the market (registered in Annex I of Regulation 1831/2003) are of microbiological origin and comprise extracted enzymes or whole cultures of yeasts or bacteria (European Commission, 2015). Mycotoxin binders aim to form non-resorbable complexes with mycotoxins in the intestinal tract of the animal, making them unavailable for absorption. Most mycotoxin binders contain clay minerals but also yeast based products are often used. The clays used as mycotoxin binders are mostly smectites (De Mil et al., 2015b), e.g. montmorillonites, which are the main constituents of bentonite. The maximum amount of bentonite allowed for aflatoxin B1 detoxification mentioned in Reguation 1060/2013 is 20 g/kg complete feed for ruminants, poultry and pigs (European Commission, 2013a), however, the inclusion rate that is applied usually varies between 1 and 2.5 g/kg complete feed. Total amounts of bentonite can reach higher levels than 1-2.5 g/kg because bentonite can also be used for other purposes such as pelletizing agents or to improve the rheological properties of feed (anticaking agent). The total amount of bentonite in complete feed is limited to 20 g/kg, with provision to indicate in the instructions for use that the simultaneous oral use with macrolides shall be avoided in any animal species, and that in poultry the simultaneous use with robenidine shall be avoided and the simultaneous use with other coccidiostats is contraindicated with a level of bentonite above 5 g/kg of complete feed (European Commission, 2013a).

Besides mycotoxin detoxifiers, coccidiostats and antimicrobials can also be mixed in the feed of broiler chickens, hence, antimicrobials/coccidiostats and mycotoxin binders may be simultaneously present in the intestinal tract and the mycotoxin binders might therefore

interact with the oral absorption of antimicrobials/coccidiostats. A number of cases of such interactions have been described previously. In 1992, the Canadian Bureau of Veterinary Drugs reported a case of lack of efficacy of tylosin (TYL) in cattle fed a bentonite supplemented feed (Canadian Bureau of Veterinary Drugs, 1992). Next, a decreased efficacy of tilmicosin (TIL) against airsacculitis was demonstrated in chickens when 2% bentonite was included in the feed (Shryock et al., 1994). In 1998, Gray et al. demonstrated that bentonite reduced the growth-promoting effect of the coccidiostats monensin and salinomycin (SAL) in broilers, but only when the coccidiostats were used below the recommended doses (Gray et al., 1998). In 2010, the European Food Safety Authority (EFSA) stated that the safety of mycotoxin binders regarding non-specific binding of oral veterinary drugs needs to be investigated (EFSA FEEDAP Panel, 2010). Devreese et al. (2012) investigated the oral bioavailability and pharmacokinetics (PK) of TYL in fasted broilers after a single oral bolus administration of TYL or TYL together with bentonite (1 g/kg feed). A relative oral bioavailability of 23.3% of TYL was observed in the test group receiving TYL together with bentonite (Devreese et al., 2012). In 2015, De Mil et al. performed a similar study using doxycycline (**DOX**) and bentonite clays in fasted broilers (10 g/kg feed) and obtained similar results (De Mil et al., 2015a). This indicates that interactions between mycotoxin binders and coccidiostats and/or antimicrobials can occur, although further studies on this subject are limited. In contrast to a reduced oral absorption, elevated plasma concentrations were seen after oral bolus administration of oxytetracycline to broilers that were fed a bentonite supplemented diet (1.5 g/kg feed) for 3 weeks (Osselaere et al., 2012). Alterations in the gut wall barrier function and surface area might explain the higher absorption of oxytetracycline (Osselaere et al., 2013c). Another reason might be the scavenging of bivalent ions by the mycotoxin binders which would otherwise form non-resorbable complexes with tetracyclines. To the authors knowledge, no studies for other veterinary medicinal products and mycotoxin binders in poultry have been published. Moreover, the published PK studies exhibit important discrepancies with the field situation such as solely acute or long term exposure to the mycotoxin binder, absence of feed or use of different inclusion rates.

This study aims to describe the influence of several mycotoxin binders on the oral bioavailability and PK properties of two antibiotics, TYL and DOX and two coccidiostats, diclazuril (**DIC**) and SAL, in broiler chickens. Mycotoxin binders were mixed in the feed and fed for 2 weeks, a setup that relates better to the field situation than previously described single bolus administration of binders in fasted broilers.

2 Materials and methods

2.1 Chemicals, mycotoxin binders and reagents

Activated carbon (Norit Carbomix®, AC) was purchased from Kela Pharma (Herentals, Belgium), and three mycotoxin binders were obtained from European wholesalers. The mycotoxin binders were labelled as Clay 1, Clay 2 and Yeast 1. Clay 1 contains montmorillonite, mica and feldspars, has a d-spacing of $12.3 \cdot 10^{-10}$ m, a cation exchange capacity (CEC) of 7.0 $cmol_c/kg$, a mineral fraction of 94.8% (m:m) and relative humidity of 5.2% (m:m). Clay 2 is a mixed layered montmorillonite and quartz, it has a d-spacing of $19.1 \cdot 10^{-10}$ m, a CEC of 51.0 $cmol_c/kg$, a mineral fraction of 78.6% (m:m) and a relative humidity of 3.4% (m:m) (De Mil et al., 2015b). Yeast 1 is a modified glucomannan fraction of inactivated yeast cells.

DOX (Soludox 15%®, 150 mg doxycycline hyclate/g) was provided by Dechravet (Heusden-Zolder, Belgium), TYL (Tylan 100 Granules®, 100 mg tylosin phosphate/g) and DIC (Vecoxan® 2.5 mg diclazuril/mL oral suspension) were purchased from Elanco Animal Health (Brussels, Belgium), and SAL (Sacox 120® microGranulate, 120 mg salinomycine sodium/g) was kindly donated by Huvepharma (Antwerp, Belgium). Water, methanol (MeOH), and acetonitrile (ACN) used for the analytical experiments and preparation of stock solutions were of high-performance liquid chromatography (HPLC)-grade and purchased from Fisher Scientific (Wijnegem, Belgium).

Analytical standards of DOX, TYL, DIC, SAL and the internal standards (IS) demethylchlortetracycline (DMCTC), valnemulin (VAL), methyldiclazuril (MeDIC) and nigericine (NIG), were purchased from Sigma-Aldrich (Bornem, Belgium). Stock solutions of 10 mg/mL were made in MeOH and stored at ≤ -15 °C. They were appropriately diluted in MeOH to obtain working solutions and stored at ≤ -15 °C. Trifluoroacetic acid (TFA) and hydrochloric acid (HCl) were purchased from Sigma-Aldrich.

2.2 Experimental design

Forty 15-days-old broilers (Ross 308, as hatched) were randomly divided in 5 groups of 8 animals, namely 1 control group and 4 test groups. They were housed in concrete floor pens of 2 m² covered with wood shavings. The animals had *ad libitum* access to feed and drinking water. Temperature and relative humidity were controlled between 15-25 °C and 40-80% respectively, the light regime was 6 hours of darkness and 18 hours of light. Housing conditions were in accordance to European Directive 2010/63 regarding housing of

experimental animals (European Commission, 2010). The feed was wheat based commercially available feed, it was finely grinded and contained following ingredients: wheat (59.49%), high protein soybean meal (17.70%), corn (8.00%), vegetable oil (5.80%), soy beans (5.00%) and premixed supplements (4.01%). The feed was analyzed for mycotoxin content using a multi–mycotoxin LC-MS/MS method adapted from Monbaliu et al. (Monbaliu et al., 2009), the method included deoxynivalenol (DON) and acetylated forms (3- and 15-acetylDON), zearalenone, aflatoxins (aflatoxins B_1, B_2, G_1 and G_2), sterigmatocystin, fumonisins (B_1, B_2 and B_3), T_2-toxin, HT_2-toxin, ochratoxin A, fusarenon-X, nivalenol, diacetoxyscirpenol, neosolaniol, altenuene, alternariol, alternariol methyl ether, fumigaclavine, enniatine B1, paxilline and roquefortine-C. Four test diets were prepared by supplementation of the feed with AC, Clay 1, Clay 2 or Yeast 1 at the conventional dose of 2 kg per ton of feed (0.2%), respectively. This mixing was done manually according to geometrical mixing procedure as previously described (Earle and Earle, 1983). The control (no binder) and test diets were fed to the control and test groups, respectively, for 15 days. Next, both the control and each test group were administered 4 veterinary medicinal products consecutively, respecting a wash-out period of 2-3 days between each administration. The 4 veterinary medicinal products were dosed using a single bolus administration directly in the crop, following order was applied: DOX, TYL, DIC and SAL. The night before each administration, 10 h of darkness was applied and 1 h before administration of the bolus, lights were turned on to ensure that the animals were in a fed state before administration of the antimicrobials or coccidiostats. The animals from the test groups also received a bolus containing the daily dose of binder, this was calculated using an average of 80 g feed intake per kg bodyweight (BW) each day. The animals from the control group received tap water. Immediately after the bolus containing the binder or water, a bolus containing the daily dose of veterinary medicinal product (DOX or TYL: both 20 mg/kg BW) or coccidiostat (DIC: 80 µg/kg BW or SAL: 4.8 mg/kg BW) was given to the animals. Blood samples were taken from the leg vein (*vena metatarsalis plantaris superficialis*) at 0 h (just before administering the bolus), 1, 2, 3, 4, 6 and 8 h post administration (p.a.) for TYL and DOX. For DIC and SAL, samples were taken at 0, 0.25, 0.5, 1, 1.5, 2, 4, 6 and 8 h p.a. Plasma was collected by centrifugation and samples were stored at ≤ -15 °C until analysis. A scheme of the actions taken is presented in table 1.

The animals were euthanized after the experiment by intravenously injecting a lethal dose of sodium pentobarbital (Kela Pharma), the experiments were approved by the ethical committee

of the Faculty of Veterinary Medicine and the Faculty of Bioscience Engineering of Ghent University (approval number: EC 2014/166).

Table 1: Scheme listing the different actions taken in the experiment.

Day (and time) of the experiment	Action
0	• Randomly allocate animals to one of the five experimental groups: Control, AC, Clay 1, Clay 2, Yeast 1
0-15	• Provide feed supplemented with: nothing (control), AC, Clay 1, Clay 2 or Yeast 1, ad libitum, inclusion rate of additives: 2 g/kg feed
15 (18:00h)	• Weighing and preparing boli of DOX and binder according to bodyweight
16 (7:00h)	• Lights turned on and feed trough refilled → animals gobble on newly supplemented feed
16 (8:00h)	• Bolus of DOX and binder was given to each animal of all groups, blood samples were taken at regular time points for the next 8 h
17-19	• Recovery
19 (18:00h)	• Weighing and preparing boli of TYL and binder according to bodyweight
20 (7:00h)	• Lights turned on and feed trough refilled → animals gobble on newly supplemented feed
20 (8:00h)	• Bolus of TYL and binder was given to each animal of all groups, blood samples were taken at regular time points for the next 8 h
21-23	• Recovery
23 (18:00h)	• Weighing and preparing boli of DIC and binder according to bodyweight
24 (7:00h)	• Lights turned on and feed trough refilled → animals gobble on newly supplemented feed
24 (8:00h)	• Bolus of DIC and binder was given to each animal of all groups, blood samples were taken at regular time points for the next 8 h
25-27	• Recovery
2 (18:00h)	• Weighing and preparing boli of SAL and binder according to bodyweight
28 (7:00h)	• Lights turned on and feed trough refilled → animals gobble on newly supplemented feed
28 (8:00h)	• Bolus of SAL and binder was given to each animal of all groups, blood samples were taken at regular time points for the next 8 h
29	• Euthanasia of the animals and necropsy

2.3 Analytical methods

DOX: An aliquot of 100 µL of plasma was supplemented with 50 µL of the IS working solution of 10 µg/mL DMCTC and thoroughly vortex mixed. Next, 15 µL of TFA and 50 µL

of HPLC-grade water were added and the tube was vortex mixed. The sample was centrifuged for 10 min at 10,800 x g (Allegra X-15R centrifuge, Beckman Coulter; Suarlée, Belgium). Two hundred µL of the supernatant was transferred to a glass vial and supplemented with 800 µL of HPLC-grade water, vortex mixed and injected onto the HPLC– tandem mass spectrometry (MS/MS) system. The LC system consisted of a Waters 2695 quaternary solvent pump and autosampler (Waters, Milford, USA). The effluent was analysed by the Quattro Ultima (Waters) MS/MS. The chromatographic conditions are presented in Table 2 and the MS parameters are displayed in Table 3.

TYL: To 100 µL of plasma, 25 µL of a working solution of the IS (25 µg/mL VAL) was added. After vortex mixing, 100 µL of ACN was added and the sample was vortex mixed again before centrifugation as described above. The supernatant was filtered through a 0.2 µm Millex-GN nylon filter (Filter Service, Eupen, Belgium) into an autosampler vial for injection onto the LC-MS/MS system. The LC-MS/MS system was the same as described for DOX. The chromatographic conditions are presented in Table 2 and the MS parameters are displayed in Table 3.

DIC: An aliquot of 100 µL of plasma was spiked with 50 µL of the IS working solution (1 µg/mL MeDIC). Next, 100 µL of a 0.5% HCl solution in ACN was added followed by vortex mixing. Next, the samples were centrifuged as described above and the supernatant was filtered through a Millex-GV PVDF 0.22 µm filter (Filter service) into an autosampler vial. The UPLC system consisted of a Acquity binary solvent manager (Waters) and an Acquity sample manager (Waters). The detector was a Quattro Premier XE (Waters) tandem MS. The chromatographic conditions as presented in Table 2 and the MS parameters are displayed in Table 3.

SAL: An aliquot of 100 µL of plasma was supplemented with 25 µL of an IS working solution containing 20 µg/mL NIG. After vortex mixing, 100 µL of ACN was added and the sample was vortex mixed again before centrifugation as described above. The supernatant was filtered through a 0.2 µm Millex-GN nylon filter (Filter service) into an autosampler vial and was injected onto the UPLC-MS/MS system. The UPLC system was the same as described for DIC. The chromatographic conditions are presented in Table 2 and the MS parameters are displayed in Table 3.

Table 2: Chromatographic conditions for the analysis of doxycycline (DOX), tylosin (TYL), diclazuril (DIC) and salinomycin (SAL) in broiler plasma.

Medicinal product	Column	Inject volume (µL)	Mobile phase	Gradient	Flow rate (mL/min)
DOX	Hypersil Gold®, Thermo, 100 x 2.1 mm, 5 µm particle size; corresponding guard column	5	A: 0.1% HCOOH in H_2O B: 0.1% HCOOH in ACN	0-0.3 min: isocratic 95% A, 5% B; 0.3-3.5 min: isocratic 10% A, 90% B; 3.5-7.5 min: isocratic 70% A, 30% B; 7.5-15 min: isocratic to 95% A, 5% B.	0.45
TYL		10	A: 0.01 M CH_3COONH_4 in H_2O, pH 3.5 using glacial acetic acid. B: ACN.	0-0.5 min: isocratic 90% A, 10% B; 0.5-8 min: linear to 10% A, 90% B; 8-9.5 min: isocratic 10% A, 90% B; 9.5 to 10 min: linear to 90% A, 10% B; 10-20 min: isocratic 90% A, 10% B.	0 to 9.5 min: 0.20; 9.5 to 9.6 min: linear to 0.5; 9.6 to 16.7 min: 0.5; 16.7 to 16.8 min: linear to 0.2; 16.8 to 17 min: 0.2
DIC	Acquity UPLC BEH C18®, Waters, 50 mm x 2.1 mm, 1.7 µm particle size; corresponding guard column	5	A: ACN B: 0.1% HCOOH in H_2O	0-3 min: 5% A, 95% B, linear to 30% A, 70% B; 3-3.5 min: linear to 90% A, 10% B; 3.5-4 min: isocratic 90% A, 10% B; 4-4.1 min: linear to 5% A, 95% B; 4.1-7 min, isocratic 5% A, 95% B.	0.30
SAL		5	A: ACN B: 0.1% HCOOH in H_2O	0-0.5 min: isocratic 90% A, 10% B; 0.5-1.5 min: linear to 1% A, 99% B; 1.5-5.0 min: isocratic 1% A, 99% B; 5.0- -5.5 min: linear to 10% A, 90% B; 5.5-10 min: isocratic 90% A, 10% B.	0 to 1.5 min: 0.15; 1.5-5.0 min: 0.3; 5-10 min: 0.15

DOX: doxycycline; TYL: tylosin; DIC: diclazuril; SAL: salinomycine; ACN: acetonitrile; HCOOH: formic acid; CH_3COONH_4: ammonium acetate

Table 3: Mass spectrometric parameters for the analysis of doxycycline (DOX), tylosin (TYL), diclazuril (DIC) and salinomycin (SAL) in broiler plasma.

Compound	*m/z* transitions for quantification	ESI mode	Collision energy (eV)	Capp. Volt. (kV)	Cone volt. (V)	Cone gas flow (L/h)	Desolv. gas flow (L/h)
DOX	445.0>428.2	+	25	3.0	40	101	895
DMCTC (IS)	465.0>448.1	+	20	3.0	30	101	895
TYL	915.9>174.7	+	35	3.0	100	23	838
VAL (IS)	564.6>263.5	+	20	3.0	100	23	838
DIC	406.9>335.7	-	20	3.5	60	87	899
MeDIC (IS)	420.9>322.8	-	26	3.5	50	87	899
SAL	773.1>431.3	+	50	3.0	55	101	896
NIG (IS)	747.1>703.5	+	60	3.0	55	101	896

IS: internal standard; eV: electronvolts; V: volts

Method validation The analytical methods were validated for both coccidiostats and both antimicrobials in plasma according to a validation protocol previously described by De Baere et al. using matrix matched calibration curves (De Baere et al., 2011). All parameters met the requirements and were in compliance with the recommendations and guidelines defined in Directive 2002/657 (European Commission, 2002b) and with international criteria described in the literature (Knecht and Stork, 1974; Heitzman, 1994; U.S. Department of Health and Human Services, 2015). The results of the validation procedure are presented in Table 4.

Table 4: Validation parameters for the analytical methods for doxycycline (DOX), tylosin (TYL), salinomycin (SAL) and diclazuril (DIC) in broiler plasma.

	Linearity: Range (ng/mL) Pearson correlation Goodness of fit (%)	Limit of Detection (ng/mL)	Quality control (ng/mL)	Accuracy and precision within day mean ± SD (n=6) (ng/mL)	Accuracy and precision between day mean ± SD (n=6) (ng/mL)
DOX (De Mil et al., 2015a)	50-7500 >0.99 3.11%	10.54	50 (LOQ)	51.2 ± 2.4 (102.4±4.8%)	/
			250	243.8 ± 5.6 (97.5±2.2%)	242.2 ± 4.6 (96.9±1.8%)
			2500	2460.4 ± 68.1 (98.4±2.7%)	2518.2 ± 59.8 (100.7±2.4%)
TYL	25-1000 >0.99 3.80%	1.62	25 (LOQ)	25.1 ± 1.3 (100.4±5.2%)	/
			50	52.0 ± 3.8 (104.0±7.6%)	54.5 ± 11.43 (109.0±22.8%)
			500	498 ± 43.8 (99.6±8.8%)	511 ± 45.7 (102.2±9.1%)
DIC	5-10000 >0.99 11.75%	0.97	5 (LOQ)	3.7 ± 0.5 (74.0±10.0%)	/
			100	95.4 ± 5.7 (95.4±5.7%)	102.3 ± 6.6 (102.3±6.6%)
			2500	2367.4 ± 220.7 (94.7±8.8%)	2519.5 ± 154.8 (100.7±6.2%)
SAL	5-1000 >0.99 9.6%	0.11	5 (LOQ)	5.5 ± 0.3 (110.0±6.0%)	/
			50	54.0 ± 1.4 (108.0±2.8%)	51.6 ± 1.8 (103.2±3.6%)
			500	518.6 ± 34.3 (103.7±6.9%)	467.5 ± 29.1 (93.5±5.9%)

LOQ: limit of quantification; SD: standard deviation

2.4 Pharmacokinetic and statistical analysis

Non-compartmental PK analysis was performed using WinNonlin® version 6.3 (Phoenix Pharsight, St. Louis, USA) and following PK parameters were calculated: area under the plasma concentration-time curve from time 0 to last sampling point and to infinity ($AUC_{0\text{-}8h}$ and $AUC_{0\text{-}inf}$, respectively), maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), elimination rate constant (k_{el}), elimination half-life ($T_{1/2el}$), volume of distribution scaled for the absolute oral bioavailability (V_d/F), and clearance scaled for the absolute oral bioavailability (Cl/F). The relative oral bioavailability of DOX, TYL, DIC and SAL (relative F) of the test groups was calculated as follows:

$$relative\ F = \frac{AUC_{0-8h,+tested\ binder}}{\overline{AUC}_{0-8h,control\ group}} \times 100$$

The effect of the different mycotoxin binders (AC, Clay 1, Clay 2 and Yeast 1) on the PK parameters was assessed using a one-way analysis of variance (ANOVA) using SPSS v22 (IBM, Brussels, Belgium) with a Bonferroni-corrected LSD post-hoc test. Significance levels (p) below 0.05 were considered significant.

3 Results

The feed contained following mycotoxins: nivalenol: 140 µg/kg, deoxynivalenol: 234 µg/kg, zearalenone: 327 µg/kg. No other mycotoxins were detected above the limit of detection (LOD; as previously described (Monbaliu et al., 2009)). The plasma concentration-time profiles of DOX, TYL, DIC and SAL are presented in Figure 1.

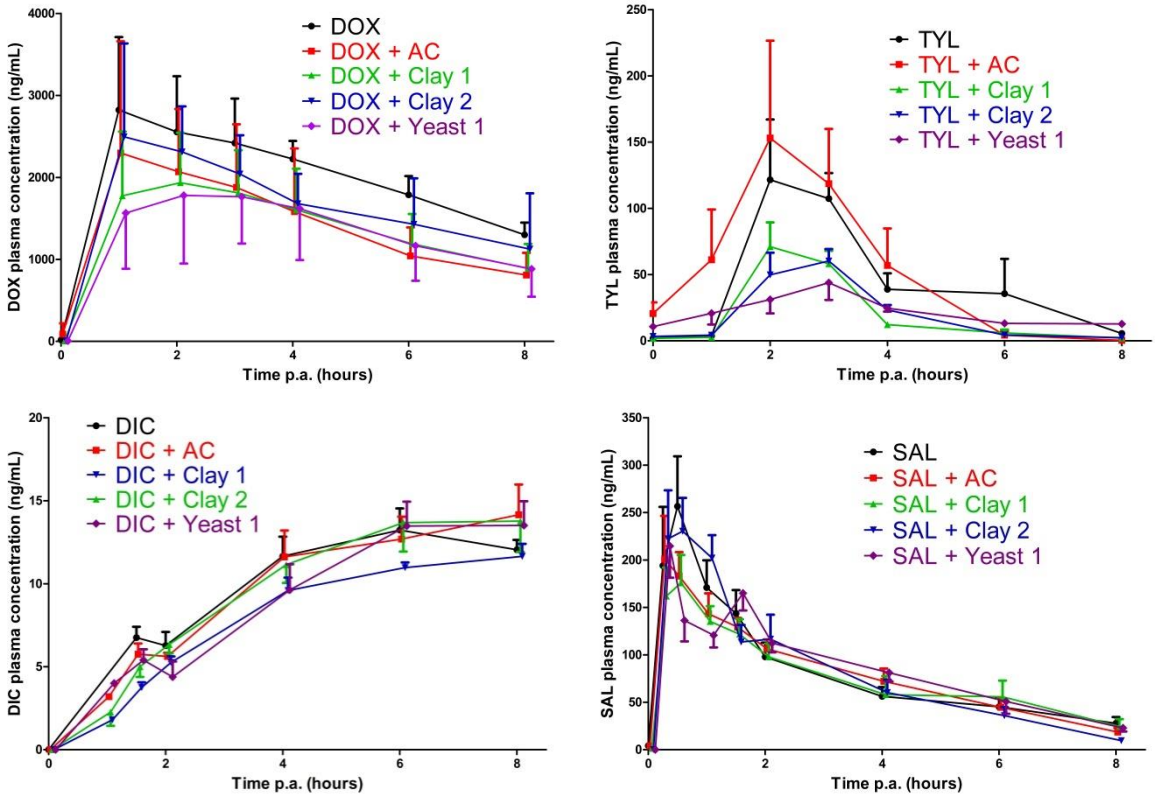

Figure 1: Plasma concentration-time profiles of doxycycline (DOX), tylosin (TYL), diclazuril (DIC) and salinomycin (SAL). Broiler chickens of the test groups were fed for two weeks with feed supplemented with 0.2% of one of the mycotoxin binders: activated carbon (AC), clay 1, clay 2 or yeast 1. The control group received no binder. Next, the control and test groups received a bolus containing water and the daily dose of mycotoxin binder, respectively, immediately followed by a bolus containing the daily dose of DOX, TYL, DIC or SAL for both the control group and test groups, at the recommended daily dose. Results are presented as mean + or - SD (n=8).

The PK parameters of all compounds are presented in Table 5. The control group of DOX exhibits a mean C_{max} and a mean $AUC_{0\text{-}inf}$ which are 45% and 70% lower respectively than previously reported for fasted broilers (De Mil et al., 2015a). The parameters k_{el} and $T_{1/2el}$ are in the same range. The $AUC_{0\text{-}inf}$ is significantly lower in the test groups compared to the control group, however discrepancy between $AUC_{0\text{-}inf}$ and $AUC_{0\text{-}8h}$ exceeded 20%, therefore

the $AUC_{0\text{-}8h}$ was preferred for interpreting bioavailability (Toutain and Bousquet-Melou, 2004). No differences in other PK parameters nor in oral bioavailability could be noted.

The control group of TYL also expressed lower plasma concentrations compared to previously reported values of TYL administered to fasted animals (Devreese et al., 2012). Although the average C_{max} and $AUC_{0\text{-}inf}$ in the Clay 1, Clay 2 and Yeast 1 group appear lower, no significant differences were demonstrated. Significant differences for $T_{1/2el}$ and V_d/F were noted for the Yeast 1 group when compared to the control group, Cl/F of the Clay 1 group differed significantly from the control group, although large interanimal variability can be seen.

The absorption of DIC was slow, reaching mean levels of 14.57 ± 3.23 ng/mL (mean ± SD, n=8) after 6 to 8 h. Only $AUC_{0\text{-}8h}$, C_{max} and T_{max} could be calculated for DIC. For the coccidiostats DIC and SAL, no significant differences could be demonstrated between the test groups and the control group.

Table 5: Pharmacokinetic parameters of doxycycline (DOX), tylosin (TYL), diclazuril (DIC) and salinomycin (SAL). Broiler chickens of the test groups were fed for two weeks with feed supplemented with one of the mycotoxin binders: activated carbon (AC), clay 1, clay 2 or yeast 1. The control group received no binder. Next, the control and test groups received a bolus containing water and the daily dose of mycotoxin binder, respectively, followed by a bolus containing the daily dose of DOX, TYL, DIC or SAL for both the control group and test groups, at the recommended daily dose. Results are presented as mean ± SD (n=8).

	Parameter	Control	AC	Clay 1	Clay 2	Yeast 1
DOX	$AUC_{0\text{-}8h}$ (h·µg/mL)	16.07 ± 2.12	11.56 ± 4.74	11.20 ± 3.41	13.35 ± 2.99	10.75 ± 4.0
	$AUC_{0\text{-}inf}$ (h·µg/mL)	28.64 ± 6.98	17.03 ± 5.71*	17.95 ± 6.02*	18.40 ± 4.87*	17.60 ± 6.42*
	Relative F (%)	100.00 ± 13.18	71.94 ± 29.52	69.71 ± 21.22	83.10 ± 18.63	66.94 ± 24.98
	T_{max} (h)	1.75 ± 1.16	1.88 ± 0.83	1.75 ± 0.89	1.75 ± 0.89	2.63 ± 0.74
	C_{max} (µg/mL)	3.07 ± 0.70	2.45 ± 1.32	2.07 ± 0.67	2.74 ± 0.89	1.95 ± 0.75
	k_{el} (1/h)	0.12 ± 0.03	0.16 ± 0.05	0.15 ± 0.04	0.18 ± 0.04	0.14 ± 0.04
	$T_{1/2el}$ (h)	9.41 ± 4.76	6.92 ± 1.82	7.33 ± 2.98	5.87 ± 1.28	7.96 ± 3.56
	Vd/F (L/kg)	6.36 ± 1.52	9.25 ± 4.34	8.90 ± 4.20	6.54 ± 1.30	11.04 ± 9.54
	CL/F (L/h/kg)	0.73 ± 0.15	1.32 ± 0.51	1.29 ± 0.62	1.15 ± 0.26	1.29 ± 0.51
TYL	$AUC_{0\text{-}8h}$ (h·ng/mL)	377.13 ± 185.57	392.34 ± 385.73	164.62 ± 78.36	161.55 ± 63.35	172.20 ± 90.46
	$AUC_{0\text{-}inf}$ (h·ng/mL)	387.55 ± 185.33	501.76 ± 402.80	168.79 ± 80.70	165.53 ± 64.16	237.62 ± 101.43
	Relative F (%)	100.00 ± 49.21	104.03 ± 102.28	43.65 ± 20.78	42.84 ± 16.80	56.31 ± 28.57
	T_{max} (h)	3.00 ± 1.41	2.50 ± 0.93	2.50 ± 0.53	2.63 ± 0.52	2.88 ± 0.83
	C_{max}(ng/mL)	155.04 ± 100.47	184.06 ± 160.77	82.59 ± 43.60	71.91 ± 43.81	50.50 ± 37.94
	k_{el} (1/h)	0.80 ± 0.42	1.15 ± 0.42	0.92 ± 0.33	0.76 ± 0.30	0.25 ± 0.04
	$T_{1/2el}$ (h)	1.71 ± 0.73	1.29 ± 1.38	1.25 ± 0.58	1.49 ± 0.55	4.05 ± 0.90*
	Vd/F (L/kg)	120.41 ± 95.01	66.34 ± 38.14	167.43 ± 68.34	191.79 ± 67.30	381.93 ± 139.92*
	CL/F (L/h/kg)	63.40 ± 30.84	71.04 ± 50.49	156.90 ± 96.26*	135.11 ± 44.87	93.14 ± 25.23
DIC	$AUC_{0\text{-}8h}$ (h·ng/mL)	75.24 ± 12.79	72.39 ± 20.99	61.49 ± 6.90	76.12 ± 22.38	68.33 ± 24.35
	Relative F (%)	100.00 ± 17.00	96.21 ± 27.90	81.73 ± 9.17	101.18 ± 29.75	90.83 ± 32.37
	T_{max} (h)	6.57 ± 1.51	7.25 ± 1.49	7.25 ± 1.49	7.00 ± 1.07	7.25 ± 1.04
	C_{max} (ng/mL)	14.57 ± 3.23	14.58 ± 4.80	11.91 ± 2.04	14.78 ± 5.63	14.20 ± 4.23
SAL	$AUC_{0\text{-}8h}$ (h·ng/mL)	694.23 ± 186.28	650.20 ± 156.37	612.51 ± 240.83	663.46 ± 191.65	717.43 ± 165.05
	$AUC_{0\text{-}inf}$ (h·ng/mL)	809.57 ± 226.39	722.52 ± 197.18	690.30 ± 277.89	692.83 ± 187.53	799.11 ± 213.93
	Relative F (%)	100.00 ± 46.61	83.97 ± 20.19	79.10 ± 31.10	85.68 ± 24.75	92.65 ± 21.31
	T_{max} (h)	0.57 ± 0.43	0.50 ± 0.42	0.46 ± 0.25	0.53 ± 0.31	0.56 ± 0.58
	C_{max} (ng/mL)	295.36 ± 138.80	246.10 ± 98.00	201.87 ± 69.71	392.96 ± 206.94	339.90 ± 318.49
	k_{el} (1/h)	0.30 ± 0.10	0.40 ± 0.167	0.37 ± 0.13	0.51 ± 0.25	0.34 ± 0.11
	$T_{1/2el}$ (h)	3.72 ± 1.75	3.05 ± 1.64	3.01 ± 1.07	2.54 ± 1.43	3.22 ± 1.11
	Vd/F (L/kg)	23.36 ± 10.17	20.47 ± 8.34	25.97 ± 18.41	21.93 ± 22.55	19.48 ± 4.62
	CL/F (L/h/kg)	6.50 ± 1.89	7.22 ± 2.26	8.42 ± 4.59	7.64 ± 3.04	6.37 ± 1.49

AUC: area under the plasma concentration-time curve from time 0 to 8h or 0 to infinity; T_{max}: time of maximum plasma concentration; C_{max}: maximum plasma concentration; k_{el}: elimination rate constant; $T_{1/2el}$: elimination half-life; V_d/F: distribution volume scaled to the absolute bioavailability; Cl/F: clearance scaled to the absolute bioavailability; * significant differences compared to the control group ($p \leq 0.05$).

4 Discussion and conclusion

In previous studies in which interactions were reported with respect to oral bioavailability and PK parameters of TYL and DOX (Devreese et al., 2012; De Mil et al., 2015a), an oral bolus model with fasted chickens was used. The conditions in those reports can be considered as a worst case scenario in which only medicinal product and binders were present in the intestinal tract after the boli were administered. Therefore, only the direct binding effect of the mycotoxin binders was evaluated without the effect of feed on this interaction. This approach is suitable when screening for interactions but shows discrepancies with the field situation regarding the duration of exposure to mycotoxin binder and the presence of feed. The setup used in this study is similar to the reported bolus studies, however, it includes the long term effects of mycotoxin binder and the presence of feed. In field conditions, the amount of feed relative to the amount of binder and medicinal product will be higher, hence this model can be considered as a more reliable approximation of field conditions for investigating the safety of mycotoxin binders.

For DOX, the C_{max} and $AUC_{0\text{-}inf}$ of the control group are lower than previously reported values in fasted chickens (Laczay et al., 2001; Ismail and El-Kattan, 2004; El-Gendi et al., 2010; De Mil et al., 2015a). This is mainly attributed to the fed status of the birds in this study, as DOX may interact with fibre rich feed or bivalent cations such as Ca^{2+} and Mg^{2+} (Davis et al., 2006). In the test groups, the relative oral bioavailability ranges from 66.9% up to 83.1%, yet the interaction is not as pronounced as reported by De Mil et al. in fasted broilers (De Mil et al., 2015a), in which relative oral bioavailabilities of 35.5% to 40.0% are reported for bentonite clays. Discrepancies with the latter study include amount of binder administered (10 g/kg feed vs. 2 g/kg feed in this study), type of mycotoxin binders nl. Clay 2 corresponds to montmorillonite 2 in a previously published paper, other mycotoxin binders differ (De Mil et al., 2015a), duration of exposure to the mycotoxin binder prior to bolus administration of DOX (0 days vs. 14 days in this study) and feed status (fasted vs. non fasted in this study). The present study more closely resembles the situation in practice, i.e. lower dose of binder, chronic exposure to the binder and presence of feed in the intestinal tract. The absence of a strong interaction between mycotoxin binders and DOX might be mainly explained by a lower dose, prandial state and other type of mycotoxin binders. The latter is partially negated by the results of an *in vitro* test reported previously (De Mil et al., 2015a), which compared the binding potential of seven mycotoxin binders to DOX in an *in vitro* experiment. DOX (100 µg/mL) was incubated for 4 h at 37 °C with 20 mg of one of following

binders in 5 mL of phosphate buffered saline solution (PBS) at pH 2.5 and 6.5. All samples expressed a binding between 80% and 100% of the added DOX. This indicates the presence of feed and the amount of binder relative to the amount of DOX are important variables for interaction. In a study in which the effect of mycotoxin binders on the oral bioavailability of DOX was studied in pigs, an interaction was seen in the groups who were fasted and received a single dose of mycotoxin binder of 10 g/kg feed. The interaction diminished when a lower dose of binder was used (2 g/kg feed). No interaction at all was seen when the animals were fed and the dose was mixed in the feed at 2 g/kg feed, this is the condition which corresponds with field conditions (De Mil et al., 2016b).

The PK values of TYL of the control group are in accordance with previously reported values in non-fasted animals (Lacoste, 2003), but were lower than reported in studies which used fasted animals (Devreese et al., 2012; Ji et al., 2014). Although a trend was noticed in the average plasma concentrations, no significantly lower relative oral bioavailabilities were demonstrated in the groups receiving Clay 1, Clay 2 or Yeast 1. For the derived parameters $T_{1/2el}$, V_d/F and Cl/F differences were noted for Clay 1 and Yeast 1, the reason for these differences remains to be elucidated although large interanimal variations were seen. The interaction between mycotoxin binders and TYL is not as pronounced as described by Devreese et al. (Devreese et al., 2012), in which a relative F of 23.3% was seen compared to a relative F of 42.8% and higher in this study. The main differences between the both studies are again the feed status of the animals (fasted and non-fasted, respectively), the type of mycotoxin binder, dose of mycotoxin binder used (1 g/kg feed and 2 g/kg feed in this study), duration of exposure to mycotoxin binder (0 days and 14 days in this study) and salt-form of TYL (tartrate and phosphate in this study). The lower plasma concentrations in the control group can be partially attributed to the use of the phosphate salt instead of the tartrate salt of TYL, which have a different oral F nl. 13.7% for the phosphate salt and 27.0% for the tatrate salt (Ji et al., 2014). Again, the main factor that can explain both the lower concentrations in the control group and the diminished interaction between the mycotoxin binders and TYL is the prandial status.

The values of the PK parameters of DIC and SAL obtained in this study are consistent with the few reports available describing the pharmacokinetics of these coccidiostats in fed birds (European commission, 1991; Henri et al., 2012; European Medicines Agency, 2013). For DIC and SAL, no differences in $AUC_{0\text{-}8h}$, relative F, T_{max} and C_{max} were seen between the different treatment groups, indicating that the mycotoxin binders have no effect on the

absorption of the coccidiostats. To date, EU (Regulation 1060/2013) prohibits the simultaneous use of the coccidiostats other than robinidine and bentonite clay when the clay is used at an inclusion rate higher or equal to 5 g/kg feed. In our study, 2 g/kg feed was included and no interactions were observed, which corresponds well with these EU regulations. For robinidine, no bentonite should be used at all (European Commission, 2013a).

It can be concluded that no significant interactions were observed between any of the mycotoxin binders and the coccidiostats, whereas a trend but no significant interactions could be noticed between some mycotoxin binders and the antimicrobials doxycycline and tylosin. Based on these results and literature, it can be concluded that both the dose as well as prandial status are important variables decisive for interaction between mycotoxin binders and oral veterinary medicinal products.

Acknowledgments

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Chapter 4: Influence of mycotoxin binders on the oral bioavailability of doxycycline in pigs

Adapted from:

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Abstract

Mycotoxin binders are feed additives that aim to adsorb mycotoxins in the gastro-intestinal tract of animals, making them unavailable for systemic absorption. The antimicrobial drug doxycycline (DOX) is often used in pigs and is administered through feed or drinking water, hence DOX can come in contact with mycotoxin binders in the gastro-intestinal tract. This paper describes the effect of four mycotoxin binders on the absorption of orally administered DOX in pigs. Two experiments were conducted, the first used a setup with bolus administration to fasted pigs at two different dosages of mycotoxin binder. In the second experiment DOX and the binders were mixed in the feed at dosages recommended by the manufacturers (=field conditions). Interactions are possible between some of the mycotoxin binders dosed at 10 g/kg feed but not at 2 g/kg feed. When applying field conditions, no influences were seen on the plasma concentrations of DOX.

Keywords: Doxycycline, mycotoxin binder, interactions, pigs, pharmacokinetics

1 Introduction

In 2009, the European Commission introduced a new category of technological feed additives titled in Regulation 386/2009: "substances for reduction of the contamination of feed by mycotoxins" (European Commission, 2009a). These 'mycotoxin detoxifiers' can be divided in two sub-categories, namely mycotoxin modifiers and mycotoxin binders. Mycotoxin modifiers aim to degrade the toxin into less toxic derivatives, whereas binders aim to adsorb mycotoxins to their surface and thereby reducing their toxicological potential (Kolosova and Stroka, 2011). The substances most frequently used as mycotoxin binder are clays and yeast derived products. The clays mainly belong to the phyllosilicates, such as montmorillonites, the main constituent of bentonites (De Mil et al., 2015b). The inclusion rate recommended by the manufacturers varies between 1 and 2.5 g/kg complete feed, the maximum amount of bentonite is described in Regulation 1060/2013 and is 20 g/kg in complete feed (European Commission, 2013a). The total amount of bentonite in compound feed can reach higher levels than 1-2.5 g/kg because of intentional/accidental overdosing, the use of bentonites as pelletizing agent or to improve the rheological properties.

In contrast to bentonite, there is no maximum set on the amount of yeast-derived mycotoxin binders as these products are usually registered as a feed ingredient. Commercially available formulations categorized as mycotoxin modifiers often also contain a substantial fraction of mycotoxin binders, i.e. a yeast derived product and/or a clay. In intensively reared livestock, administration of antimicrobials and coccidiostats is primarily done through the feed or drinking water. Non-specific interactions between mycotoxin binders and these veterinary medicinal products and/or nutrients (e.g. vitamins) should therefore be studied (EFSA FEEDAP Panel, 2010). Interactions between macrolide antibiotics (tylosin, TYL, and tilmicosin) and clay-based mycotoxin binders were previously reported in cattle and broilers (Canadian Bureau of Veterinary Drugs, 1992; Shryock et al., 1994). Also lincomycine showed an interaction in broilers, although no specifications of the anti-mycotoxin agent were presented (Amer, 2005). The efficacy of the coccidiostats monensin and salinomycin may be affected by mycotoxin binders in chicken feed (Gray et al., 1998; Nesic et al., 2003). Accordingly, Regulation 1060/2013 stated that the simultaneous oral use of bentonite with macrolides should be avoided. Moreover, for poultry, the simultaneous use with robenidine should be avoided and the use with coccidiostats other than robenidine is contraindicated at a level of bentonite above 5 g/kg complete feed (European Commission, 2013a).

Previous pharmacokinetic (PK) studies have also reported interactions. In an experiment with pigs, the effects of mycotoxins (T-2 toxin or deoxynivalenol) and a yeast derived mycotoxin binder on the oral absorption of doxycycline (DOX) and paromomycin were assessed. A significant higher area under the plasma concentration-time curve (AUC) was seen for DOX in the groups fed mycotoxin-contaminated feed supplemented with mycotoxin binder, compared to the control group receiving no mycotoxin nor binder. In contrast, no significant differences were seen in the binder group or mycotoxin exposed groups only, when compared to the control group (Goossens et al., 2012). In a study with broiler chickens, elevated plasma concentrations after bolus administration of oxytetracycline were detected after feeding a bentonite-based mycotoxin binder upgraded with a yeast for three weeks (Osselaere et al., 2012). Although the mechanisms for these observations are still unclear, this might point towards an indirect effect of the binder on the intestinal barrier. The latter was demonstrated by the observation of longer villi over the entire length of the small intestine after three weeks of feeding a clay-based binder (Osselaere et al., 2013c). Devreese et al. studied the PK properties of TYL in fasted broiler chickens, using an oral bolus model in which TYL or TYL in combination with a bentonite-based mycotoxin binder was administered. The dose of bentonite was the daily dose corresponding to an inclusion rate of 1 g/kg feed. A relative oral bioavailability of only 23% was found (Devreese et al., 2012). Recently, our group performed a similar experiment with DOX and montmorillonite-based mycotoxin binders at an inclusion rate of 10 g/kg feed in fasted broiler chickens, and a relative oral bioavailability of 40% was noted (De Mil et al., 2015a). These PK experiments included only one dosage of the mycotoxin binder and were executed in fasted animals. Although, the parameters dosage and prandial state, might be major influencing factors.

To the authors knowledge, no other studies have been performed in pigs with respect to the effects of mycotoxin binders on the absorption of orally administered veterinary drugs. Also, studies using field conditions to investigate possible interactions are lacking. Tetracycline antibiotics are frequently used in pig production, they comprised 37% and 23% of the total use of antimicrobials for food producing animals in Europe in 2012 (European Medicines Agency, 2014) and in Belgium in 2014 (Dewulf et al., 2015), respectively. In 97.7% of the cases, these drugs are mixed in the feed or drinking water (European Medicines Agency, 2014), hence, they can come in contact with mycotoxin binders before the main absorption site - i.e. small intestine - is reached. DOX is one of the most commonly used tetracycline antibiotics in pig rearing, mainly because of its higher oral bioavailability compared to other

tetracylines (Bergstrom et al., 2003). DOX is a broad spectrum, semisynthetic tetracycline used in pigs for respiratory tract infections caused by susceptible pathogens such as *Pasteurella multocida, Bordetella bronchiseptica, Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae (Pijpers et al., 1989).*

The aim of present study was to investigate the interaction between frequently used mycotoxin binders and the oral absorption of DOX in pigs, taking into account the inclusion rate of binder and prandial state of the animals.

2 Materials and methods

2.1 Chemicals, mycotoxin binders and reagents

DOX and demethylchlortetracycline (DMCTC, internal standard, IS), used for the analytical experiments, were obtained from Sigma-Aldrich (Bornem, Belgium). Water, methanol (MeOH), and acetonitrile (ACN) used for the analytical experiments and preparation of stock solutions were of high-performance liquid chromatography (HPLC)-grade and purchased from Fisher Scientific (Wijnegem, Belgium). Stock solutions of DOX and DMCTC of 10 mg/mL were made in MeOH and stored at ≤ -15 °C. They were further diluted in MeOH to obtain working solutions of appropriate concentration and were also stored at ≤ -15 °C. Formic acid (HCOOH) and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany) and were of HPLC-grade quality. Four mycotoxin binders were obtained from several European wholesalers, they are referred to as Clay 1 to 3 and Yeast 1. The physico-chemical properties of the clay-based mycotoxin binders were described previously (De Mil et al., 2015b). Clay 1 contained a mixed layered montmorillonite and quartz, it had a d-spacing of $19.1 \cdot 10^{-10}$ m, a cation exchange capacity (CEC) of 51.0 $cmol_c/kg$, a mineral fraction of 78.6% (m:m) and a relative humidity of 3.4% (m:m). Clay 2 contained montmorillonite, mica and feldspars, had a d-spacing of $12.3 \cdot 10^{-10}$ m, a CEC of 7.0 $cmol_c/kg$, a mineral fraction of 94.8% (m:m) and relative humidity of 5.2% (m:m). Clay 3 only contained montmorillonite, had a d-spacing of $12.7 \cdot 10^{-10}$ m, a CEC of 111.7 $cmol_c/kg$, a mineral fraction of 86.8% (m:m) and a relative humidity of 13.2% (m:m). Doxycycline hyclate (Doxylin® 50% WSP) used for the animal studies was supplied by Dopharma (Raamsdonkveer, The Netherlands).

2.2 Experimental design

2.2.1 Oral bolus design, fasted animals

In the bolus experiment, thirty healthy pigs (Belgian Landrace, 10 weeks old, mixed male/female, 26.1 ± 3.4 kg average BW ± SD) were randomly allocated in five treatment groups (each n=6), one control group and four test groups (Clay 1 to 3 and Yeast 1 group). The pigs were housed on a 50/50 concrete floor/grids. Temperature and air humidity were climate controlled at 25 ± 3 °C (average ± SD) and 25-60% respectively. Water was supplied *ad libitum*. The feed was a meal-based, commercially available feed (Optipro®, Aveve, Meigem, Belgium), which contained no mycotoxin binders and no clay- or yeast-based feed additives, it was given in two administrations per day. Six samples of the feed were analysed

for the presence of mycotoxins according to a multi-mycotoxin LC-MS/MS method adapted from Monbaliu et al. (Monbaliu et al., 2009). The feed contained different cereals (maize, wheat, barley, cabbage-, rapeseed- and sunflower seeds), animal fat, beet molasses and soybeans. It had a raw ashes content of 5.51% (m:m), raw protein content of 16.0% (m:m), fatty components of 5.75% (m:m) and a crude fibre content of 4.50% (m:m). After three days of acclimatisation all animals were fasted for 12 h. Next, a lubricated rubber tube was inserted into the stomach through which a single dose of one of the four mycotoxin binders (Clay 1 – 3, Yeast 1), dispersed in tap water, was administered. The control group received only tap water. Immediately after the mycotoxin binder (or water), a single dose of Doxylin®, dissolved in tap water, was administered to all groups. The tube was rinsed with 50 mL of tap water and 50 mL of air to remove remaining mycotoxin binder or Doxylin®. The dose of mycotoxin binders corresponded with the daily intake and was estimated using a 2 g/kg inclusion rate and a daily feed consumption of 1.6 kg per animal (low exposure, LE). The dose of Doxylin® was the daily dose as recommended by the manufacturer, i.e. 10 mg DOX/kg BW. Blood samples (ca. 4 mL) were collected by puncturing the jugular vein and collected in ethylenediaminetetraacetic acid (EDTA)-containing vacuum tubes (Vacutest Kima, Arzergrande, Italy). Blood samples were taken at 0 h (just before onset of the experiment), and at 1, 2, 3, 4, 6, 8 and 12 h after administration of the boli (p.a.).

After a wash-out period of three days, the experiment was repeated with two of the five groups, namely the Clay 1 and 3 group. This time, the dose of binder was calculated using an inclusion rate of 10 g/kg feed instead of 2 g/kg feed (high exposure, HE). All other procedures were the same as described above.

2.2.2 Steady state design, field conditions

Thirty other healthy pigs (Belgian Landrace, mixed male/female, 26.4 ± 4.1 kg average BW ± SD) were randomly allocated to five treatment groups: one control group and four test groups. All groups received feed of the same batch as described for the bolus experiment. For the test groups, this feed was supplemented with one of the four mycotoxin binders (Clay 1 – 3, Yeast 1) at an inclusion rate of 2 g/kg feed, as recommended by the manufacturers. The mixing was done according a three-stage mixing procedure (Earle and Earle, 1983), the first stage (up to 5 kg) was done manually, the second stage (up to 30 kg) in a construction mixer used solely for this purpose, and the third stage (up to 250 kg) in a vertical screw feed mixer. The feed of the control group contained no mycotoxin binder. This feed regime was maintained for two

weeks, and after this period all animals received medicated feed. Doxylin® was mixed in the feed of all groups according to instructions of the manufacturer. The dose was 270 mg DOX/kg feed, corresponding to a dose of 10 mg DOX/kg BW. It was mixed using a ten-stage geometrical mixing procedure (Earle and Earle, 1983). The first six stages (up to 2 kg) were done manually, the last four stages (up to 30 kg) were done in a construction mixer. The medicated feed was given in two administrations per day, at 8 am and 6 pm, for three consecutive days. Blood samples were taken daily at 10 am, 2 pm and 8 pm, corresponding to 2 h and 6 h after the first administration and 2 h after the second feed administration. The blood samples were taken as described for the bolus experiment.

These animal experiments were approved by the ethical committee of the Faculty of Veterinary Medicine and the Faculty of Bioscience Engineering of Ghent University, approval number EC 2015/09.

2.3 LC-MS/MS analysis

Plasma was collected by centrifuging the blood samples (524 × *g*, 10 min, 4 °C, New Brunswick Scientific, Rotselaar, Belgium) and stored at ≤ -15 °C until analysis. To 250 µL of plasma, 50 µL of a 3 µg/mL working solution of IS was added, next, 50 µL of HPLC-grade water was added. The samples were vortex mixed for 5 seconds, and supplemented with 25 µL of TFA, vortex mixed again for 15 seconds and centrifuged (10800 × *g*, 15 min, 4 °C, Kendro technologies, Osterode, Germany). Next, 150 µL of supernatant was mixed with 150 µL of HPLC-grade water and 5 µL was injected into the LC-MS/MS instrument.

The LC-MS/MS method was similar to the method described in a previous paper for broiler plasma analysis (De Mil et al., 2015a). The method was validated in pig plasma for a range between 25 ng/mL (= limit of quantification, LOQ) and 7500 ng/mL. Validation parameters were as following: linear calibration curves were weighted 1/x; Pearson correlation was >0.99 and goodness of fit below 10%. Within-run accuracy and precision was determined using blank samples fortified with DOX at 25, 250 and 2500 ng/mL. For each concentration level six samples were spiked, recovery rates were 23.9 ± 1.3 ng/mL, 259 ± 13.1 ng/mL and 2640 ± 86.7 ng/mL respectively. Between-run accuracy and precision was determined on a similar manner at 250 and 2500 ng/mL, for three consecutive days a total of nine samples were tested for each concentration. Recovery rates were 247 ± 14.1 ng/mL and 2580 ± 123 ng/mL. Limit of detection was based on a signal-to-noise ratio of 3/1 of six blank samples spiked with DOX at 25 ng/mL (=LOQ) and was found to be 0.96 ng/mL.

2.4 Pharmacokinetic and statistical analysis

The following PK parameters were determined for the bolus experiment for each pig by non-compartmental analysis using Phoenix WinNonlin® (St. Louis, United States of America): area under the plasma concentration-time curve from time 0 to last sampling point and to infinity ($AUC_{0\text{-}12h}$ and $AUC_{0\text{-}inf}$), maximum plasma concentration (C_{max}) and time to maximum plasma concentration (T_{max}), elimination rate constant (k_{el}), elimination half-life ($T_{1/2el}$), volume of distribution relative to the absolute oral bioavailability (V_d/F), and clearance relative to the absolute oral bioavailability (Cl/F). The relative oral bioavailability (relative F) of the test groups was calculated as follows:

$$relative\ F = \frac{AUC_{0-inf,DOX+tested\ binder}}{\overline{AUC}_{0-inf,DOX\ control\ group}} \times 100$$

For the steady state experiment, the AUC until the last sampling point ($AUC_{0\text{-}58h}$) was calculated using WinNonlin®, steady state concentration (C_{ss}) was calculated as the average of the last six plasma concentrations. Total body clearance (Cl) whether or not corrected for absolute oral bioavailability (F) was calculated by: input rate/C_{ss}. The input rate was assumed to be the daily dose per 24 h. The PK parameters of the different test groups were compared to the control group using one-way analysis of variance with a Least Significant Difference (LSD) with Bonferroni correction as post-hoc test. Significance levels (p) below 0.05 were considered significantly different.

3 Results and discussion

The results of the mycotoxin analysis of the feed samples are shown in supplementary Table 1. Deoxynivalenol (DON), 3-acetyl-DON, 15-acetyl-DON, HT-2 toxin, fumonisin B1, sterigmatocystin and zearalenone could be detected, however all samples complied with the recommended maximum levels according to the Directive 2002/32, Recommendation 2006/576 and Recommendation 2013/165 (European Commission, 2002c, 2006, 2013b). The other mycotoxins included in the screening method, aflatoxins (aflatoxins B_1, B_2, G_1 and G_2), fumonisins (B_2 and B_3), T-2 toxin, ochratoxin A, fusarenon-X, nivalenol, diacetoxyscirpenol, neosolaniol, altenuene, alternariol, alternariol methyl ether and roquefortine-C, were below the decision limit and thus also compliant with the European legislation, if available.

3.1 Oral bolus design (LE and HE)

The plasma concentration-time profiles of DOX are shown in Figure 1, the PK parameters are presented in Table 1 and 2 for LE and HE groups, respectively. In the LE groups no significant differences between the test groups and the control group were observed. In contrast, in the HE groups, both the Clay 1 and Clay 3 group displayed significantly lower plasma concentrations of DOX in comparison to the control group, as reflected by a lower values for $AUC_{0\text{-}12h}$, $AUC_{0\text{-}inf}$, relative F, T_{max} and C_{max}.

Pigs receiving the high dose of binder (~ 10 g/kg feed) expressed lower plasma concentration levels compared to pigs receiving the lower dose (~ 2 g/kg feed). All PK parameters of the test groups, except T_{max}, k_{el}, $T_{1/2el}$ and V_d/F for Clay 1 HE were significantly different from those of the control group. Differences in oral bioavailability might explain the differences seen in V_d/F and Cl/F, although this cannot be calculated with the available information. The relative F-values in the high exposure groups were about 20%, indicating the systemic exposure to DOX is significantly lower in the high exposure groups. These results are consistent with the previously reported interactions between DOX and mycotoxin binders in broiler chickens (De Mil et al., 2015a), where a relative F of ≤ 40% was seen in fasted chickens when a binder at 10 g/kg feed was administered together with DOX. The groups that received the lower dose of mycotoxin binder showed no significant difference compared to the control group. The control group expressed a mean C_{max} of 2.01 µg/mL and a mean $AUC_{0\text{-}inf}$ of 10.56 h·µg/mL, which is similar to previously reported results (Baert et al., 2000). The latter authors reported a C_{max} of 1.52 µg/mL and an AUC of 13.79 h·µg/mL after oral

administration of 10.5 mg/kg BW to fasted piglets. A second maximum in plasma concentration was present at about 4 h p.a. and might be due to enterohepatic recycling, a phenomenon previously described for DOX in pigs (Riond and Riviere, 1990).

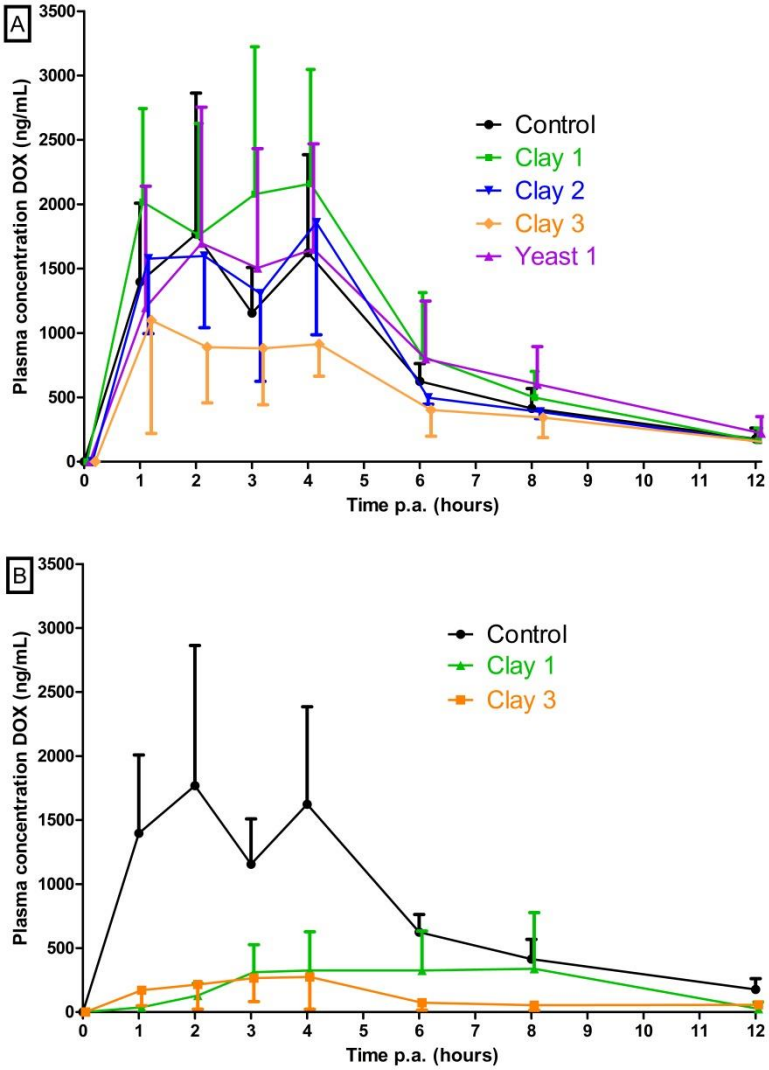

Figure 1: Plasma concentration-time profiles of doxycycline (DOX) in pigs. A: each test group was fasted and was given a bolus with mycotoxin binder at a dose corresponding to an inclusion rate of 2 g/kg feed, the control group received water. Immediately thereafter, all groups received a bolus containing Doxylin® (10 mg DOX/kg bodyweight). B: Same as A but using an inclusion rate of binder of 10 g/kg feed. The results are presented as the mean + or - SD (n = 6).

Table 1: Main Pharmacokinetic Parameters Of Doxycycline (DOX) For Fasted Pigs Following Bolus Administration of Doxylin® 10 mg DOX/kg BW And Mycotoxin Binder using an Inclusion Rate Of 2 g/kg Feed (low exposure, LE). Results Are Presented As Mean ± SD (n = 6).

Treatment group	Control	Clay 1 LE	Clay 2 LE	Clay 3 LE	Yeast 1 LE
AUC_{0-12h} (h·µg/mL)[a]	9.60 ± 3.49	12.29 ± 4.57	9.73 ± 2.35	6.37 ± 2.63	10.72 ± 5.59
AUC_{0-inf} (h·µg/mL)[a]	10.56 ± 3.97	12.90 ± 4.84	10.43 ± 2.42	7.54 ± 2.67	11.78 ± 6.04
Relative F (%)	100.00 ± 37.61	122.31 ± 45.87	98.91 ± 22.95	71.50 ± 25.34	111.69 ± 57.28
T_{max} (h)[b]	2.00 ± 1.10	2.83 ± 1.47	2.50 ± 1.64	2.17 ± 1.47	2.50 ± 1.22
C_{max} (µg/mL)[c]	2.01 ± 0.91	2.46 ± 0.90	2.12 ± 0.77	1.31 ± 0.75	1.93 ± 1.06
k_{el} (1/h)[d]	0.23 ± 0.08	0.29 ± 0.05	0.22 ± 0.04	0.21 ± 0.13	0.23 ± 0.06
$T_{1/2el}$ (h)[e]	4.83 ± 1.74	3.57 ± 0.52	4.69 ± 0.87	6.59 ± 3.95	4.60 ± 1.11
Vd/F (L/kg)[f]	4.77 ± 1.35	3.07 ± 1.12	4.66 ± 1.14	9.26 ± 5.81	5.36 ± 3.92
Cl/F (L/h/kg)[g]	1.09 ± 0.46	0.88 ± 0.33	1.00 ± 0.23	1.45 ± 0.43	1.15 ± 0.76

[a] AUC: area under the plasma concentration-time curve from time 0 to 12h or 0 to infinity; [b] T_{max}: time of maximum plasma concentration; [c] C_{max}: maximum plasma concentration; [d] k_{el}: elimination rate constant; [e] $T_{1/2}$: elimination half-life; [f] V_d/F: distribution volume relative to the absolute bioavailability; [g] Cl/F: clearance relative to the absolute bioavailability.

Table 2: Main Pharmacokinetic Parameters Of Doxycycline (DOX) For Fasted Pigs Following Bolus Administration of Doxylin® 10 mg DOX/kg BW And Mycotoxin Binder using an Inclusion Rate Of 10 g/kg Feed (high exposure, HE). Results Are Presented As Mean ± SD (n = 6).

Treatment group	Control	Clay 1 HE	Clay 3 HE
AUC_{0-12h} (h·µg/mL)[a]	9.60 ± 3.49	2.26 ± 2.16 *[h]	1.49 ± 0.94 †[h]
AUC_{0-inf} (h·µg/mL)[a]	10.56 ± 3.97	2.28 ± 2.55 *[h]	2.07 ± 1.02 *[h]
Relative F (%)	100.00 ± 37.61	21.62 ± 24.14 *[h]	19.67 ± 9.64 *[h]
T_{max} (h)[b]	2.00 ± 1.10	4.50 ± 2.07	4.50 ± 3.83
C_{max} (µg/mL)[c]	2.01 ± 0.91	0.45 ± 0.40 *[h]	0.32 ± 0.21 *[h]
k_{el} (1/h)[d]	0.23 ± 0.08	0.33 ± 0.16	0.15 ± 0.06
$T_{1/2el}$ (h)[e]	4.83 ± 1.74	3.44 ± 1.19	7.44 ± 2.44
Vd/F (L/kg)[f]	4.77 ± 1.35	26.77 ± 18.28	45.46 ± 32.81 ‡[h]
Cl/F (L/h/kg)[g]	1.09 ± 0.46	8.16 ± 4.80 ‡[h]	6.11 ± 3.51 ‡[h]

[a] AUC: area under the plasma concentration-time curve from time 0 to 12h or 0 to infinity; [b] T_{max}: time of maximum plasma concentration; [c] C_{max}: maximum plasma concentration; [d] k_{el}: elimination rate constant; [e] $T_{1/2}$: elimination half-life; [f] V_d/F: distribution volume relative to the absolute bioavailability; [g] Cl/F: clearance relative to the absolute bioavailability; [h] *, † and ‡ indicate significant differences compared to the control group, respectively at the 0.05, 0.01 and 0.001 p-level.

3.2 Steady state design, field conditions

The plasma concentration-time profiles of the steady state experiment are shown in Figure 2. The values for the main PK parameters are listed in Table 3. The steady state was reached after about 12 h. Following, C_{ss} reached values between 100 and 175 ng/mL. No significant differences between the test- and the control group were observed.

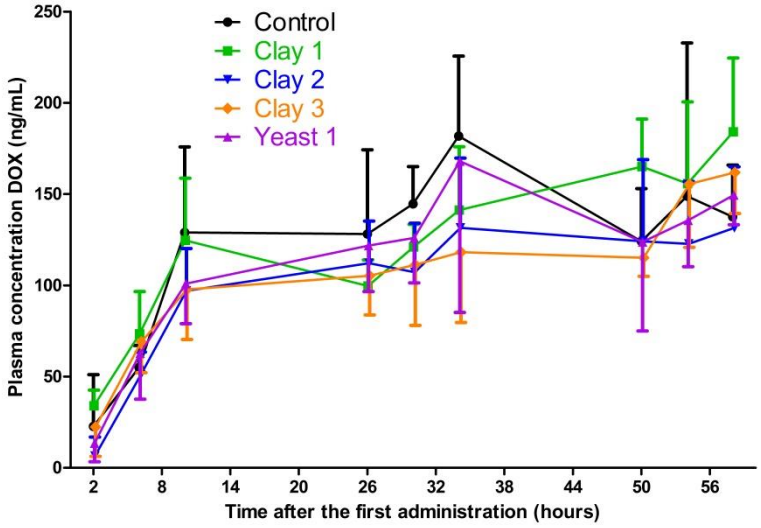

Figure 2: Plasma concentration-time profile of doxycycline (DOX) in pigs during continuous administration of Doxylin® in the feed at the recommended dose of 270 mg/kg feed, corresponding to a daily dose of 10 mg DOX/kg bodyweight. Mycotoxin binders were mixed in the feed of the test groups using an inclusion rate of 2 g/kg feed. Results are presented as mean + or - SD (n=6).

Table 3: Plasma concentration of doxycycline (DOX) at steady state (C_{ss}), the area under the plasma concentration-time curve (AUC_{0-58h}) and total body clearance (Cl) whether or not corrected for absolute oral bioavailability (F) in pigs during continuous administration of Doxylin® in the feed at 270 mg/kg feed, corresponding to a daily dose of 10 mg DOX/kg bodyweight. Mycotoxin binders were mixed in the feed of the test groups using an inclusion rate of 2 g/kg feed. Results are presented as mean ± SD (n=6).

Treatment	C_{ss} (ng/mL)	AUC_{0-58h} (h·µg/mL)	Cl/F (L/24h/kg BW)	Cl (F=21.2%)* (L/24h/kg BW)
Control	143.02 ± 29.48	7.34 ± 1.35	72.60 ± 15.84	3.36 ± 1.37
Clay 1	144.46 ± 24.32	7.18 ± 1.22	71.03 ± 12.88	2.73 ± 1.11
Clay 2	121.50 ± 28.53	6.05 ± 1.28	85.51 ± 16.68	3.54 ± 1.44
Clay 3	127.86 ± 15.89	6.10 ± 1.04	79.22 ± 9.74	2.06 ± 0.84
Yeast 1	137.23 ± 27.37	6.76 ± 1.60	74.99 ± 12.86	2.73 ± 1.11

C_{ss}: concentration at steady state; AUC_{0-58h}: area under the plasma concentration-time curve; Cl: Clearance. No significant differences were observed between the test groups and the control group.
* F was adopted from Baert et al. (2000).

The recommended inclusion rate for bentonite-based mycotoxin binders is in the range of 1 and 2.5 g/kg complete feed. However, higher levels of bentonites in feed might be reached due to the simultaneous use of bentonites for other purposes such as pelletizing agent or to improve rheological properties. The maximum level of bentonite is set in Regulation 1060/2013 at 20 g/kg feed (European Commission, 2013a). No differences were seen in the DOX plasma concentrations between the test groups and the control group, indicating that adding mycotoxin binder to the feed at an inclusion rate of 2 g/kg feed has no influence on the oral bioavailability of DOX. Although, the steady state concentrations were lower than previously reported values, where a steady state concentration of 1.21 µg/mL (Bousquet et al., 1998) and 1.06 µg/mL (Pijpers et al., 1991) was measured at 12 h after start of administration of comparable doses of DOX in the feed. The discrepancy might be attributed to differences in breed, bodyweight, type of feed, spillage, light cycles, formulation of the feed or the commercial formulation of DOX.

3.2 General

Bolus studies in fasted pigs of the HE groups indicate that an interaction is possible between different clay-based mycotoxin binders and DOX. Studies in fasted broiler chickens using a similar setup also indicated a potential risk when using mycotoxin binders in combination with DOX (De Mil et al., 2015a). The current study indicates that the results obtained in fasted animals should be nuanced. The interaction depends upon the inclusion rate of mycotoxin binder and the presence of feed in the gastro-intestinal tract, an important factor not present in the bolus experiments using fasted pigs in this study and in the cited study with DOX in broilers. A lower oral bioavailability of DOX was seen when a clay-based mycotoxin binder, in a dose corresponding to 10 g/kg feed, was co-administered with DOX in fasted pigs. When applying field conditions, using an inclusion rate of mycotoxin binders of 2 g/kg feed and a DOX dose of 270 mg/kg in feed, no influence was seen on the plasma concentrations of DOX. This indicates the use of DOX is safe when the feed is supplemented with mycotoxin binders at the conditions described above. When using higher inclusion rates of DOX and/or binders, either accidental (inhomogeneous mixing) or deliberate, interactions cannot be excluded.

Acknowledgments

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Supplementary Table 1: Concentration of mycotoxins in the feed, results are presented as mean ± SD (n=6)

Mycotoxin	Concen-tration (µg/kg)	Decision limit (CCα) (µg/kg)	Detection capability (CCβ) (µg/kg)	Maximum level for pig feed (µg/kg)[a]
Aflatoxin G_1	< CCα	1.93	4.19	N.a.
Aflatoxin G_2	< CCα	2.39	4.79	N.a.
Aflatoxin B_1	< CCα	6.75	8.08	20[b]
Aflatoxin B_2	< CCα	1.53	2.57	N.a.
Ochratoxin A	< CCα	3.44	8.93	50
Sterigmatocystin	5.5 ± 2.0	4.75	8.99	N.a.
Fumonisin B_1	33.1 ± 3.9	31.84	64.34	Σ 5000
Fumonisin B_2	< CCα	24.37	54.69	
Fumonisin B_3	< CCα	23.18	42.67	N.a.
T_2-toxin	< CCα	9.38	19.47	Σ 250
HT_2-toxin	11 ± 4	9.23	19.58	
Diacetoxyscirpenol	< CCα	0.67	1.36	N.a.
Neosolaniol	< CCα	8.60	17.53	N.a.
Nivalenol	< CCα	36.22	71.41	N.a.
Deoxynivalenol (DON)	554 ± 160	60.61	128.29	900
3-ADON	16 ± 6	4.90	9.80	N.a.
15-ADON	53 ± 20	3.07	5.29	N.a.
Fusarenon-X	< CCα	16.58	34.62	N.a.
Zearalenone	156 ± 50	17.85	35.99	250 or 100[c]
Roquefortine C	< CCα	1.08	2.45	N.a.
Alternariol	< CCα	11.98	23.23	N.a.
Alternariol methylether	< CCα	17.75	39.00	N.a.
Altenuene	< CCα	4.54	8.89	N.a.

[a] Guidance for maximum level is provided by the European Commission: Directive 2002/32/EC, Recommendation 2006/576/EC and Recommendation 2013/165/EU; [b] Not applicable for young animals; [c] 250 µg/kg for sows and fattening pigs, 100 µg/kg for piglets and gilts; N.a. Not available.

Chapter 5: *In vitro* model to assess the adsorption of oral veterinary drugs to mycotoxin binders in a feed-containing buffered matrix

Adapted from:

Thomas De Mil, Mathias Devreese, Patrick De Backer and Siska Croubels (2016a). *In vitro* model to assess the adsorption of oral veterinary drugs to mycotoxin binders in a feed-containing buffered matrix. Submitted to Animal Feed Science and Technology.

Abstract

Mycotoxin binders are feed additives which are mixed in the feed to adsorb mycotoxins and thereby reducing their toxic effects on animals. Interactions with orally administered veterinary medicinal products, such as antimicrobials or coccidiostats, have been reported previously. This paper describes an *in vitro* model to screen for interactions between mycotoxin binders and veterinary drugs with respect to the non-specific binding of drugs. It is designed as a static setup using single concentration of drug and binder in a feed-containing matrix, buffered at different pHs. The model was applied to two frequently used antimicrobials in veterinary medicine, doxycycline (DOX) and tylosin (TYL) and four mycotoxin binders. Proportions of feed, DOX or TYL and binder are equivalent to the *in vivo* situation for broiler chickens, and pH and volume of the buffer are representative for the gastrointestinal tract of chickens as well. Similar results were obtained for DOX and TYL, more specifically up to an inclusion rate of 20 g binder/kg feed, no significant binding was demonstrated, determined as the free concentration of DOX and TYL. One exception was noticed for TYL and a bentonite based mycotoxin binder, for which no significant interaction could be demonstrated up to 10 g/kg instead of 20 g/kg.

Keywords: Mycotoxin binder, doxycycline, tylosin, adsorption, *in vitro,* safety assessment

1 Introduction

Mycotoxin binders are feed additives which are often mixed in feed to counter the harmful effects of mycotoxins by adsorbing the toxin to their surface and thereby reducing their toxicological potential (European Commission, 2009a). The mechanism was described in detail for the binding of aflatoxin B1 (AFB1) to montmorillonite (Deng et al., 2010). To date, bentonite, a clay which contains mainly montmorillonite, is the only substance registered in the EU to bind AFB1 (European Commission, 2015). Bentonites are usually mixed in the feed at a concentration of 1 to 2.5 g/kg, but levels up to 20 g/kg are allowed in complete feed for ruminants, poultry and pigs to bind AFB1 (European Commission, 2013a). Besides binding of AFB1, bentonites are also used for other purposes such as pelletizing agent or to improve the rheological properties of bulk feed (anticaking agent), hence levels higher than 2.5 g/kg can be reached. The total amount of bentonite allowed in feed for these other purposes is 20 g/kg as well (European Commission, 2013a). For yeast-derived mycotoxin binders no maximum level is provided by European legislation because most yeast-based mycotoxin binders are registered as a feed ingredient. Concerns about non-specific binding of other feed compounds such as vitamins or medicinal products, and thus the safety of binders, were expressed by the European Commission in 2009 (EFSA, 2009). The binding of mycotoxins is indeed deemed to be non-specific and different *in vivo* interactions between mycotoxin binders and oral veterinary medicinal products have been described (Shryock et al., 1994; Devreese et al., 2012; De Mil et al., 2015a). Therefore, the European Commission provided to indicate in the instructions for use of bentonites that the simultaneous oral use with macrolides shall be avoided in any animal species, and that in poultry the simultaneous use with robenidine should be avoided and the simultaneous use with coccidiostats other than robenidine is contraindicated with a level of bentonite above 5 g/kg of complete feed (European Commission, 2013a).

In vitro models to assess the efficacy of mycotoxin binders to adsorb mycotoxins include static and dynamic models. Dynamic models are usually composed of different compartments and several parameters can be altered during the experiment, thereby mimicking different segments of the gastro-intestinal (GI) tract (Avantaggiato et al., 2003; Blanquet et al., 2004). These models are rather time consuming and require many resources, hence, they are not suitable for screening experiments. Static models are more cost and labour efficient and thereby more suited for screening experiments, however, they are less representative for the

GI-tract and the results should be interpreted carefully. Static models include adsorption-isotherm (Ramos et al., 1996) or single-concentration studies (Sabater-Vilar et al., 2007; Vekiru et al., 2007; Devreese et al., 2013b). The European Commission stated that for the authorisation of a bentonite as a mycotoxin binder, efficacy testing needs to be carried out using a static adsorption test in a buffered matrix of pH 5.0 with a concentration of 4 mg/L for AFB1, and 0.02 % (w/v) for the feed additive (European Commission, 2013a).

In vitro models to assess the safety with respect to binding potential of medicinal products are very scarce and they are usually derived from models to assess efficacy (Devreese et al., 2013b; De Mil et al., 2015a). Furthermore, these models do not include feed, although feed is always present in the field situation and can be a major factor influencing the bioavailability of a drug (Marasanapalle et al., 2011). Indeed, some drugs may have a lower oral bioavailability when administered with feed as the drug can undergo non-specific binding with feed compounds. Poorly soluble drugs, i.e. drugs classified as a type II drug according to the Biopharmaceutics Classification System (BCS), express a higher bioavailability when administered with food.

To the authors knowledge, no static models with feed are available to assess interactions between mycotoxin binders and veterinary drugs with respect to the non-specific binding of drugs. Doxycycline (DOX) and tylosin (TYL) are frequently used antimicrobials to treat pulmonary and gastro-intestinal infections in poultry and swine and are therefore used in the present setup. The aim of this study was to develop a static adsorption model to assess the binding of veterinary drugs, with DOX and TYL as model compounds, to various mycotoxin binders in a buffered matrix with relevant pH ranges and containing feed.

2 Materials and methods

2.1 Mycotoxin binders and veterinary drugs

The mycotoxin binders were purchased from Sigma-Aldrich (Bornem, Belgium) or were obtained from European wholesalers. The mycotoxin binders were labelled as Clay 1, Clay 2, Clay 3 and Yeast 1. Physico-chemical characterisation of the clays is described in a previous report, and clay sample numbers 27, 6 and 24 were included in this study (De Mil et al., 2015b). Yeast 1 is a modified glucomannan fraction of inactivated yeast cells. The feed was wheat based, commercially available finely grinded feed for broiler chickens (Aveve, Meigem, Belgium), it contained following ingredients: wheat (59.49%), high protein soybean meal (17.70%), corn (8.00%), vegetable oil (5.80%), soy beans (5.00%) and premixed supplements (4.01%), no mycotoxin binders or clay-based feed additives were present in the feed. Analysis according to a previously validated method (Monbaliu et al., 2010) indicated the feed contained following mycotoxins: nivalenol: 140 µg/kg, deoxynivalenol: 234 µg/kg, zearalenone: 327 µg/kg. No other mycotoxins were detected above the limit of detection, hence the feed is compliant to European Directives (European Commission, 2002c), Recommendations (European Commission, 2006) and indicative levels (European Commission, 2013b) for poultry. Reference standards of DOX, TYL, demethylchlortetracycline (DMCTC) and valnemulin (VAL) were purchased from Sigma-Aldrich.

2.2 Experimental setup

Ten gram of feed was supplemented with one of the four mycotoxin binders to a concentration of 0, 1.0, 2.5, 5.0, 10, 20, 50 or 100 g/kg feed. Next, 19 mL of phosphate buffered salt (PBS) solution and 1 mL of working solution of DOX or TYL were added. To obtain the PBS, a salt solution containing 8.0 g/L NaCl (VWR, Leuven, Belgium), 0.1 g/L $MgCl_2 \cdot 6H_2O$ (Sigma-Aldrich), 0.2 g/L KCl (Sigma-Aldrich) and 0.13 g/L $CaCl_2 \cdot 2H_2O$ (VWR) was supplemented with a phosphate buffer system to the pH's of 2.5, 6.5 and 8.0 to achieve a total osmolarity of 9.6 mmol/L (De Mil et al., 2015a).

The working solution of DOX was prepared by mixing Doxylin 50% WSP® (Dopharma, Raamsdonkveer, The Netherlands) with HPLC-grade water (Thermo Fisher Scientific, Wijnegem, Belgium) to a concentration of 5 mg/mL, this concentration was calculated using a daily feed intake of 80 g/kg bodyweight (BW)/day and a DOX dose of 20 mg/kg BW/day. For

the working solution of TYL, Tylan Soluble 100%® (Elanco Animal Health, Brussels, Belgium) was mixed with HPLC-grade water to a concentration of 3 mg/mL, which corresponds to a dose of 24 mg/kg BW/day when 80 g feed/kg BW/day is consumed.

The volume of 20 mL (PBS + working solution DOX/TYL) relative to the amount of feed (10 g) corresponds to the water:feed ratio applicable for broilers of 2:1 (Pesti et al., 1985). The model includes three different acidity levels of PBS, namely pH 2.5, 6.5 and 8.0, which comprise the range of acidity levels in the GI-tract of mammals and poultry in general (Maresca, 2013).

Next, the tubes which contained the feed, buffer and TYL or DOX, were horizontally shaken (150 rpm) for 4 h at 37 °C (New Brunswick Scientific, Rotselaar, Belgium). The pH of the samples was checked with litmus paper to verify no major changes in Ph occurred during the experiment. The samples were centrifuged (3,724 × g, 15 min, 25 °C) and 250 µL of the supernatant was transferred to an Eppendorf cup. Three replicates were analysed per inclusion rate, per pH, per mycotoxin binder, and per antimicrobial.

2.3 Analysis of DOX and TYL

For DOX, 50 µL of a 100 µg/mL working solution of the internal standard, DMCTC in high-performance liquid chromatography (HPLC)-grade methanol (MeOH, Thermo Fisher Scientific), was added followed by 50 µL of MeOH. After vortex mixing, 25 µL of trifluoroacetic acid (Sigma-Aldrich) was added and the sample was thoroughly vortex mixed again for 30 sec. The sample was centrifuged at 10,800 × g for 15 min at 4 °C and 18 µL of the supernatant was supplemented with 282 µL of HPLC-grade water prior to analysis. The LC-MS/MS analysis was executed as previously described (De Mil et al., 2015a).

The samples for TYL were supplemented with 50 µL of a 250 µg/mL working solution of the internal standard, VAL in HPLC-grade acetonitrile (ACN, Filter Service, Eupen, Belgium). Next, 950 µL of ACN was added, the sample was thoroughly vortexed for 30 seconds and centrifuged at 10,800 × g for 15 min at 4 °C. Next, 25 µL of the supernatant was supplemented with 275 µL of an aqueous 0.01 M ammonium acetate buffer (Sigma-Aldrich). The HPLC equipment consisted of a Waters 2690 pump and autosampler (Waters, Milford, USA), chromatographic separation was achieved on a Purospher RP-18 LiChroCART column and corresponding guard column (Merck, Darmstadt, Germany). Mobile phase A was ACN supplemented with 0.01% HPLC-grade formic acid (Merck), mobile phase B was water

supplemented with 0.01% formic acid. The gradient was isocratic 50:50 (A:B, v/v), and the flow rate was set at 0.6 mL/min. The HPLC effluent was interfaced to a Quattro Ultima tandem mass spectrometer (Waters, Manchester, United Kingdom). For TYL, following reaction was monitored for quantification: *m/z* 915.8>174.2 and a collision energy of 34 eV and cone voltage of 35 V were applied. The analysis was adapted from (Devreese et al., 2012) and validated as described by De Baere et al. (2011). For VAL, the monitored reaction was *m/z* 564.2>263.1, a collision energy of 23 eV and a cone voltage of 30 V were applied. The method was validated for a range between 1 and 150 µg/mL according to a matrix-matched validation protocol described by De Baere et al. (2011), which is compliant with European guidelines (European Commission, 2002b) and international guidelines (U.S. Department of Health and Human Services, 2015).

2.4 Statistical analysis

Each inclusion rate was analysed in triplicate per mycotoxin binder; the results were compared to the respective control (no binder) using one-way analysis of variance (ANOVA) followed by a post hoc, Bonferroni-corrected, LSD-test. Significance levels (p) below 0.05 were considered significant.

3 Results

The results are presented in figure 1. For DOX, no significant difference could be demonstrated with clay binders up to an inclusion rate of 20 g/kg. The results obtained with the yeast derived mycotoxin binder did not differ from the control samples throughout the entire inclusion range. For DOX, the experiment could not be executed at pH 8.0 because of a high variability in the results, which might be caused by a difference in keto-enol tautomerism at higher pH-levels (Weng et al., 1993).

For TYL, similar results were obtained, namely no significant interaction throughout the entire inclusion rates tested for the yeast derived binder and for the clay binders up to an inclusion rate of 20 g/kg feed. Except for Clay 3, for which the lowest concentration for which no significant interaction could be demonstrated is 10 g/kg. The experiment could not be executed for pH 2.5 because TYL is not stable below a pH of 4 (Paesen et al., 1995).

Although, *in vivo* trials with fasted broiler chickens already demonstrated interactions for TYL and DOX with clay based binders at 2 g/kg and 10 g/kg inclusion rate, respectively (Devreese et al., 2012; De Mil et al., 2015a). On the other hand, when performing *in vivo* trials in fed broiler chickens, no interactions between TYL and the clay based binders were observed after oral administration of TYL and 2 g/kg clay based binder (De Mil et al., 2016c), which corresponds with the *in vitro* results obtained in this study. Therefore, both prandial status of the animals and inclusion rate of the binders used must be taken into account in both *in vitro* and *in vivo* studies. European legislation discourages the simultaneous use of macrolides and bentonite clays (European Commission, 2013a), which also corresponds with our findings for TYL.

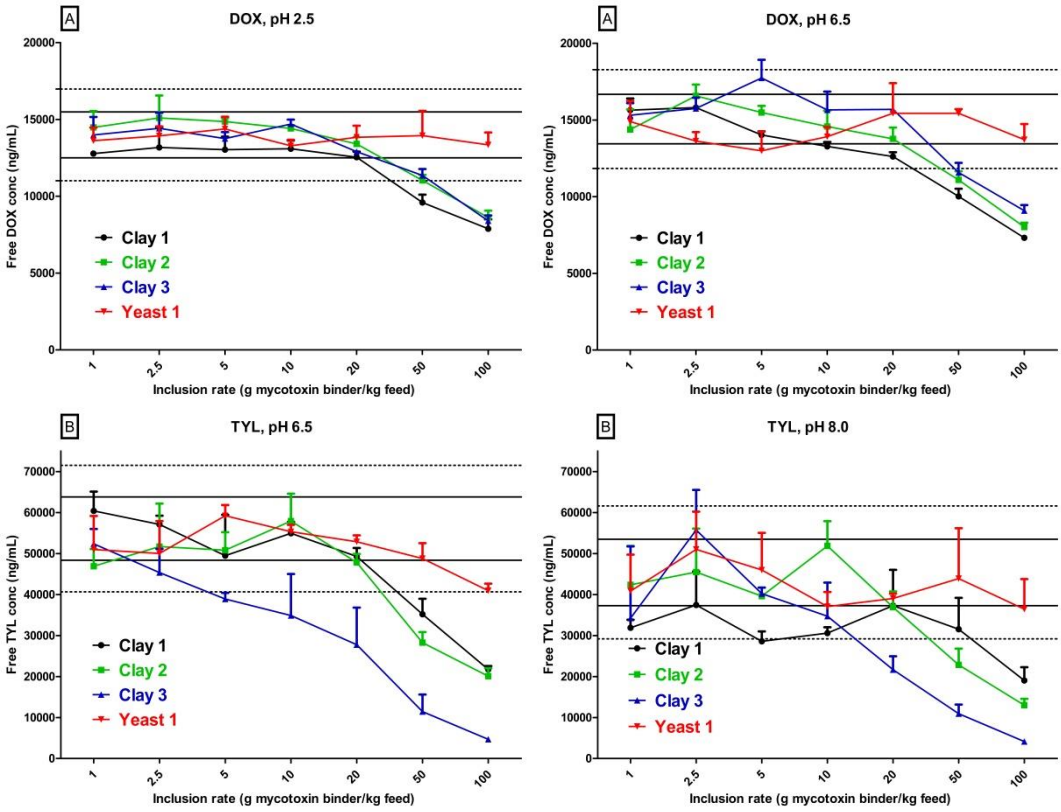

Figure 1: Free doxycycline (DOX) (A) and tylosin (TYL) (B) concentrations for different inclusion rates of the respective mycotoxin binder at two different pH's (presented as mean + SD). The samples (n = 3 per inclusion rate per mycotoxin binder) were incubated for 4 h in 20 mL of phosphate buffered saline solution containing 10 g of finely grinded feed supplemented with mycotoxin binder at different inclusion rates. The horizontal lines represent the average free concentration ± 1 SD (full line) or ± 2 SD (dotted line) of DOX or TYL in the control samples without mycotoxin binder (n = 12).

4 Discussion and conclusions

This model approximates the *in vivo* situation better than available static single concentration models because of the inclusion of feed. Standardized protocols for *in vitro* models that establish fixed values for variables such as buffer choice, duration of the experiment, total volumes used, …, are urgently needed to enable comparison and evaluation between results of different experiments. This is already the case for the efficacy testing protocol for the binding AFB1 by bentonites (European Commission, 2013a), but not for safety testing with regard to non-specific interactions. If the analytical method is adjusted properly, this model can also be used for screening the interaction with other medicinal products, vitamins or mycotoxins. Further refinements may include the use of (simulated) gastric juices to mimic the *in vivo* situations. The model has now been developed using pH values representative for the GIT of poultry and with minor adjustments this model can also be appropriate for monogastrica. In conclusion, the model presented here may be well suited to evaluate and screen other mycotoxin binders for interactions with oral veterinary drugs if the analytical method is adjusted accordingly.

Acknowledgments

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GENERAL DISCUSSION

Mycotoxins are frequent feed contaminants which can impair animal health and performance. Both pre- and post-harvest methods are applied to reduce the deleterious effects caused by mycotoxins. One of the most commonly used methods is the use of mycotoxin binders. These feed additives claim to bind mycotoxins in the gastro-intestinal tract and consequently remove them from the animal along with the faeces and thereby reducing their toxicological effects. The binding of mycotoxin binders is deemed to be non-specific, a number of literature studies report the binding of veterinary medicinal products to these mycotoxin binders when administered orally (mixing in the feed or drinking water). To date, the use of mycotoxin binders is prohibited when used concomitantly with macrolide antibiotics in all species. Its use is restricted when used in poultry with robenidine, a non-ionophoric coccidiostat. Information for other categories of veterinary medicinal products is largely lacking, and the models used in the available reports, are not always suitable for their intended purpose.

The aim of this thesis was to investigate the safety of mycotoxin binders regarding the possibility of non-specific binding of veterinary medicinal products. Therefore, suitable *in vitro* and *in vivo* models to screen for interactions between veterinary medicinal products and mycotoxin binders were developed and applied.

Although promising attemts are made (e.g. Physiologically Based Pharmacokinetic modelling), the pharmacokinetics of a veterinary drug cannot be accurately predicted without *in vivo* experiments. This is because of the complexity of the target organism and pharmacokinetic processes, hence no model can yield completely reliable predictions regarding the interactions between mycotoxin binders and veterinary drugs. Final confirmation of risk assessment should be carried out in the target species whilst applying field conditions. Aberrations from field conditions result in less reliable predictions, but are sometimes inevitable. In this case, there are too many possible combinations of veterinary drugs and mycotoxin binders to test in animals. The main reasons are ethical, practical and financial. Therefore, binder-drug combinations are funnelled through different stages, each stage reducing the number of combinations to be tested. A first selection is made by selecting the veterinary medicinal products to be tested and collecting representative samples of mycotoxin binders of which also a selection is made. Next, combinations are tested *in vitro* and *in vivo*. Figure 1 shows an overview of the progress, methodologies and main results obtained in this doctoral thesis.

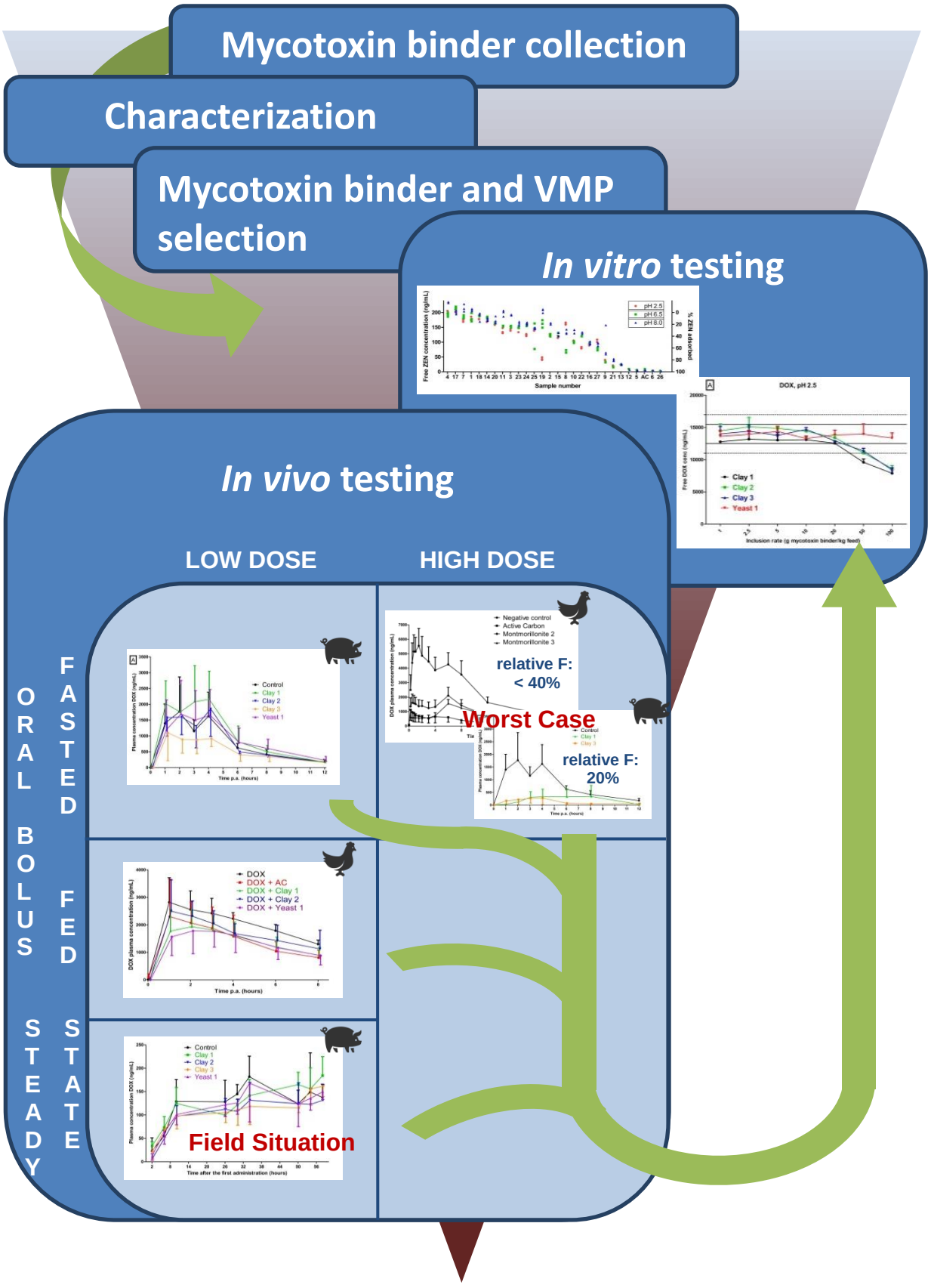

Figure 1: Overview of the methodologies and main results obtained in this doctoral thesis. VMP: veterinary medicinal product; relative F: relative oral bioavailability.

Veterinary medicinal product selection

The most commonly used veterinary medicinal products mixed in pig and poultry feed are antimicrobials and coccidiostats (European Medicines Agency, 2014). The products of interest are those that are exposed to mycotoxin binders before the site of absorption is reached. The risk associated with the binding of an antimicrobial by a mycotoxin binder is a decline in oral bioavailability. This decreased systemic exposure can be detrimental for the therapeutic efficacy and, in the case of antimicrobials, can lead to altered resistance selection. In case the use of mycotoxin binders leads to a higher bioavailability, maximum residue limits in animal products may be violated. Otherwise, there is a report of elevated oral bioavailability after prolonged exposure to a mycotoxin binder (Osselaere et al., 2012). To reduce the risk attributed to the combined use of mycotoxin binders and veterinary medicinal products, competent authorities restricted or prohibited the use of certain antimicrobials and coccidiostats when mycotoxin binders are applied. In turn, setting (too stringent) regulations also has detrimental effects: besides the economic losses suffered by the producers of the medicinal products and mycotoxin binders, these regulations can be an important constraint in the selection of a medicinal therapy for livestock, resulting in less therapies to choose from. Also potential beneficial effects of the mycotoxin binders may be discarded by such restrictions.

Characterization and mycotoxin binder selection

The parameters to characterise the mycotoxin binders (Chapter 1) were chosen pragmatically with respect to the adsorption of veterinary drugs. Together with the X-ray diffraction (XRD) results, they provide a relatively thorough identification of the mycotoxin binders that are on the market. XRD is a technique capable of measuring the distance between layers of molecules in a mineral or crystal. Provided correct calibration and adequate reference materials, the type of mineral can be identified using this technique. Also mixtures of different minerals can be analysed, however, the precision and comprehensiveness of the results are often compromised depending on the complexity of the sample. Overall, XRD is an essential technique for understanding mineral mycotoxin binders, however, much information cannot be elucidated by this technique and it should be complemented by other tests.

The characterization tests were executed to handle the inaudibility originating from both the complexity of the minerals and the compounded nature of commercially available mycotoxin

binders. They were used in this thesis to justify the selection of the mycotoxin binders used in further studies. For further studies, samples were selected based on the (mineral) content, physicochemical properties and their relevance in the agricultural sector. Figure 2 illustrates the different categories of mycotoxin binders. The groups were obtained by extracting principle components out of all of the variables that described the mycotoxin binder samples, and subjecting them to cluster analysis. The two most important principle components are plotted on the graph along with the different clusters. Each group is represented in the samples used in further studies.

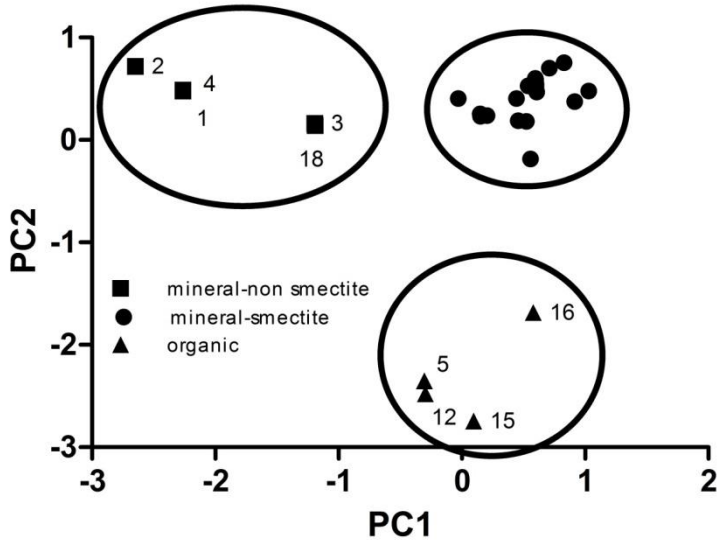

Figure 2: Categories of mycotoxin binders obtained by subjecting the characterisation and XRD-data to principle component and cluster analysis. PC1 and 2 are the two most important principle components, the numbers of the samples correspond with sample numbers used in Chapter 1.

Montmorillonite is the most encountered substance in commercially available mycotoxin binders but also many other minerals are found. Bentonite can be registered as mycotoxin binder, it is defined in Regulation 1060/2013 as having a montmorillonite (dioctahedral smectite) content of at least 70% (w:w) (European Commission, 2013a). However, many types of montmorillonite exist, each exerting different physicochemical and binding properties (Uddin, 2008). The main differences between different montmorillonites are the crystal composition (mixed layers), substitutions of Al^{3+} or Mg^{2+} ions in the silicate sheets, surface tension, type of exchangeable cation and degree of saturation. Furthermore, treatments using acid and/or heat may alter the physicochemical properties. So, even if the mycotoxin

binder is ‘pure’ and defined as a montmorillonite clay, many variations are possible regarding their physicochemical properties and perhaps their binding capabilities.

Organic substances found in mycotoxin binders were of the humic/fulvic acid type or derived yeast products. The latter distinction is made based upon the information provided by the manufacturer because the XRD and other characterization (mainly mineral fraction) tests only indicate the presence of organic material. The nature of the organic substance is even more complex as the variety seen in mineral substances, and in this thesis, no further distinction was made regarding their physiochemical characteristics. In the context of adsorption of veterinary medicinal products, they were considered as a diverse collection of hydrophobic and hydrophilic binding sites.

The binding properties regarding veterinary drugs of the minerals found in mycotoxin binders are not known or only poorly investigated (EFSA FEEDAP Panel, 2013a). Studies regarding the binding properties of minerals other than montmorillonite were executed in contexts such as purification of waste water (Beall, 2003) or mobility of an antibiotic in soil (Kulshrestha et al., 2004). The presence of substances, other than montmorillonite, in bentonite registered as mycotoxin binder, is permitted up to a level of 30% (w:w), provided they do not violate regulations regarding (limits for) undesired substances in feed or feed additives described in Regulation 2002/32 (European Commission, 2002c).

Zearalenone binding

Determining the physicochemical properties of the mycotoxin binders is not only important for selection of representative samples, they are also important for comparing with other binders. Certainly when the binding properties ought to be assessed, the physicochemical properties are utmost important. The assumption underlying former statement, is that the binding is correlated to a certain physicochemical parameter, which might not be the case when a very specific stereochemical interaction is needed for binding. The correlation of binding and physicochemical properties was assessed for zearalenone (ZEN), a mycotoxin for which both high and low binding to mycotoxin binders is described (Bueno et al., 2005). A correlation could be demonstrated with the mineral fraction and the d-spacing, a measure for the distances between two adjacent silicate layers in a clay. For the smectite-like mycotoxin binders, the correlation was not of the linear form but more like a cut-off. Indicating these parameters are more related to a restraining factor rather than to the binding mechanism.

Remarkable is the absence of correlation with the cation exchange capacity (CEC), a parameter that is often cited when discussing the binding properties of mycotoxin binders. The reason why this parameter is cited, is the facilitation role of exchangeable cations in the adsorption of aflatoxin B1 (AFB1) to smectite clays (Deng et al., 2010). For the mineral fraction, an inverse correlation was noted, indicating a correlation between the organic fraction and ZEN adsorption. This might be attributed to the hydrophobic binding sites organic substances can offer (Picollo, 1999).

More extensive characterisation might unveil parameters which correlated in a linear manner with the adsorption. Candidate parameters include, but are not limited to: specific surface, surface tension, chemical composition of clays, intra-layer substitutions, etc. To elucidate the binding mechanism completely, samples and ZEN-saturated samples should be subjected to infra-red spectrometry. This information would be very useful when searching for an agent to bind a specific target (i.e. mycotoxins), however for purpose of safety testing with regard to non-specific binding, detailed information on the exact binding mechanism would be redundant.

This experiment does illustrate the potential of this model to compare binding between different mycotoxin binders. Furthermore it is fast, cheap and can be executed using basic laboratory materials.

In vitro binding of veterinary medicinal products

The purpose of the *in vitro* part of the thesis was to identify a number of combinations which have a high potential for exerting an interaction. Apart from an adequate sensitivity and specificity, additional requirements include a high throughput, low cost and simple setup so it can be executed using basic lab equipment. Such an *in vitro* model was previously described by Sabater-Vilar (Sabater-Vilar et al., 2007). This model is more sophisticated than the model required for efficacy testing for AFB1 adsorption, described in Regulation 1060/2013 (European Commission, 2013a) because of the addition of salts in the matrix and the use of different acidity levels. It was slightly adapted and applied to ZEN (Chapter 1) and to doxycycline (Chapter 2). Besides doxycycline, also other veterinary drugs (sulfadiazine, trimethoprim and tylosin) and coccidiostats (diclazuril, lasalocid and salinomycin) were tested with this setup, only the method of analysis of the drug differed. The results are presented in Figure 3.

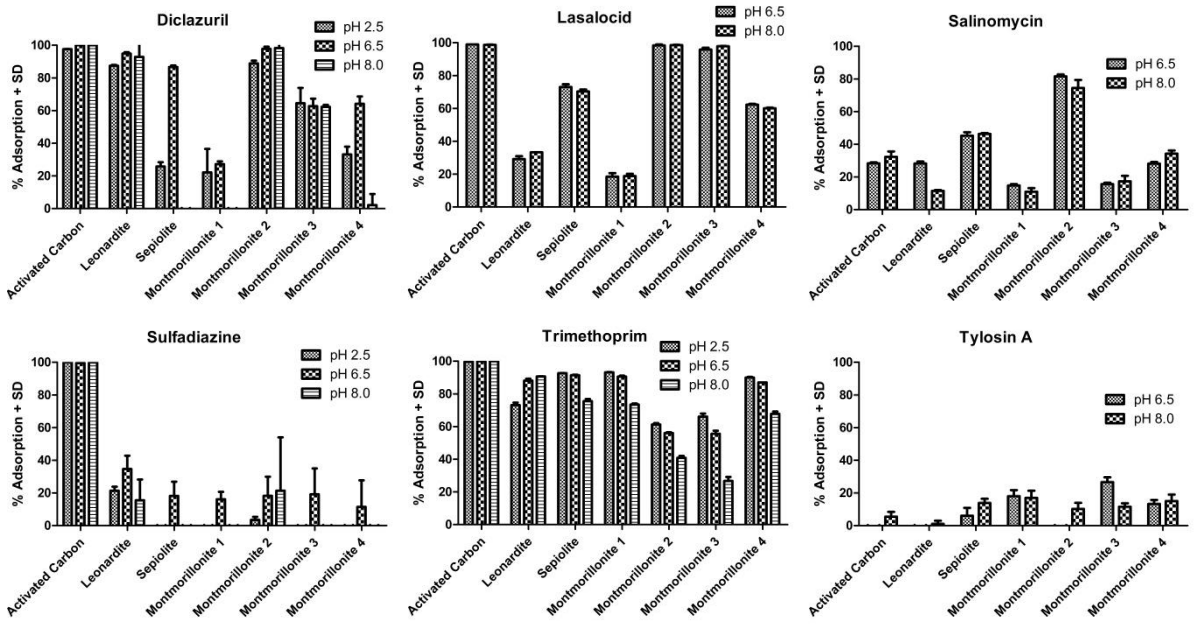

Figure 3: *In vitro* binding of selected veterinary medicinal products according to the protocol described in Chapters 1 and 2. Concentrations that were used are (in µg/mL): diclazuril: 2, lasalocid: 50, salinomycin: 150, sulfadiazine: 100, trimethoprim: 50, tylosin A: 2200.

There are many factors that can alter the outcome and can be set arbitrary. An overview of the factors that determine the outcome are presented in Figure 4. The factors that depend on the setup include total volume in which the reaction takes place, amount of drug and mycotoxin binder used, temperature, stir/shake, duration of the experiment, matrix: solvents, other components (feed, salts, toxins, …), pH (buffer), etc. Some of them (e.g. temperature, pH, ratio drug/binder, …) can be set according to physiological analogies. The use of (simulated) gastric fluids might improve similarity with the *in vivo* situation. Others factors, such as total volume, duration of the experiment, etc., are difficult to decide upon and can have significant impact. **There is an urgent need to standardize these parameters in order to correlate *in vitro* results to one another.** Because of the lack of standardization, the binding of the different veterinary medicinal products can only be compared between combinations in the same experimental setup. Regarding the results obtained in this thesis, doxycycline and trimethoprim are adsorbed better than salinomycin, sulfadiazine and tylosin A, which are, in general, poorly adsorbed by the tested mycotoxin binders. It is remarkable that activated carbon is not a good adsorbent for all veterinary medicinal products. It is also remarkable that tylosin A was only poorly adsorbed, whereas previously reported *in vivo* results with the same mycotoxin binder indicate a strong interaction (Devreese et al., 2012). Overall, the results indicate that interactions are possible between the tested mycotoxin binders and some

veterinary drugs *in vitro* and that further *in vivo* studies are needed to investigate the potential interactions.

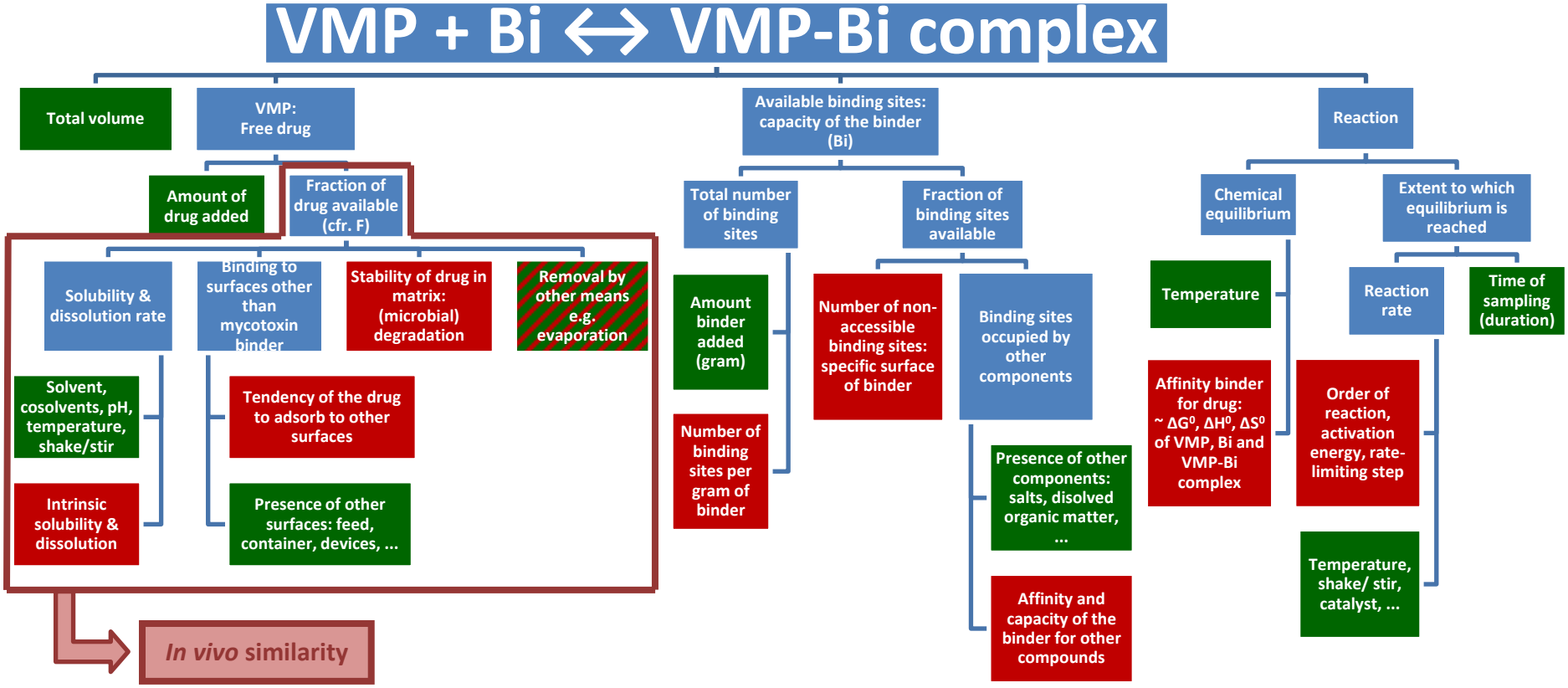

Figure 4: Factors determining the outcome of a static, *in vitro* adsorption screening experiment. Top: adsorption process presented as a chemical reaction. Factors in green: controlled for by the experimental setup: total volume, amount of drug and mycotoxin binder used, temperature, stir/shake, matrix: solvents, other components (feed, salts, toxins, …), pH (buffer), etc. Factors in red: depend on the binder and veterinary medicinal product to be tested, cannot be controlled. VMP: veterinary medicinal product; Bi: mycotoxin binder. ΔG^0: Gibbs free energy of the binding reaction; ΔH^0: bond-dissociation energy; ΔS^0: entropy of the binding reaction

In vivo experiments and refinement of the *in vitro* model

The pharmacokinetic (PK) approach for studying the effects of mycotoxin binders on the absorption of orally administered veterinary medicinal products is the method of choice, mainly because the objectivity of the method, i.e. not based on subjective scoring of clinical symptoms to assess the efficacy of pharmacological therapy. It is also the method recommended by the European Food Safety Authority, EFSA (EFSA FEEDAP Panel, 2010), which states oral bioavailability of the drug should be determined, preferably by monitoring the plasma concentration (EFSA FEEDAP Panel, 2010). Several *in vivo* PK approaches are possible to assess the oral bioavailability of veterinary drugs, the bolus model is the most used model for this purpose. The factors determining the outcome are presented in Figure 5.

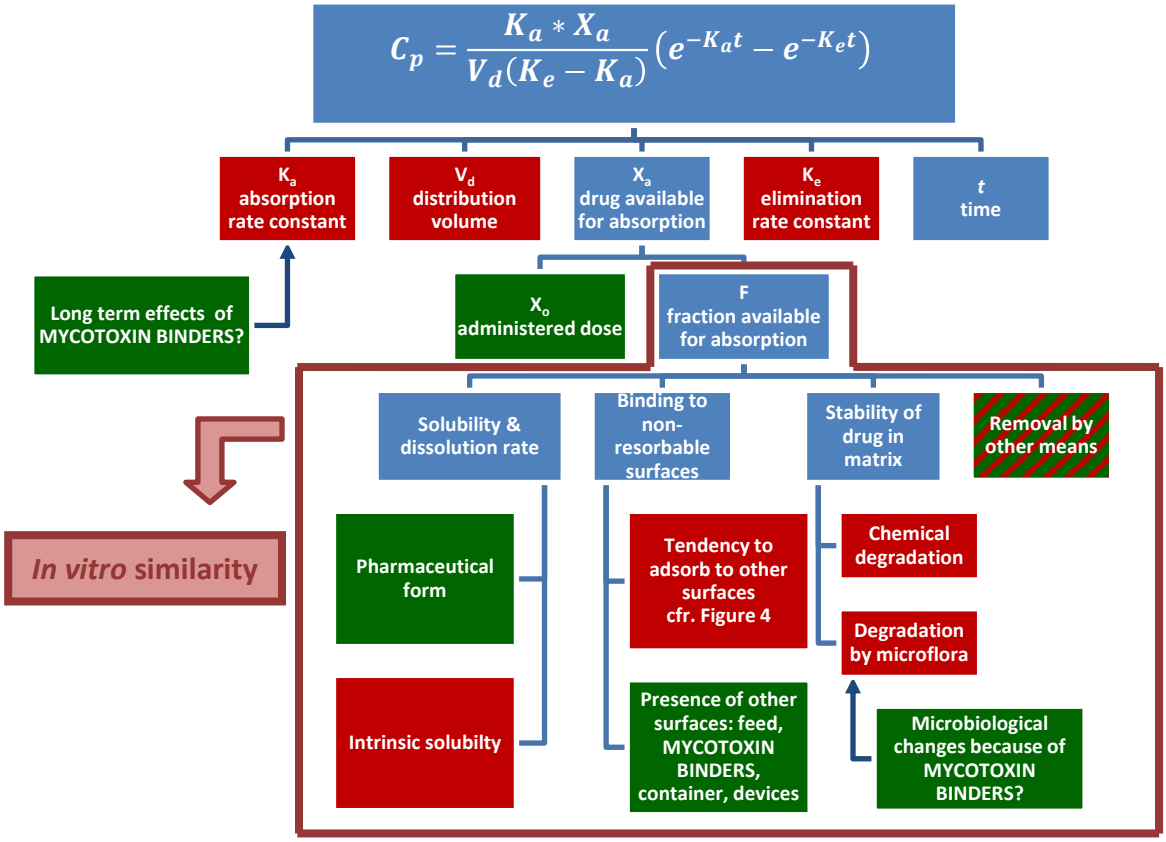

Figure 5: Factors determining the outcome of an *in vivo* pharmacokinetic oral bolus experiment in which pharmacokinetics can be described by a 1-compartmental model. C_p: Plasma concentration at time t, K_a: absorption rate constant, K_e: elimination rate constant, X_a: drug available for absorption, X_0: dose of drug administered, F: fraction available for absorption. Factors in green: controlled for by the experimental setup. Factors in red: depend on the binder and veterinary medicinal product and test animal, cannot be controlled for.

Similarities can be seen with the factors determining the *in vitro* outcome, nevertheless, also differences are noted. They include the kinetic nature of the system, namely dynamic and static. In the animals, drugs are usually constantly removed from the system, i.e. a dynamic system, whereas in the *in vitro* model, the total amount of drug remains constant throughout the experiment. Another discrepancy is the presence of microbiota in live animals. The effect of mycotoxin binders on the microbiota is poorly understood. It is expected that direct effects of mycotoxin binders on bacteria will depend on the dose, type of binder and time of exposure. Indirect effects may arise by alleviation of negative effects of mycotoxins on bacteria. In case the drug is subject to microbial degradation, alterations in gut microbiota may have an influence. Finally, mycotoxin binders can have an influence on the morphology and physiology of the gut wall (Osselaere et al., 2013c; Pinton and Oswald, 2014; Antonissen et al., 2015), possibly resulting in alterations of the rate and extent of absorption of the drug. Long term effects on the microbiota and gut wall are most described for yeast derived mycotoxin binders (Newman, 1994; de los Santos et al., 2007; Goossens et al., 2012), whereas no data is available for mineral binders.

The *in vitro* model described in Chapter 5 is a refinement of the model described in Chapter 1 and 2. It is basically the same setup, but with an important alteration, namely the presence of feed in the system. The results should be interpreted with the same prudence as with the first model, but they are better aligned with the *in vivo* results obtained in this thesis and previously reported by other authors. To illustrate this, an overview of these studies are presented in Table 1. These results indicate that the presence of feed and the inclusion rate are decisive for interaction. For tylosin, (clinical) interactions were seen when including a high dose in the feed (estimated 5%) (Canadian Bureau of Veterinary Drugs, 1992) or when using a low dose (0.1%) in fasted chickens. However, no interactions were seen when using fed chickens and using a dose of 0.2% binder. The *in vitro* model, with inclusion of feed, indicated that an interaction can be expected as from an inclusion rate of 1% binder. This finding is in accordance with the results of the *in vivo* experiment in fed chickens using a dose of 0.2% binder, since no interaction was noted. It is also in accordance with the reported *in vivo* interaction, which included a low dose (0.1%) but no feed was present. For doxycycline, the *in vitro* tests indicate an interaction as from 2% inclusion rate onwards, which is also in accordance with the *in vivo* experiments. Because mycotoxin binders are included in the feed, the latter is always present in the matrix of interactions between mycotoxin binders and oral medicinal products. Therefore, it is **advised to always include feed in safety testing models**

and experiments, both *in vitro* and *in vivo*. For the combinations tested in this thesis, the risk for interaction is low when using doses of 0.2%, which is generally recommended by the manufacturer. When using higher doses, interaction cannot be excluded.

Indications for specific (stereochemical) interactions, i.e. having a high capacity/affinity to that extent they can overcome the challenges raised by the presence of feed or other matrix components, were not seen in the combinations studied in this thesis.

Table 6: Overview of the available results obtained from literature and the presented doctoral thesis for interactions between veterinary medicinal products and mycotoxin binders.

Medicinal product	Species/ *in vitro*	Mycotoxin binder type	Presence of feed	Inclusion rate or equivalent	Outcome	Reference
Tylosin	Cattle	?	Yes	? (>5%)	Clinical interaction	(Canadian Bureau of Veterinary Drugs, 1992)
Tilmicosin	Broiler	Clay	Yes	≥ 2%	Clinical interaction	(Shryock et al., 1994)
Monensin	Chicken	Clay	Yes	0,5%	Clinical interaction (if dose drugs < recommended)	(Gray et al., 1998)
Salinomycin	Chicken	Clay	Yes	0,5%		
Salinomycin	Chicken	Clay	Yes	0,5%	Clinical interaction	(Nesic et al., 2003)
Lincomycin	Broiler	?	?	?	Interaction	(Amer, 2005)
Sulfadiazine	Broiler	Clay	Yes	0.2%	No interaction	(De Mil et al, non-published results)
Trimethoprim	Broiler	Clay	Yes	0.2%		
Doxycycline	*In vitro*	Clay	No	1%	Adsorption	(De Mil et al., 2015a)
Doxycycline	Broiler	Clay	No	1%	PK interaction	
Tylosin	Broiler	Clay	No	0.1%	PK interaction	(Devreese et al., 2012)
Doxycycline	Broiler	Clay and yeast	Yes	0.2%	No significant PK interaction	(De Mil et al., 2016c)
Tylosin	Broiler	Clay and yeast	Yes	0.2%	caution is advised	
Diclazuril	Broiler	Clay and yeast	Yes	0.2%	No interaction	
Diclazuril	Broiler	Clay and yeast	Yes	0.2%	No interaction	
Doxycycline	Pig	Clay and yeast	No	0.2%	Not significant	(De Mil et al., 2016b)
Doxycycline	Pig	Clay	No	1%	PK interaction	
Doxycycline	Pig	Clay and yeast	Yes (SS)	0.2%	No interaction	
Tylosin	*In vitro*	Clay and yeast	Yes	0 – 10%	Adsorption as from 2%	(De Mil et al., 2016a)
Doxycyline	*In vitro*	Clay and yeast	Yes	0 – 10%		

PK: Pharmacokinetic; SS: Steady State

Conclusion and future perspectives

For the veterinary medicinal products and the mycotoxin binders included in this thesis, no interactions are expected provided they are used at the recommended level of 0.2% feed. Caution is needed to ensure this inclusion rate is respected because the clays, registered as mycotoxin binders, can also be added for other purposes. No indications were noted of highly specific (stereochemical) interactions. This does not exclude the possibility for these kind of interactions for other combinations of oral veterinary drugs and/or mycotoxin binders. Although it is advisable to evaluate interaction with veterinary medicinal products on an individual basis, extrapolation of the results can probably be done to veterinary medicinal products belonging to the same class and having with similar physicochemical properties (e.g. doxycycline to other tetracyclines).

Therefore, screening for potential interactions should be carried out in the context of registration of new mycotoxin binders. In case a highly specific interaction is suspected, the binding mechanism is essential to assess the risks and benefits of the mycotoxin binder. The models used for this screening, *in vitro* or *in vivo*, should include feed as an important constituent of the matrix in which the screening is executed. Furthermore, they should be standardized to enable comparison between independently conducted research. Exploration of *in silico* models such as the physiologically based pharmacokinetic (PBPK) modeling, might contribute to this field.

A topic that was not covered in this thesis but which is highly relevant to the field are the long term effects of feeding mycotoxin binders to farm animals, in relation to absorption of xenobiotics such as antimicrobials, coccidiostats, but also vitamins (Papaioannou et al., 2002; Afriyie-Gyawu et al., 2008), micronutrients or other contaminants. To date they are poorly investigated but could be significant. Direct long term effects may include morphological- (Gonzalez et al., 2004; Goossens et al., 2012), metabolic- (Newman, 1994), digestibility changes and/or effects on the integrity of the barrier function of the GIT (Osselaere et al., 2013c). Indirect long term impact of mycotoxin binders may result from alterations in microbiota (Hu et al., 2004; Xia et al., 2004; Trckova et al., 2009), effects of nutrients in the mycotoxin binder (Reichardt et al., 2012), or scavenging low doses of (endo)toxins might also be important (Patterson and Staszak, 1977; Gilardi et al., 1999; Szajewska et al., 2006). For the latter, the long term effects of these (endo)toxins needs to be elucidated.

Another valuable contribution to the field would be a cost-efficacy study of the deployment of mycotoxin binders compared to other measures to reduce the damage caused by mycotoxins, such as Good Agricultural Practices and diverse treatments of feedstuffs. This is a very challenging task, especially to include all the potential effects of these additives. Another threshold is to understand the total impact of mycotoxins, a scientific area still in development. Tools to conduct cost effectiveness assessments, such as the incremental cost effectiveness ratio (ICER) (Russell et al., 1996), are available and frequently used in human medicine. Barring appropriate adaptations of these frameworks, they should yield valuable information to assess these additives compared to other measures to reduce damage caused by mycotoxins.

Finally, the effect of mycotoxin binders on the extractability of mycotoxins or veterinary medicinal products should be investigated in the context of analysis of these compounds in feed. It is important that these compounds can be quantified accurately, however, this may not be the case if mycotoxin binders e.g. alter the efficacy of sample clean-up and preparation.

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SUMMARY

Mycotoxins are secondary metabolites of fungi and contamination of food and animal feed with these compounds is a well-known problem in the agricultural sector. Many mycotoxins can impair human and animal health when they are ingested. European legislation and guidelines aim to prevent that highly contaminated feed enters the market. Therefore, the number of cases of clinical intoxication in animals (mycotoxicosis) is low in the European Union. Nevertheless, chronic exposure to low concentrations of mycotoxins can cause significant economic losses by reducing the zootechnical performance of food producing animals. To counteract the effects of low concentrations of mycotoxins, various strategies are being used. A frequently used method is the inclusion of special additives in the feed, called mycotoxin binders and - modifiers. Mycotoxin binders aim to adsorb mycotoxins to their surface in the gastro-intestinal tract and subsequently remove them with the excreta. In case veterinary medicinal products are adsorbed instead of mycotoxins, less is available to be absorbed by the target animal with a reduced pharmacological action as a consequence, and therefore the therapeutic efficacy may be in jeopardy.

The European Food Safety Authority (EFSA) recommends investigating the safety of mycotoxin binders regarding non-specific binding of other compounds such as orally administered veterinary drugs. To date, only a limited number of studies have been conducted with respect to this safety assessment. The approach and design of these studies are not aligned, for example, the inclusion rates of binders range from 0.1% up to 6%. This results in a pool of fragmented information from which no general conclusions can be deducted.

The **General Introduction** gives an overview of the various aspects of the risks associated with mycotoxins in animal feed. Both the toxicological properties and exposure are discussed for the main mycotoxins. Furthermore, an overview of the legislation and pre- and post-harvest measures against the deleterious effects of mycotoxins on animals is presented. The second part of the introduction is dedicated to the mycotoxin binders. An overview of the molecular structure of the registered binders is presented. Specific attention is drawn to the diversity and physicochemical properties of clays. Next, the European legislation of mycotoxin binders is discussed. Finally, an overview of the hitherto available *in vitro* and *in vivo* models for the efficacy and safety assessment of binders is discussed, including their advantages and weaknesses in light of this research.

The **General Objective** of this thesis is to investigate the influence of mycotoxin binders on the pharmacokinetics of orally administered veterinary medicinal products in broiler chickens and pigs using appropriate models. These species were selected because veterinary medicinal

products, such as antimicrobials and coccidiostats, are mainly administered through feed or drinking water in these species.

In **Chapter 1**, the physicochemical properties of 27 commercially available mycotoxin binders were determined. An *in vitro* screening model was validated for binding with zearalenone (ZEN), a mycotoxin which has – based on the available literature –shown a large diversity in terms of binding to mycotoxin binders. The model comprised mixing ZEN and mycotoxin binder in a buffer system, representative for the various pH values found in the gastro-intestinal tract. After 4 h of incubation, the free concentration of ZEN was determined. Finally, the physicochemical properties were correlated to the extent of binding of ZEN. There was a significant inverse correlation with the percentage of mineral fraction of the mycotoxin binders. A positive correlation between binding and the 'd-spacing' of clays, a measure of the distance between two successive layers of a clay, was also established.

Chapter 2 describes the use of the *in vitro* model, developed in Chapter 1, to evaluate the binding of doxycycline (DOX), a widely used antimicrobial agent, to a selection of mycotoxin binders. Based on the results, three mycotoxin binders were selected and tested *in vivo* in broiler chickens, using an oral bolus model with fasted broilers and an inclusion rate of binder equivalent to the expected daily intake when 10 g/kg is included in the feed. The results demonstrated a significant decrease in systemic exposure to DOX for the chickens in the test groups compared to the control group, which received no binder. The relative oral bioavailability in the test groups amounted 40% or less. This indicates a strong interaction between the tested mycotoxin binders and DOX in fasted broiler chickens.

In **Chapter 3**, the effects of four different mycotoxin binders were studied on the oral absorption of two antimicrobials (DOX and tylosin, TYL) and two coccidiostats (salinomycin, SAL, and diclazuril, DIC) in broiler chickens. A similar bolus design was used as in Chapter 2, however, the animals were non-fasted and were given an oral bolus with a lower dose of mycotoxin binder, equivalent to the daily dose for an inclusion rate of 2 g/kg, which is the recommended dose according to most manufacturers of binders. Pharmacokinetic analysis revealed a trend to lower plasma concentrations of DOX and TYL in the test groups in comparison with the control group. However, the observed interactions were not significant and not as pronounced as observed in Chapter 2 for DOX. It can be concluded that the feeding status and/or inclusion rate of mycotoxin binder are major factors influencing possible interactions.

In **Chapter 4**, two experiments in pigs were conducted. In the first experiment, the influence of four mycotoxin binders on the oral bioavailability of DOX was determined. For this, the bolus model was applied as described in Chapter 2 using fasted animals. In order to verify the effect of the inclusion rate of mycotoxin binder, two different dosages were tested, corresponding to 2 and 10 g/kg feed. Again, there was a clear effect of the inclusion rate noted, with a relative oral bioavailability of DOX of only 20% in the group that received the high dose, compared to a relative oral bioavailability of 100% in the group that received the low dose.

In the second experiment, the mycotoxin binder was added to a rate of 2 g/kg feed, and DOX was also mixed in the feed at the recommended dose. The conditions in this study were thus the same as those in the field situation. However, no difference in oral bioavailability of DOX was recorded between the test groups and the control group. These *in vivo* experiments demonstrate that also in pigs, both the inclusion rate and the feeding status are two decisive variables for interaction between binders and veterinary drugs.

The goal of **Chapter 5** was to examine as from which inclusion rate onwards there is a potential risk of interaction in an *in vitro* setup. The experimental design showed some important differences compared to the setup in Chapter 1. Mycotoxin binders and DOX or TYL were incubated in buffer in which also feed was present and wherein the amount of mycotoxin binder ranged from 1 g/kg to 100 g/kg feed.

For most of the mycotoxin binders, both for DOX as for TYL, a no interaction could be detected up to an inclusion rate of 20 g/kg. For one bentonite-based binder, an interaction was observed with TYL at an inclusion rate of 5 (pH 6.5) or 10 g/kg (pH 8.0) feed. The European guideline advises a maximum inclusion rate for bentonite of 20 g/kg. These findings further demonstrate that interaction between these antimicrobials and mycotoxin binders is *inter alia* dependent on the inclusion rate.

In the **General Discussion and Conclusions** of this doctoral thesis, the used models are related to each other and attention is paid to the practical applicability and relevance to the field situation.

For the veterinary medicinal products and mycotoxin binders studied in this thesis, no interaction is expected when used at the recommended inclusion rate of 2 g/kg feed in fed pigs or broiler chickens. At higher inclusion rates, interactions cannot be excluded. Although it is possible that this type of interaction may occur with other combinations of oral veterinary medicinal products and mycotoxin binders. Therefore, it is necessary to screen for possible

interactions in the registration process of new mycotoxin binders. The models developed in this thesis, both *in vitro* and *in vivo*, may contribute to this purpose and should include feed as an important factor.

SAMENVATTING

Mycotoxinen zijn secundaire metabolieten van schimmels en contaminatie van voedsel en diervoeder met deze verbindingen is een gekend probleem in de landbouw. Diverse mycotoxinen kunnen de gezondheid van mens en dier aantasten wanneer ze worden opgenomen. De Europese wetgeving en richtlijnen hebben als doel te voorkomen dat sterk gecontamineerde voeders op de markt gebracht worden. Het aantal gevallen van acute intoxicatie bij dieren (mycotoxicose) is bijgevolg beperkt in de Europese Unie. Desalniettemin kan chronische blootstelling aan lage concentraties van mycotoxinen aanzienlijke economische schade veroorzaken door de zoötechnische prestaties van voedselproducerende dieren te verminderen. Om de effecten van lage concentraties van mycotoxines tegen te gaan worden diverse strategieën toegepast. Een veel gebruikte methode is de toevoeging van speciale additieven in voeder, genoemd mycotoxinebinders en -modifiers. Mycotoxinebinders hebben als doel mycotoxinen te adsorberen aan hun oppervlak in het gastro-intestinaal kanaal om ze vervolgens met de uitwerpselen te verwijderen. Indien echter diergeneeskundige geneesmiddelen geadsorbeerd worden in plaats van mycotoxinen, is er minder geneesmiddel beschikbaar om te worden geabsorbeerd door het doeldier met een daling van de farmacologische werking tot gevolg, waardoor de therapeutische werkzaamheid van het geneesmiddel in het gedrang komt.

Het Europees Agentschap voor Voedselveiligheid (EFSA) adviseert om onderzoek te verrichten naar de veiligheid van mycotoxinebinders betreffende de niet-specifieke binding van andere componenten, zoals o.a. oraal toegediende diergeneesmiddelen. Tot op heden werden slechts een beperkt aantal studies uitgevoerd met betrekking tot dit aspect. De aanpak en opzet van de beschikbare studies zijn bovendien niet op elkaar afgestemd, bijvoorbeeld de gebruikte inclusieratio van binder varieert van 0,1% tot 6%. Dit resulteert in gefragmenteerde informatie waaruit geen algemene conclusies kunnen worden getrokken.

In de **Algemene Inleiding** wordt een overzicht gegeven van de verschillende aspecten van de risico's verbonden aan contaminatie van diervoeders met mycotoxinen. Zowel de toxicologische eigenschappen als de blootstelling worden besproken voor de belangrijkste mycotoxinen. Verder wordt een overzicht gegeven van de wetgeving en de mogelijke maatregelen, zowel voor als na de oogst, tegen de schadelijke effecten van mycotoxinen. Het tweede deel van de inleiding is gewijd aan de mycotoxinebinders zelf. Een overzicht van de moleculaire structuur van de geregistreerde mycotoxinebinders wordt gegeven. Specifieke aandacht wordt gevestigd op de diversiteit en de fysicochemische eigenschappen van kleien. Vervolgens wordt de Europese wetgeving van mycotoxinebinders besproken. Tenslotte wordt

een overzicht gegeven van de bestaande *in vitro* en *in vivo* modellen om de efficaciteit en veiligheid ervan te onderzoeken, en hun voordelen en tekortkomingen worden besproken in het licht van dit onderzoek.

De **Algemene Doelstelling** van dit proefschrift is om de invloed van mycotoxinebinders op de farmacokinetiek van oraal toegediende geneesmiddelen voor diergeneeskundig gebruik te onderzoeken bij vleeskippen en varkens aan de hand van geschikte modellen. Deze diersoorten werden geselecteerd omdat geneesmiddelen zoals antimicrobiële middelen en coccidiostatica, vooral worden toegediend via voeder of drinkwater bij deze diersoorten.

In **Hoofdstuk 1** werden de fysicochemische eigenschappen van 27 commercieel beschikbare mycotoxinebinders bepaald. Een *in vitro* screening model werd gevalideerd voor binding met zearalenone (ZEN), een mycotoxine dat - op basis van de beschikbare literatuur - een grote diversiteit in binding aan verschillende mycotoxinebinders vertoont. Het model omvat het mengen ZEN en de mycotoxinebinders in een buffersysteem, representatief voor de verschillende pH-waarden in het gastro-intestinaal kanaal. Na 4 uur incubatie werd de vrije concentratie van ZEN bepaald. Tenslotte werden de fysicochemische eigenschappen gecorreleerd met de mate van binding van ZEN. Er was een significante omgekeerde correlatie met het percentage minerale fractie van mycotoxinebinders. Een positieve correlatie tussen mate van binding en de ‘d-spacing’ van kleien, een maat voor de afstand tussen twee opeenvolgende lagen van een klei, kon eveneens worden vastgesteld.

Hoofdstuk 2 beschrijft het gebruik van het *in vitro* model, ontwikkeld in Hoofdstuk 1, teneinde de binding van doxycycline (DOX) te evalueren bij een aantal mycotoxinebinders. Op basis van de resultaten werden drie mycotoxinebinders geselecteerd en *in vivo* getest aan de hand van een oraal bolus model bij uitgevaste vleeskippen, met een inclusieratio van binder overeenkomstig met 10 g/kg voeder. De resultaten toonden een significante daling in systemische blootstelling van DOX bij de kippen in de testgroepen in vergelijking met de controlegroep, die geen binder verstrekt kreeg. De relatieve orale biologische beschikbaarheid bedroeg 40% of minder. Dit duidt op een sterke interactie tussen de geteste mycotoxinebinders en DOX bij nuchtere vleeskippen.

In **Hoofdstuk 3** werden de effecten van vier verschillende mycotoxinebinders bestudeerd op de orale opname van twee antimicrobiële middelen (DOX en tylosine, TYL) en twee coccidiostatica (salinomycine, SAL, en diclazuril, DIC) bij vleeskippen. Een gelijkaardige orale bolus proefopzet werd gebruikt als in Hoofdstuk 2, maar de dieren waren in gevoede toestand en kregen een bolus met een lagere inclusieratio aan mycotoxinebinder,

overeenkomend met de dagelijkse dosis bij een inclusie van 2 g/kg voeder, hetgeen de aanbevolen dosering is volgens de meeste fabrikanten van mycotoxinebinders. Farmacokinetische analyse toonde een trend tot lagere plasmaconcentraties van DOX en TYL bij de testgroepen in vergelijking met de controlegroep. Echter, de waargenomen interacties waren niet significant en niet zo uitgesproken als deze gezien in Hoofdstuk 2 voor DOX. Er kan geconcludeerd worden dat de prandiale status en/of dosering van mycotoxinebinders bepalende factoren zijn voor mogelijke interacties.

In **Hoofdstuk 4** werden twee experimenten bij varkens uitgevoerd. In het eerste experiment werd de invloed van vier mycotoxinebinders op de biologische beschikbaarheid van DOX bepaald. Hiervoor werd opnieuw het bolus model toegepast zoals beschreven in Hoofdstuk 2 bij uitgevaste dieren. Om het effect van de inclusieratio mycotoxinebinder te verifiëren, werden twee verschillende doseringen getest, overeenkomend met 2 en 10 g/kg voeder. Opnieuw werd er een significante daling in de relatieve orale biologische beschikbaarheid van DOX vastgesteld. Deze bedroeg slechts 20% in de groep die de hoge dosis kreeg, vergeleken met een relatieve orale biologische beschikbaarheid van 100% in de groep die de lagere dosis toegediend kreeg.

In het tweede experiment werden de mycotoxinebinders toegevoegd aan een inclusieratio van 2 g/kg voeder, en werd DOX eveneens gemengd in het voeder aan de aanbevolen dosering. De omstandigheden in deze studie zijn bijgevolg dezelfde als deze in de veldsituatie. Er kon geen verschil in orale biologische beschikbaarheid van DOX waargenomen worden tussen de testgroepen en de controlegroep. Deze *in vivo* experimenten tonen aan dat ook bij varkens zowel de inclusieratio als prandiale status twee beslissende variabelen zijn voor het optreden van interacties tussen binders en geneesmiddelen.

Het doel van **Hoofdstuk 5** was om te onderzoeken vanaf welke inclusieratio er een potentieel risico is tot interactie, dit in een *in vitro* model. De experimentele opzet vertoonde enkele belangrijke verschillen met deze in Hoofdstuk 1. Mycotoxinebinders en DOX of TYL werden in een buffer gebracht waarin ook voeder aanwezig was. De hoeveelheid mycotoxinebinder varieerde van 1 g/kg tot 100 g/kg voeder.

Voor de meeste mycotoxinebinders, zowel voor DOX als voor TYL kon geen interactie waargenomen tot en met een inclusieratio van 20 g/kg voeder. Voor één bentoniet-gebaseerde mycotoxinebinder werd een interactie waargenomen met TYL vanaf een inclusieratio van 10 (pH 6,5) of 20 g/kg (pH 8,0) voeder. De Europese richtlijn voor het gebruik van bentoniet adviseert een maximum inclusieratio van 20 g/kg. Deze bevindingen tonen eveneens aan dat

de interactie tussen mycotoxinebinders en antimicrobiële middelen onder meer afhankelijk is van de inclusieratio.

In de **Algemene Discussie en Conclusie** van dit proefschrift worden de gebruikte *in vitro* en *in vivo* modellen aan elkaar gerelateerd en wordt aandacht besteed aan de praktische toepasbaarheid en de relevantie van de resultaten voor de veldsituatie.

Voor de antimicrobiële middelen, coccidiostatica en mycotoxinebinders bestudeerd in dit proefschrift, worden er geen interacties verwacht bij gebruik aan de aanbevolen inclusieratio van 2 g/kg voeder in niet-gevaste varkens en vleeskippen. Bij gebruik van hogere inclusieratios kunnen interacties niet worden uitgesloten. Het is evenwel mogelijk dit soort interactie kan optreden met andere combinaties van orale geneesmiddelen voor diergeneeskundig gebruik en mycotoxinebinders. Daarom is het steeds noodzakelijk om te screenen op mogelijke interacties in de registratieprocedure van nieuwe mycotoxinebinders. De ontwikkelde modellen in dit proefschrift, zowel *in vitro* als *in vivo*, kunnen bijdragen aan dit doel en dienen bij voorkeur voeder in te sluiten als een belangrijke factor.

CURRICULUM VITAE

Thomas De Mil was born in Ghent on April 18, 1987. He finished high school in the Don Bosco College in Zwijnaarde in 2005. Driven by a broad scientific interest, he took up studies industrial engineering at the Katholieke Hogeschool Sint-Lieven. During this year, his general scientific interest became more specific, namely chemistry and healthcare. Therefore, after the successful completion of the first year undergraduate industrial engineer, he started the studies pharmaceutical sciences at UGent. He graduated in 2011 as Master of Science in Drug Development. Following he completed a Master General Management at the Vlerick Leuven Gent Management School in Ghent in 2012. Immediately afterwards, he began as a doctoral researcher at the Department of Pharmacology, Toxicology and Biochemistry of the Faculty of Veterinary Medicine at UGent. His doctoral research investigated the impact of mycotoxin binders on the pharmacokinetics of orally administered drugs for veterinary use in pigs and poultry (acronym: MYTOXBIND). It was funded by the Federal Public Service Health, Food Chain Safety and Environment. In 2015 the MYTOXBIND project was followed by a finalising doctoral fellowship from the Special Research Fund of UGent.

Thomas is author and co-author of several scientific publications in international peer-reviewed journals. He gave presentations at various national and international congresses and supervised several students in completing their master thesis or Honours program. He completed the Doctoral Training Program of the Doctoral School of Life Sciences and Medicine of UGent in 2016.

Thomas De Mil werd geboren in Gent op 18 april 1987. Hij beëindigde de middelbare school in het Don Bosco College in Zwijnaarde in 2005. Gedreven door een brede wetenschappelijke belangstelling, vatte hij de studies industrieel ingenieur aan op de Katholieke Hogeschool Sint-Lieven. Gedurende dit jaar, werd zijn algemene wetenschappelijke interesse meer specifiek, nl. in chemie en gezondheidszorg. Na het succesvol afronden van de eerste bachelor industrieel ingenieur vatte hij vervolgens de studies farmaceutische wetenschappen aan de UGent aan. Hij behaalde in 2011 het diploma van Master of Science in de Geneesmiddelenontwikkeling. Vervolgens studeerde hij in 2012 af als master General Management aan de Vlerick Business School in Gent. Onmiddellijk na afstuderen begon hij als doctoraatsbursaal bij de vakgroep Farmacologie, Toxicologie en Biochemie van de faculteit Diergeneeskunde van de UGent. Het doctoraatsproject handelde over de invloed van mycotoxinebinders op de farmacokinetiek van oraal toegediende geneesmiddelen voor diergeneeskundig gebruik bij varkens en pluimvee (acroniem: MYTOXBIND). Het werd gefinancierd door de Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu. In 2015 werd het MYTOXBIND project opgevolgd door een finaliserende doctoraatsbeurs van het Bijzonder Onderzoeksfonds van de UGent.

Thomas is auteur en coauteur van verschillende wetenschappelijke publicaties in peer-reviewed internationale tijdschriften. Hij gaf presentaties op diverse nationale en internationale congressen en begeleidde diverse bachelor en master studenten bij het voltooien van hun masterproef of Honours programma. Hij vervolmaakte de doctoraatsopleiding van de Doctoral School of Life Sciences and Medicine van de UGent in 2016.

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