Mycotoxins

General Information, Emerging Mycotoxins and Co-Occurrence

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Mycotoxins are secondary metabolites that are produced by certain filamentous fungi. They are found to be common contaminants of food and feed sources. There are three main genera of fungi associated with the production of mycotoxins, which are *Aspergillus*, *Fusarium* and *Penicillium* (CAST, 2003). In 2019, it was shown in a survey that 88% of all tested feed samples contained at least one mycotoxin (Gruber-Dorninger et al. 2019). Therefore, the economic impact of mycotoxins is severe. Economic effects can be related to animal mortality, disease and reduced production, but also crop losses and mycotoxin analysis (Zain, M.E. 2011). The mycotoxins that are most relevant for animal production are aflatoxins, fumonisins, zearalenone, trichothecenes (deoxynivalenol, T-2 toxin) and ochratoxin (Richard, J.L. 2007).

Emerging mycotoxins can be defined as mycotoxins that are not routinely determined, nor regulated by legislation. Even though little attention is given to this group of mycotoxins, their incidence is increasing (Vaclavikova et al. 2013). Based on this definition, it can be expected that many mycotoxins with (potential) toxicity are part of this group. Some of the most studied emerging mycotoxins appear to be enniatins, beauvericin, fusaproliferin, culmorin, moniliformin, fusaric acid, emodin, alternariol, butanolide, tenuazonic acid, sterigmatocystin, mycophenolic acid and alternariol monomethyl ether. For many of the members of the emerging mycotoxin group, there are knowledge gaps, highlighting the need for more research and attention towards these mycotoxins, so that a risk assessment can be done (Gruber-Dorninger, 2017). When risk assessments of these mycotoxins have been made, it will be possible to set legislation and guidance levels around these mycotoxins (Kovalsky et al. 2016). The knowledge gaps are still present in all three main factors for risk assessment; occurrence, toxicity and toxicokinetics. For some of the emerging mycotoxins, such as enniatins, alternariol or moniliformin, toxicity data is available. However, this data mostly originates from in vitro studies, while in vivo data remains limited (Fraeyman et al. 2017).

It is possible that a feed sample is contaminated with more than one mycotoxin at a time, this is called a co-occurrence. There can be different reasons for co-occurrence; several fungi are capable of producing more than one mycotoxin at the same time, feed (ingredients) can be contaminated with more than one fungi at a time, and finished feed usually consists out of several ingredients (which can all contain different mycotoxins) (Smith et al. 2016). A recent survey showed that about 48% of all feed samples (7,049 in total) contained two or more mycotoxins (Rodrigues and Nachrer, 2012). Most studies show synergistic or additive effects when there is co-occurrence of multiple mycotoxins, and these effects are generally regarding decreased animal performance. (Grenier and Oswald, 2011).

In general, it is considered difficult to interpret toxicity data of co-occurring mycotoxins. It is expected that mycotoxins which have similar toxicity mechanisms cause synergistic and additive effects (Speijers and Speijers, 2004). However, besides the toxicity of each mycotoxin separately, there are many factors that could influence the final toxicity of the cooccurring mycotoxins such as toxicokinetics, toxicodynamics, mechanism and chemistry in cells, and experimental design (Lee and Ryu, 2017). It was shown, based on data from literature including 127 feed samples, that the most frequently occurring combinations of mycotoxins are co-occurrence of aflatoxins and ochratoxin A (21%), aflatoxins and fumonisins (20%) and DON and zearalenone (13%). Other combinations of mycotoxins were also shown to co-occur but are less frequent (Smith et al. 2016).

In-feed solutions

A wide variety of products, intended to reduce the negative effects of mycotoxins, are globally available. A common mode of action for such products is the binding of mycotoxins, also called adsorption (Kolosova and Stroka, 2011). Clay minerals are considered to be natural adsorbents of mycotoxins and are generally available at quite low prices. Montmorillonite, bentonite and zeolite are examples of clays that have the capacity to bind aflatoxins in the gastrointestinal tract, and therefore reduce the absorption of aflatoxins. Such clays are generally non-nutritive and non-toxic for the animals but allow for a protective effect against certain mycotoxins (Oguz and Kurtoglu, 2000). Aside from the inorganic adsorbents, such as zeolites, bentonites and other clays, organic binders can be used, for example, yeast cell wall constituents (Kolosova and Stroka, 2011). In general, it can be stated that mycotoxin binders can vary in efficiency, depending on the mycotoxins in the feed, as well as on the binder itself. Polarity, size, solubility, shape and charge are important characteristics of the mycotoxins, that determine the efficacy to which they can be adsorbed by the binder product. Also, the external pH, of the environment to which the mycotoxin binder is added, is important in the adsorption process (Dakovic et al. 2005). Clays can be defined as naturally occurring minerals with a particle size

smaller than 2µm (Subramaniam and Kim, 2015). Most types of clays are phyllosilicates, meaning that they consist of layers. Such phyllosilicates can be subdivided into two groups, depending on the number, type and charge of the layers.

The kaolin group (for example nacrite, kaolinite and dickite) have a 1:1 structure, with a tetrahedral Si sheet that is linked to an octahedral Al sheet via a covalent bond (Subramaniam and Kim, 2015). Another type of structure is the 2:1 group, consisting out of one octahedral sheet (generally Mg, Al or the combination of Mg and Al) fitting in the middle of two tetrahedral Si sheets. This type of structure is called the smectite group (for example montmorillonite, saponite or hectorite) (Subramaniam and Kim, 2015). Next to phyllosilicates, another type of clay can occur with a three-dimensional structure. This group is called the zeolites, and the three-dimensional structure (of SiO_4^{4-}, and AlO_4^{5-}) is linked via shared oxygen atoms (Subramaniam and Kim, 2015). Via the three-dimensional pores, exchange of cations and water can occur. These clays are negatively charged, making them very effective cation exchangers for positively charged toxins. Due to the small pore size of zeolites, they are considered to be very selective in adsorption, with a high affinity for toxins and other contaminants (Subramaniam and Kim, 2015).

Orffa developed Excential Toxin A as a single-spectrum solution, containing one ingredient and aiming at binding the mycotoxins in the feed. The product consists of one specific zeolite, tectosilicate (clinoptilolite), which is mined in Europe. As a zeolite, it has the typical 3-dimensional framework with small pores, as mentioned above. The focus of Excential Toxin A is to bind the mycotoxins in the gastrointestinal tract of the animal, in order to prevent the mycotoxin from being absorbed on an intestinal level and entering the bloodstream. The product has been shown to have complete binding of aflatoxins (>90%), both at pH 3 (simulating the stomach environment) as well as pH 7 (simulating the intestinal environment). Also, for the enniatins, one of the emerging mycotoxins, binding was shown to be over 90% in the full pH range of the gastrointestinal tract. For fumonisins, the product shows complete binding at pH 3, but strongly reduced binding at pH 7. When, besides aflatoxins, fumonisins or enniatins, a) other types of mycotoxins are present in the feed, b) contamination levels are high or c) there's co-contamination, it's advised to switch from Excential Toxin A to Excential Toxin Plus.

Excential Toxin Plus is Orffa's broad-spectrum mycotoxin adsorbent, aimed at the full spectrum of mycotoxins. This product consists of five ingredients and focuses on five functions to reduce the negative effects of mycotoxins.

First, the product includes organic acids aimed at preventing the growth of mould and mycotoxins in stored feed. Literature shows that the inclusion of calcium propionate can reduce the growth of Aspergillus flavus and reduce the production of AFB1 by Aspergillus flavus (Alam et al. 2009).

A second function of the product is that it aims for the adsorption of mycotoxins in the gastrointestinal tract of animals, via the inclusion of two aluminosilicates and yeast in the product. The combination of these three ingredients allows for a synergistic effect, with high binding efficacy for different types of mycotoxins.

The third function is the strengthening of the intestinal barrier via the inclusion of betaine in Excential Toxin Plus. Many mycotoxins, such as DON, reduce villi length intestinal barrier function (Pinton et al. 2012), causing the animal to be more susceptible to pathogens. Betaine can accumulate in intestinal cells and is known for having a protective effect on the intestinal cells during a challenge (Kettunen et al. 2001). In research published by Kettunen et al. (2001), it was shown that betaine can reduce the harmful effect of a challenge on intestinal villi, allowing for longer villi during challenges compared to the challenged group without betaine.

The liver is an organ that is often negatively affected by mycotoxins in the diet (Domijan and Peraica, 2010). Besides accumulation in intestinal cells, betaine is also known to accumulate in liver cells (Kettunen et al. 2001). Wen et al. (2021) showed that betaine allows for improved liver health in broilers fed mycotoxin (zearalenone) contaminated feed. The fourth function of Excential Toxin Plus can therefore be stated as the hepatoprotection by betaine.

Finally, as described in the first section of this paper, mycotoxins often reduce immune function (Sobrova et al. 2010, Tao et al. 2018). Yeast has been extensively described in the literature for its immune stimulating effects (El-Boshy et al. 2010). As such, the fifth function of Excential Toxin Plus is the strengthening of the overall immune function through the inclusion of yeast. This yeast has a dual function in the product; adsorbing mycotoxins and immune support.

Some mycotoxins, such as the trichothecenes, are known to be difficult to bind. For such mycotoxins, it is important to include other strategies that contribute to reducing the negative effects of these mycotoxins. Excential Toxin Plus is a broad-spectrum solution, with functions not only aimed at adsorption but also aimed at prevention, intestinal support, hepatoprotection and strengthening of the immune system. It can therefore provide protection against the full range of mycotoxins.

Binding efficacy trial

In collaboration with the Centre of Excellence in Mycotoxicology and Public Health at the University of Ghent (Belgium), Orffa designed an in vitro model to analyse the binding capacity of different commercial mycotoxin adsorbents, using liquid chromatography with tandem mass spectrometry (LC-MS/ MS). The assay mimics the conditions in the gastrointestinal tract by testing the compounds both at pH 3 (which represents the pH level in the stomach) as well as pH 3-7 (resembling the pH in the intestine). This trial compared the binding capacity of the 8 premium mycotoxin binders that are globally

Table 1: Mycotoxin binding efficacy of Excential Toxin Plus compared to 8 premium mycotoxin binders

	1	2	3	4	5	6	7	8	Excential Toxin Plus
DON pH3	+	+	+	0	0	+	0	+	0
DON pH3-7	+	0	0	0	0	0	+	+	+
HT-2 pH3	+	0	0	0	+	0	+	++	+
HT-2 pH3-7	++	0	0	0	0	0	+	++	+
T-2 pH3	+	0	+	0	+	+	+	+	++
T-2 pH3-7	+	0	0	0	0	+	+	+	+
ZEN pH3	++	+	++	+	+	++	++	++	+++
ZEN pH3-7	++	+	+	+	+	++	++	0	++
AFB1 pH3	+++	+++	+++	+++	+++	+++	+++	+++	+++
AFB1 pH3-7	+++	+++	+++	+++	+++	+++	+++	+++	+++
AFB2 pH3	+++	+++	+++	+++	+++	+++	+++	+++	+++
AFB2 pH3-7	+++	+++	+++	+++	+++	+++	+++	+++	+++
AFG1 pH3	+++	+++	+++	+++	+++	+++	+++	+++	+++
AFG1 pH3-7	++	+++	+++	+++	+++	+++	+++	+++	+++
AFG2 pH3	++	+++	+++	+++	+++	+++	+++	+++	+++
AFG2 pH3-7	+	++	++	++	+++	+++	+++	++	+++
OTA pH3	++	0	++	+	++	++	+++	++	+++
OTA pH3-7	0	0	0	+	0	+	+	0	+
FUM B1 pH3	0	+	+++	+++	+++	++	+++	++	+++
FUM B1 pH3-7	0	0	0	0	+	+	0	0	+
FUM B2 pH3	++	+	+++	+++	+++	++	+++	++	+++
FUM B2 pH3-7	0	++	0	0	+	++	0	0	++

Complete binding ("+++" >90%)
Partial binding ("++" >50%; < 90%)
Limited binding ("+" >10%; <50%)
No significant binding ("0" <10%)

available, to the binding efficacy of Excential Toxin Plus for different mycotoxins: trichothecenes (DON, HT-2, T-2), zearalenone (ZEN), aflatoxins (AFB1, AFB2, AFG1, AFG2), ochratoxins (OTA), and fumonisins (FUM B1, FUM B2) (published at the World Mycotoxin Forum Amsterdam, 2018).

The binding efficacy for the trichothecenes (DON, HT-2, T-2) is considered to be low, which is also shown in the results of this trial (Table 1). It is shown that the adsorption capacity for the trichothecenes is low for all tested products. Aflatoxins, on the other hand, are shown to be bound to a high extent for all of the tested products, both at pH 3 as well as pH 3-7. Some of the tested products are shown to bind ochratoxins, but in general binding capacity at pH 7 is quite low for this type of mycotoxin. For fumonisins, similar results were found, also showing that some products have high binding capacity at pH 3, but low binding at pH 7. When considering Excential Toxin Plus, the product shows equal or better binding efficacy compared to 8 of the commercially available premium mycotoxin binders.

Trial in laying hens

In collaboration with the CERSA and the University of Lomé in Togo, a trial in laying hens was performed to study the effects of Excential Toxin Plus in an aflatoxin-contaminated layer diet (published at World Poultry Congress, Paris 2022). The trial included 840 hens, in cages (3 per cage), in open houses, that were randomly assigned to 24 pens. There were two treatments; T1: control diet, T2: control diet + 1.5 kg of Excential Toxin Plus/tonne of feed. It was shown (Table 2) that Excential Toxin Plus significantly improved egg production, especially in old hens (+8.7%, $p<0.05$); +2.4% for young hens), and significantly reduced FCR for both old (-9.2%, $p<0.05$) and young (-4.6%, $p<0.05$) hens. In old hens, Excential Toxin Plus was shown

Table 2: Effect of Excential Toxin Plus on performance and egg parameters in hens with different ages (young hens 47-59 weeks, old hens 62-74 weeks)

	Young hens		Old hens	
	Control, T1	ETP, T2	Control, T1	ETP, T2
Performance				
Average daily feed intake, g/d	112.5	110.1	112.5	110.9
Egg production rate, %	70.1 ^{b}	71.8 ^{b}	64.5 ^{a}	70.1 ^{b}
Feed conversion ratio, kg/kg	2.642 ^{b}	2.520 ^{a}	2.881 ^{b}	2.615 ^{a}
Egg parameters				
Egg weight, g	59.0	60.8	59.4	63.4
Yolk weight, g	15.5	15.4	15.2	15.6
Albumen weight, g	35.6 ^{a}	37.6 ^{ab}	36.3 ^{a}	39.6 ^{b}
Shell weight, g	7.9 ^{a}	7.7 ^{a}	7.9 ^{a}	8.5 ^{b}

ETP = Excential Toxin Plus
Row with different superscript (^{a,b}) differ significantly ($p < 0.05$).

to improve egg weight by +4.0 g, due to a significant improvement in albumen and shell weight ($p<0.05$).

Trial in swine

A recent trial (published at World Mycotoxin Forum Parma, 2022), performed on a commercial farm in the Philippines, investigated the effects of three different broad-spectrum mycotoxin binders on reducing the effects of zearalenone on growth performance and incidence of Vulva Hypertrophy (VH) in gilts. The study consisted out of two trials, T1 performed in nursery gilts and T2 in growing gilts. Both trials started when the gilts showed signs of VH. Gilts from the same batch were allocated into three groups and fed three different types of multi-component mycotoxin adsorbents A, B or Excential Toxin Plus. For both trials, weight and feed intake were recorded and feed conversion ratio (FCR) was calculated. Vulva hypertrophy was noted per animal, initially and at the end of the trial period. Both trials lasted until the gilts were 180 days old and were selected, either as replacement gilts or sold as finishers, with selection criteria being the occurrence of oestrous during these first 180 days.

In both trials, considering the overall period, gilts from the Excential Toxin Plus group had higher final body weight and daily gain, and lower FCR compared to groups A and B (Figure 1). The Excential Toxin Plus group also resulted in the highest reduction of VH cases (-54%) in T1. In T2, groups B and Excential Toxin Plus reduced VH cases in more than 30%, with group A increasing appearance of VH.

Conclusions

Mycotoxins are already present as a major challenge for animal production worldwide, accounting for substantial economic losses. Due to the changing climate and intensive agricultural practices, the mycotoxin challenge is expected to increase in the coming years. Not only the most commonly known mycotoxins, such as aflatoxins, fumonisins, zearalenone, ochratoxins, DON, T-2 and HT-2 present a challenge, but also the emerging mycotoxins. This highlights the need for more research into toxicity mechanisms and also into means for mycotoxin prevention and reduction.

The market for mycotoxin solutions already includes different commercially available products. Orffa developed Excential Toxin A, a single spectrum solution focussed mainly on the binding of aflatoxins and fumonisins. A second product, Excential Toxin Plus, is a broad spectrum solution with high in vitro binding efficacy to different types of mycotoxins, and on top, prevention, intestinal support, hepatoprotection and strengthening of the immune system. Both products have been demonstrated to reduce the negative effects of mycotoxins.

Figure 1: Performance results in A) body weight (BW), B) average daily gain (ADG), C) feed conversion ratio (FCR), and D) reduction percentage of VH cases, receiving three different commercial mycotoxin binders; A, B or Excential Toxin Plus.