Mycotoxins: *in vitro* methodologies to assess detoxifying products

The contamination of feed by mycotoxins is a global safety issue, leading to substantial economic losses worldwide.

Mycotoxins are small and stable secondary metabolites produced by fungi which are extremely difficult to eradicate. Consumption of mycotoxin-contaminated diets may induce adverse effects on animal and human health, resulting in teratogenic, carcinogenic, oestrogenic or immune-suppressive effects.

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Physical or chemical treatments for mycotoxin decontamination often alter the nutritional value of the feed. That is why biological methods are usually considered among the most reliable strategies to reduce animal exposure to mycotoxins.

Decreasing mycotoxin availability

The objective is to decrease mycotoxin bioavailability by including detoxifying agents in the feed, which leads to a reduction of mycotoxin uptake and distribution to the blood and to the organs.

Detoxifying agents are usually classified in two different groups: mycotoxin binders and biotransforming agents.

The efficacy of those products for their adsorbing or degrading ability has to be tested. In vitro analysis is a powerful tool to screen and select detoxifying agents, before further in vivo studies, which are expensive and should be reduced as much as possible in accordance with the 3Rs principle (Replacement, Reduction and Refinement of animals used for research).

Many models have also been developed, ranging from single-concentration studies to more elaborate models, which mimic the gastrointestinal tract. Therefore, the objective of this article is to investigate the existing methods for in vitro testing to evaluate the mycotoxin detoxifying agents and identify the best prescreening strategies.

Mycotoxin detoxifying agents

There is no international consensus to evaluate the efficiency of mycotoxin detoxifying agents. In the European Union, the commission regulation (EC) No 386/2009, the mycotoxin detoxifying agents are defined in a functional group of feed additives 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 modes of action'. The feed additives belonging to this regulation are precisely defined in terms of chemical formula, description and methods of analysis and are detailed in several legal texts published in different issues of the Official Journal of the European Union.

Due to their physico-chemical and structural natures, action mechanisms and also methodologies used to evaluate mycotoxins detoxification potential vary according to the types of agents.

- **Adsorbing agents**

Adsorbing agents, also called binders, can be silica-based inorganic compounds or carbon-based organic polymers. The inorganic adsorbing agents consist of natural clay products (hydrated sodium calcium aluminosilicate, zeolites, bentonites), along with synthetic polymers. Organic agents involve some yeast cell wall components, as well as activated charcoal, synthetic polymers, humic substances and dietary fibres.

Adsorbing agents should be able to bind the mycotoxins without dissociating in the gastrointestinal tract of the animal. In this way, the mycotoxin-adsorbing agent complex passes through the animal and is eliminated via the faeces, and exposure of animals to mycotoxins is limited.

Different types of intermolecular interactions can be involved in the same adsorption process (hydrophobic, electrostatic, molecular recognition, etc).

- **Biotransforming agents**

Biotransforming agents include a great number of micro-organisms and pure enzymes that can degrade or detoxify several mycotoxins, such as bacteria, yeast, fungi, protozoa and enzymes. When selecting detoxifying and adsorbing agents, both qualitative and quantitative aspects (for example affinity, capacity and selectivity) must be observed. As much as possible, quantitative aspects should be measured and expressed in a way that makes it possible to compare adsorbing agents and predict their actions in different circumstances.

Review of experimental studies

In vitro preliminary tests of mycotoxin adsorption are regarded as great tools to screen potential mycotoxin-detoxifying agents. In practice, if no adsorption occurs in vitro, it is very unlikely that it will happen in vivo.

Considering adsorbing agents, different types of experiments can be performed in order to measure potential of products to counteract mycotoxins effect.

- **'Single-concentration' studies.**

These are the most widely used in vitro methods. It determines the adsorption of a pure synthetic toxin present in an aqueous medium at a fixed concentration after adding a specific concentration of binder. This method is simple to perform, cheap and not wasteful of toxin. It is useful to compare binders with similar mechanisms of action. However, results are strongly dependent on adsorbing agent concentration and correlation with in vivo is difficult to establish as it is not representative of real gastro-intestinal (GI) conditions.

- **'Isotherm' studies**

These aim to obtain a curve representing the amount of toxin adsorbed per unit of binder weight as compared to the concentration of toxin initially present in the medium. It usually gives the adsorption curve at a constant temperature for one concentration of binder and different concentrations of pure toxin. The opposite can also be done.

Adsorption isotherms can be performed in aqueous medium but also in the presence and absence of a feed matrix. The results of these studies are usually examined to assess whether a matrix commonly associated with mycotoxin contamination can affect adsorption efficiency.

Adsorption studies give a better idea of efficacy because it considers the reversibility of the adsorption process (equilibrium). It is considered as one of the best assays to determine total capacity of toxin adsorption by the binder.

Compared to 'single-concentration' tests, these methods are more complicated to perform, take more time to complete comparative studies and are more expensive.

- **'Static and dynamic GI' models**

These are in vitro approaches using gastro-intestinal models that try to mimic physiological in vivo conditions, taking into account the presence of digestive enzymes and their concentrations, pH, digestion time, and salt concentrations, among other factors.

Whereas 'static' models used fixed concentrations of enzymes, salt, bile acids etc at each step of digestion, 'dynamic' ones allow simulation of the different compartments of the GI tract (stomach/rumen), duodenum, jejunum and ileum) connected with peristaltic valves and with dynamic supply of digestive juices involved in digestion. It may include feed with and without natural contamination.

A dynamic model is the most appropriate method to compare with in vivo complexity and also allows bioaccessibility of the mycotoxins to be studied. Indeed, this study can be completed by simulation or model of intestinal barrier (cell culture).

This method determines the

transfer of mycotoxin through a lamina of jejunal epithelial cell (for example IPEC-J2 from swine), which is exposed to the mycotoxin alone or in combination with the binder. It is also used to evaluate potential interferences of the binder with nutrients and/or therapeutic substances. The integrity of the cell membrane can also be evaluated.

It gives complementary information to classical in vitro experimental studies about the metabolism of the mycotoxin, which is the most critical step. However, these tests require good expertise in cellular biology and are expensive.

Concerning biotransforming agents, their mycotoxins-detoxifying properties are usually evaluated in vitro by 'incubation studies'.

Micro-organisms (bacteria, fungi, yeast) are mixed with the mycotoxins in buffered or specific broth culture under favourable conditions for micro-organism growth (temperature, agitation, aerobic or anaerobic conditions and at physiologically relevant pH values).

Some variant broth cultures have been tested using complex matrix such as rumen fluid, digesta or including feed. In the same way, for enzymes, incubation studies are used, in general in buffer solution and incubation times varying from a few minutes to several days.

Temperature and pH conditions can also vary to get close to optimal conditions for enzymatic activities. Important considerations to take into account are the ability of tested agents to degrade mycotoxins without producing metabolites with potential toxic effects.

Indeed, every reaction does not necessary lead to a real detoxification, as the metabolised mycotoxin can acquire greater toxic properties than the parent compound. This is the case, for example, of zearalenone (ZEN) biotransformation performed in vivo by yeasts, which reduce the toxin to α-zearalenol, which is actually more oestrogenic than ZEN.

Quantitative aspects

Whatever the type of detoxifying agents tested and experimental conditions used, a mycotoxin quantification system is needed.

The most common methodologies are liquid chromatograpy (LC) or gas chromatography (GC) and immunochemical methods such as Enzyme-Linked Immunosorbent Assay (ELISA).

The type of analytical tools as well as methodologies used for sample preparation (extraction, purification) are highly dependent on the structure of mycotoxins studied.

HPLC is widely accepted as an official method for the determination of toxins. It is applied in conjunction with UV, fluorescence, amperometric or spectrofluorimetric detection. Both normal and reverse-phase HPLC are used for separation and purification.

A number of mycotoxins already have natural fluorescence (ochratoxin) and thus can be detected directly by HPLC-fluorescence (HPLC-FD). Others, such as fumonisin, can require an upstream quantification step such as derivatisation that can be performed by employing o-phthalaldehyde or 9-(fluorenylmethyl) chloroformate.

GC is a technique applicable to the compounds that are volatile and thermostable.

Detection is achieved by linking the system to mass-spectrometry (MS), flame ionisation or Fourier transform infrared spectroscopy. Most mycotoxins are not volatile and therefore also need an upstream quantification step.

Chromatographic methods are considered the most accurate and reliable methods of analysing mycotoxins.

Nevertheless, the expensive equipment, the necessity of skilled operators and the time required for analysis can be limiting factors when the number of analysis is high when various parameters are tested in vitro.

The need for accurate, sensitive, easier to use, less expensive, and more rapid tests led to the development of immunological methods for mycotoxins quantification.

These methods are based on the ability of specific monoclonal or polyclonal antibodies to distinguish the three-dimensional structure of a specific mycotoxin (antigens).

These can be performed as immunoaffinity column-based analysis (IAC) or ELISA.

This latest method is often used in commercially available kits (Ridascreen, Aflatoxin Cup, etc).

Several commercial kits are available on the market and can be used for in vitro tests. They are often designed for one type of mycotoxin, with application scope, specificity and cross reactivity, accuracy and precision, and measurement range.

All these parameters are important to take into account to choose the kits for in vitro tests.

In addition to ELISA, rapid screening tests and a number of new techniques such as biosensors are emerging.

They consist of a recognition element, commonly of biological origin, that produces a quantifiable response in a signal transduction element when in contact with the target analyte. Most signal transduction mechanisms are optical (colorimetric, fluorescence, enhanced chemiluminescence), electrochemical or surface plasmon resonance. Nowadays, most of these promising technologies are in experimental or development scale and are not currently used to quantify mycotoxins in a context of in vitro mycotoxin-detoxifying agents studies.

Conclusion

There is a great potential for the use of detoxifying agents to counter mycotoxins and various in vitro methodologies can be used to assess their efficacy (Fig. 1).

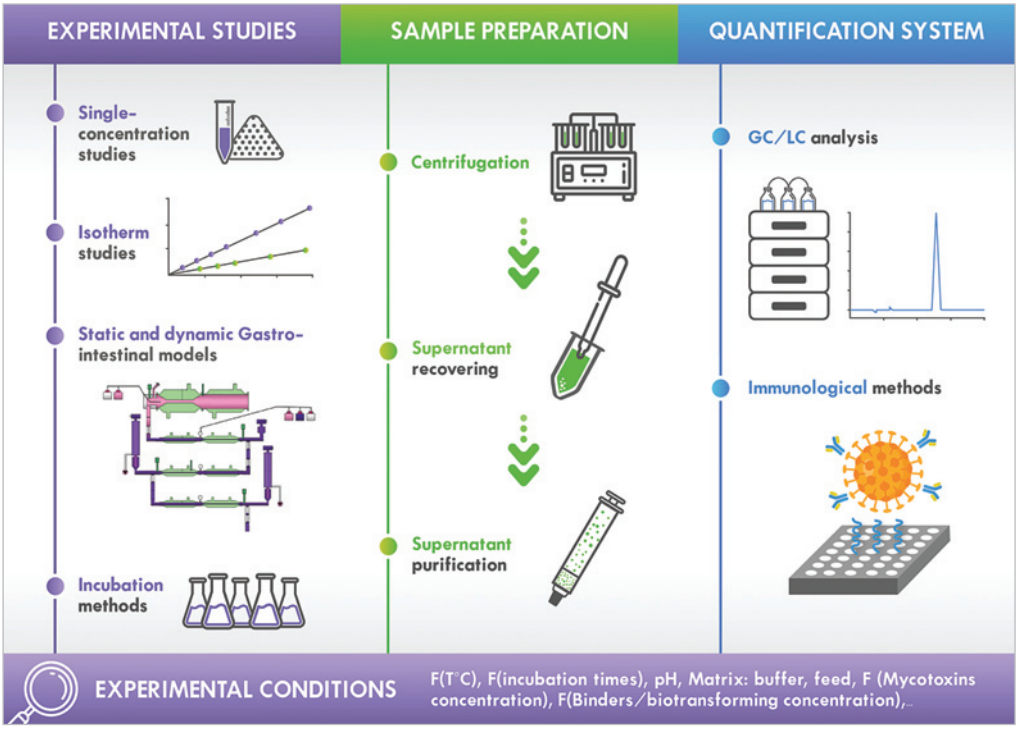
In vitro laboratory studies, ranging from simple-concentration studies to more complex studies simulating the gastro-intestinal tract are considered a useful prescreening strategy to select the promising adsorbing materials or products.

The more complex studies with addition of feed matrix will give a more reliable picture. It is strongly recommended to study the influence of digestive fluids and, if possible, to apply a dynamic gastro-intestinal tract model, before making a decision on the in vitro properties of the adsorbing agents and considering further tests in vivo.

Considering the multitude of parameters which can be used when in vitro tests are performed, it is important to determine what type of information is required (screening, binders efficacy optimisation, bio accessibility, gastro-intestinal metabolism) to choose the most adapted experimental conditions or use several of them simultaneously.

Finally, new research trends in this field should be devoted to the development of emerging methods such as biosensors and optimisation of experimental procedures allowing, in the meantime, a better correlation with in vivo trials. ■

Fig. 1. Summary of available tools (experimental studies and conditions, sample preparation and quantification systems) to measure the potential of products that may counteract the effect of mycotoxins.



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