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PREVENTION AND CONTROL OF MYCOTOXICOSES USING MODIFIED CLINOPTILOLITE

INTRODUCTION

Fungi may develop in stored grains following field inoculation. During the process of host colonization fungi utilize plant nutrients, and may also produce toxins that are harmful for humans and animals (Viera, 2003). Fluctuations in animal performance are relatively common, and sudden losses in feed conversion, as well as in other performances, are in general attributed to mycotoxin contaminated feeds.

It is assumed that at least 25% of grains are contaminated with known mycotoxins and probably more with still unidentified toxins (Leibetseder, 1989). Regarding those facts, presence of mycotoxins in animal feed represents a great problem for animal production in our country. During 1999—2000 presence of zearalenone, ochratoxin A, aflatoxin B_1 and T-2 toxin was faund out in 72.3—74.5, 41.2—63.6, 20.1—21.65 and 29.7—45.1% of scrutinized feed samples (Bočarov-Stančić et al., 2000). Moreover, laboratory data demonstrate that over 70% of feed samples are contaminated with two or more mycotoxins in amounts above maximal tolerable levels (Šefer et al., 1994; Mašić et al., 2002).

Based on a risk assessment, the prevention and control of mould development and mycotoxin in the food chain can be undertaken at consecutive levels. Basically, the best way to minimize the risk for a mycotoxin to come into food chain would be to prevent its formation in crop production and or during storage of feedstuffs. The use of contaminated feeds requires either the dilution with non-contaminated grains to an acceptable level or uses some of known methods to alleviate detrimental effects of mycotoxins, but financial, nutritional and toxicological considerations will temper this decision (Danicke, 2002).

PREVENTION AND CONTROL OF MYCOTOXINS

From a practical standpoint, the best means of restricting mycotoxin contamination is by prevention, in particular, by excluding or reducing toxigenic mould growth in the raw and processed material. When mycotoxin contaminated material is suspected or identified, it may be salvaged by the removal of contaminated material by mechanical separation techniques, by chemical extraction or by decontamination/detoxification of the material by physical, chemical or biological techniques (Smith and Moss, 1985).

Prevention. Mycotoxins can be produced by toxigenic moulds growing on (1) living plants, (2) decaying plant material and (3) stored products. Thus, mycotoxin prevention is based on making unfavourable conditions for moulds growing (Sinovec et al., 2000).

Pre-harvest treatment is based on methods of cultivating to improve plant vigor, the judicious use of insecticides and fungicides to reduce insect and fungal infestation, irrigation to avoid drought condition and, more recently, genetically methods to develop commercially acceptable varieties of crops that will be more resistant to toxigenic moulds or will inhibit toxin production.

Post-harvest treatment is mainly based on physical and chemical methods (Frisvad and Samson, 1991). *Physical methods*, in order to prevent or to delay mould spoilage of feeds, include heating, pasteurization and sterilization, cooling, vacuum packing, canning, drying and irradiation. Chemical methods are based on using preservatives (substances which inhibit or kill microorganisms in feeds) and, in practice, organic acids (propionic, sorbic, formic, etc.) are the most common group of chemical used.

Elimination. Once a product is contaminated with mycotoxins there are only two options if it is to be used for animal nutrition: (1) the toxin can be removed and (2) the toxin can be degraded into less toxic or non-toxic compounds (Smith and Moss, 1985).

Physical separation is connected with taking apart loose-shelled kernels, shriveled kernels and discolored or off-colored kernels. Such kernels can be separated either by hand or by color sorters.

Chemical separation includes numerous processes that have been developed to remove toxin from contaminated materials by various extraction techniques. Several procedures can be used to remove toxin and include extraction of toxin with appropriate solvents, simultaneous solvent extraction of oil and toxin and selective extraction of toxins from oil.

Degradation or detoxification are the methods of choice when mycotoxin contaminated material is suspected or identified. Making toxin less toxic or non-toxic (degradation) or extracting from contaminated materials (detoxification) seems to be the most promising way in a combat with mycotoxins. Many physical, chemical and biological methods have been investigated to eliminate (degrade or detoxify) *in situ* mycotoxin in raw material.

Physical methods include mainly heating and irradiation, but the adverse effects of heat treatment on the appearance and nutritive value of the products

makes the practical application of these methods highly doubtful. Better results could be achieved by combination of heating and increasing moisture content (e. g. hydro-thermic treatment).

Chemical methods include a wide range of chemicals that have been tested as reagents for the destruction of toxins such as acids, alkalis, aldehydes, oxidizing agents and several gases. Although technical treatments of contaminated feedstuffs have been proven to successfully degrade several mycotoxins, most of the procedures are expensive or were only labtested. Besides, limitations associated with decontamination process (changing nutritive value) and insufficient degradation makes this process untenable.

Biological methods are based on the natural ability of some microorganisms (bacteria, yeasts) to degrade most molecules of mycotoxins. Some investigations clearly show that where marked reduction in toxin presence in feedstuffs after inoculation of some microorganisms (P a s t e i n e r, 1998). Whether this is due to inhibition of production or breakdown of the toxin has not yet been determinated. The possibility of producing mycotoxin-degrading enzymes would have considerable commercial interest and application.

Nutritive approach. As the ingestion of contaminated feed is the main way of input mycotoxins in to animal body, a nutritional approach is the logical way to reduce impact of mycotoxins. Nutritive treatment is based primarily on raising quality of feed (increasing nutrients) in order to avoid or alleviate negative effects after mycotoxin ingestion (P i v a and G a l v a n o, 1999).

Due to protein synthesis inhibition as prime mode of action, increasing the protein content by 1—2% in the ratio could prevent mycotoxin effects. Besides, adding methionine (glutathione detoxification), phenylalanine (ochratoxin A) or neutral amino acids (competition with triptophane) is recommended in some cases. Increasing antioxidating nutrients (selenium, vitamin E, A, C) is the approach to reduce cell wall altering by action of free radicals.

However, most of these procedures are expensive, but more elegant and inexpensive solution would be a feed additive, which would detoxify the contaminated feeds *in situ*, i. e. during passage through the digestive tract. For this purpose, several adsorbents are used in practice.

Adsorbents are the substances, which are not absorbed from intestines, and they have the ability to physically bind some chemical structures decreasing their absorption. Many adsorbents were tested for binding capacity, and the degree of adsorption depends on the chemical structure of the mycotoxin in relation to the surface properties, and geometry of the adsorbent, as well as their polarity.

Several commercially available adsorbents claimed to bind mycotoxins were tested for adsorption of some mycotoxins. Most studies have used aluminosilicates such as hydrated sodium calcium aluminosilicates and zeolites, or even natural clays containing them. Similarly, the same effects could be achieved by addition of cell wall mannanoligosaccharide obtained from *S. cerevisaea* (D e v e g o w d a et al., 1998).

ZEOLITES

Zeolites are natural hydrated aluminosilicates with unlimited three-dimensional crystalline structure with large surface and negative polarity. Utilization of natural zeolites depends more or less upon their physical and chemical characteristics, like cation exchange, adsorption, hidratation/dehidratation, size and shape of mineral granules, porosity and hardness. Specific adsorption (chemisorptions), as well as other physical and chemical properties of zeolites (gas and fumes adsorption, hydratation, ion exchange, catalytic ability, etc.) is basically determined by crystal structure with pores system, acting as a sieve of molecular range. Natural zeolites, unlike other aluminosilicates, exhibit high adsorption ability even when low concentration of adsorbing material is present. According to the research data, zeolites can adsorb and inactivate about 35% of ochratoxin (Dumić et al., 1998).

The modified mineral adsorbents acquire wider role in human and veterinary medicine (Rodrigues et al., 1997). Changeable cations in zeolite minerals are bound by weak forces into tethraedric structure, with the possibility to be relatively easy removed or replaced by other cations from the solution. Changeable cations are positioned into zeolite canals of clinoptilolite, thus by ion exchange minerals with new characteristics could be produced, without disturbing original clinoptilolite structure (Tomašević-Čanović et al., 1997, 1998). Mineral adsorbent based upon clinoptilolite structure, with NH_4^+ changeable cation was created by technological manufacture of natural zeolites. Roentgen diffraction of natural (Ca-dominant) and modified (NH_4^+ — dominant) clinoptilolite were identical, while results of thermal analysis were different because the part NH_4^+ — ions were liberated upon heating.

In vitro trial. Two studies were conducted under *in vitro* conditions, particularly the feasibility of utilizing a clinoptilolite, aimed at alleviating and/or preventing harmful effects of AFB_1 and OTA. For experimental purposes MC was obtained by technological processing of natural zeolites, bearing a dominant Ca ion in exchangeable position. In order to contain a reversible NH_4^+ cation mineral adsorbent was grinding on particles under 63 μ, mixing with 10% solution of 1M NH_4Cl and keeping at 20°C during 3 subsequent days.

First study of MC binding capacity for AFB_1 was done in electrolyte made by the addition of 0.1M HCl/dm^3 and 0.05M NaCl/dm^3 in water solution, as well as 0.1M NaOH for achieving 2 or 7 pH of solution (Resanović, 2000). The 99% pure crystallyne AFB_1 (Sigma, 1162-62-8) was diluted in methanol in concentration of 0.1 mg AFB_1/mL and after that water was adjoined up to 100 mL. Experimental solutions were carried at 37°C and gentle shaking after modified clinoptilolite addition in the amount of 10 mg/cm^3. Index of absorption was calculated relative to the determined amount of pure AFB_1 in solution, followed the adding modified clinoptilolite after 5 and 30 min, as well as after 6, 24 and 48 hours.

On both pH values adsorbtion of AFB_1 by MC began with fast reaction and almost all AFB_1 were adsorbed in a few first minutes of contact. Later,

between 1—48 h, adsorbtion is slower and significantly less amounts of AFB_1 were binding to active center of mineral adsorbent, which is in agreement with other findings (Philips et al., 1995). An obtained result clearly shows that saturation of all MC binding center were not achieved by used AFB_1 concentration. AFB_1 adsorption index upon modified clinoptilolite was satisfying, ranging between 90 and 95% on both pH values (2 and 7) in the concentration range of 50—300 μg/g.

Second study of MC binding capacity for ochratoxin A was done in electrolyte made by the addition of 0.1 M HCl/dm^3 and 0.05M $NaCl/dm^3$ in water solution as well as 0.1 M NaOH for achieving 3.8 pH of solution (Zurovac-Kuzman, 2002). The 99% pure crystalline OTA (Sigma, O-1877) was diluted in methanol in the concentration of 0.2 mg OTA/mL and after that water was adjoined up to 100 mL. Experimental solutions were carried at 37°C and gentle shaking after addition modified clinoptilolite in the amount of 10 mg/cm^3. Index of absorption was calculated relative to the determined amount of pure OTA in solution followed the adding modified clinoptilolite after 120 min.

MC exhibits high adsorption ability even when low concentration of adsorbing material is present. According to the research data, zeolites can adsorb and inactivate about 35% of ochratoxin (Dumic et al., 1998).

The achieved results show that MC could very efficiently remove AFB_1 or OTA from the water solution. The obtained data are comparable with similar results done by Phillips et al. (1995), Kubena et al. (1990, 1993) and Harvey at al. (1993) that there was reached high level mycotoxin adsorption by different adsorbents (zeolites, silicates, phylosilicates) on different pH values, as well as different temperature. It has to be pointed that it is not possible to totally compare achieved results with other findings (Phillips et al., 1995), because accurate physical and chemical characteristics of used adsorbents were not cited.

In vivo trials. Many adsorbents in *in vitro* conditions show the ability to bind and adsorb different mycotoxins, but there are only a few references describing *in vivo* results. Thus, after preliminary study, further investigations were performed in an *in vivo* trial.

The aim of the first study was to examine the protective effects of modified clinoptilolite (MC) on the adverse effects of aflatoxin B_1 (AFB_1) on poultry (Resanović et al., 2001). The three-week long trial was performed on 21-day-old Hybro broilers divided into three groups. The control, as well as experimental groups was fed with mashes without AFB_1, while 0.5% MC was added to the feed for the second experimental group. The AFB_1 was administrated per os to both experimental groups in dose of 0.1 mg/kg BW daily. At the end of the trial all broilers were sacrificed and samples of livers were taken for pathohistological examination, as well as for determination of AFB_1 residues using TLC.

The liver of the treated broilers was enlarged, dark yellow colored and tender in consistence. In some cases punctiformes and maculoses extravasation could be seen. Varied amounts of fatty droplets could be detected in hepatocytes. Progressive fatty vacuolization, i. e. a different degree of fatty meta-

morphosis were spread centrolobularly or panlobularly. In altered areas focuses of extensive necrosis could be seen. Hyperplasia of the intrahepatic bile ducts was also prominent. Residues of AFB_1 were detected in all liver samples.

In the liver samples of the control group, as well as the group offered feed with added MC, no histopathological alteration or presence of AFB_1 residues was detected. The obtained results suggest that modified clinoptilolite represent a strong binding agent for AFB_1 and could prevent the adverse effects of AFB_1.

The aim of the second study was to examine the protective effects of MC on the adverse effects of OTA in poultry (Nedeljković-Trailović et al., 2001). The 42-day long trial was performed on 36 day-old Hybro broilers divided into three groups. After a 14-day long preexperimental period, the experimental groups were offered feed contaminated with OTA in the amount of 1.0 mg/kg, while 0.5% MC was added to the feed for the second experimental group. At the end of the trial all broilers were sacrificed and samples of kidneys were taken for pathohistological examination, as well as for determination of OTA residues using TLC.

Proximal tubules were predominantly affected, while glomerules were chiefly preserved. Cytoplasm of tubulocytes was microgranulated and the nuclei were masked. Vacuolization was noticed in a certain number of altered cells. Foci of acute tubular necrosis were noticed in a few tubules. In some cases weak hemorrhage could be seen in affected areas. Residues of OTA in the amount of 3.23±0.80 ppm were detected in all kidney samples.

In the kidney samples of the group offered contaminated feed with added MC morphological alterations were expressed in the form of intracellular edema. Epithelial cells of proximal tubules were enlarged with opaque cytoplasm, which caused tubule lumen stenosis. Apoptotic body could be noticed between some tubulocytes. Residues of OTA in the amount of 1.43±0.39 ppm were detected in all kidney samples.

CONCLUSIONS

During the process of host colonization fungi utilize plant nutrients, and also may produce toxins that are harmful for humans and animals. The extent of mould growth determines the degree of depletion in the nutrient content of the feedstuff. Mould mainly uses its host as a source of energy, and besides carbohydrates, fat utilization during mould development may be extensive. Reduction in performance of animals is usually credited only to mycotoxin effects, but it would be more accurate to accept an interaction between the reduction in nutrient content and the mycotoxin effects, especially when low levels of mycotoxin are present.

Based on a risk assessment, the prevention and control of mould development and mycotoxin in the food chain can be undertaken at consecutive levels. The use of contaminated feeds requires either the dilution with non-contaminated grains to an acceptable level or uses some of known methods to alleviate

detrimental effects of mycotoxins, but financial, nutritional and toxicological considerations will temper these decisions.

A new approach to mycotoxin control is the use of selective chemisorbents which added to feed are capable of forming irreversible complexes with mycotoxin molecules in the intestinal lumen. Such complexes are not digestible, pass down the digestive tract and are excreted in the feces. The net effect is to reduce the dose of adsorbed toxin to the point that it is below biological threshold. This allows contaminated feed to be fed with minimal losses in performance. The presented data clearly indicated that using modified clinoptilolite as adsorbent might reduce the negative impact of mycotoxins. Adsorptive capacity of modified clinoptilolite, obtained by technological processing of natural zeolites, was significantly increased by changing molecular arrangement and polarity.

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ПРЕВЕНЦИЈА И КОНТРОЛА МИКОТОКСИКОЗА КОРИШЋЕЊЕМ МОДИФИКОВАНОГ КЛИНОПТИЛОЛИТА

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Резиме

Микотоксини представљају сталну опасност у узгоју животиња јер су присутни у хранивима и негативно утичу на здравствено стање и производне резултате чак и у малим количинама. Поремећаји производних резултата животиња су врло чести, а слабија конверзија хране, као и слабији параметри производње, генерално се везују за коришћење хране контаминиране микотоксинима.

С обзиром да је главни пут уношења микотоксина ингестија контаминиране хране, оптимално решење је превенција контаминације спречавањем или редукцијом раста токсин-продукујућих плесни на хранивима. Са друге стране, у

случајевима када се посумња или идентификује присуство микотоксина у храниивима и/или храни елиминација се може извршити механичком сепарацијом, хемијском екстракцијом и деконтаминацијом или детоксификацијом коришћењем физичке, хемијске или биолошке методе.

Ефикасни методи за деконтаминацију хране веома су скупи и непрактични, посебно ако се ради о великој количини хране. Зато могућност избора представља коришћење адсорбената у циљу ублажавања негативних ефеката микотоксина на производне резултате животиња. Адсорбенти имају могућност да вежу микотоксине током пасаже хране кроз дигестивни тракт, односно да смање ресорпцију микотоксина присутних у храни.

Истраживања изведена последњих година, као и резултати сопствене лабораторије, указују да је степен контаминације хране веома висок са тенденцијом раста, што указује на чињеницу да ће микотоксини још дуго бити један од примарних проблема у исхрани животиња. Досадашња искуства, експериментална и практична, указују да коришћење адсорбената може да ублажи и/или превенира негативне ефекте микотоксина. Адсорпциони капацитет модификованог клиноптилолита, добијеног технолошком прерадом зеолита, значајно расте модификацијом молекулске структуре и поларности.