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ORIGINAL ARTICLE

Reduction of Ochratoxin A in Chicken Feed Using Probiotic

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Abstract

Mycotoxins present in fodders may evoke health problems of animals and people. The data published by FAO in 2001 show that 25% of raw materials are contaminated with mycotoxins, while their type and concentration are to a great extent dependable on the climatic zone. Biological detoxification of mycotoxins by the use of microorganisms is one of the well-known strategies for the management of mycotoxins in foods and feeds. The aim of this study was to determine the influence of spontaneous fermentation and that with the use of probiotic bacteria and yeast on ochratoxin A (OTA) concentration and the microbiota pattern during fermentation. The probiotic preparation is a natural product containing bacteria resistant to gastric juice and bile: *Lactobacillus paracasei* LOCK 0920, *Lactobacillus brevis* LOCK 0944, *Lactobacillus plantarum* LOCK 0945, as well as live yeasts *Saccharomyces cerevisiae* LOCK 0140 of high fermenting capacity. After 6-hour fermentation with the probiotic, in feed with a low concentration of ochratoxin A (1 mg/kg) the amount of ochratoxin A decreased by 73%. In the case of high a concentration (5 mg/kg) the decrease in ochratoxin A was lower at about 55%. This tendency was sustained during the following hours of incubation (12th and 24th hours). The application of probiotic bacteria and yeasts resulted in the reduction of aerobic spore forming bacteria. It can be concluded that the probiotic preparation containing bacteria of *Lactobacillus* strains and yeasts *Saccharomyces cerevisiae* used in the study was conducive to detoxification of ochratoxin A added to a feed.

Key Words

ochratoxin A; detoxification; probiotic, feed

INTRODUCTION

Ochratoxin A (OTA) is a contaminant often present in feeds and foods of plant and animal origin. It is mainly produced by several species of the genera *Aspergillus* and *Penicillum* [1]. This toxin can be formed during various phases of food production, already during the vegetation of plants on the fields, during harvest and in storage. The ability to synthesize mycotoxins is conditioned genetically, but it is also determined by environmental factors that include the following elements: substrate composition, its consistence, humidity, temperature and the presence of competitive microflora [2].

The data published by FAO in 2001 show that 25% of raw materials are contaminated with mycotoxins, while their type and concentration are to a great extent dependable on the climatic zone. In spite of the fact that in Europe we can observe less favorable conditions for synthesis of mycotoxins than for instance in North America or Asia, the problem of the presence of mycotoxins in grains is also very important in numerous European countries (primarily the Scandinavian countries, the southern parts of Germany, as well as Austria and Italy) [3]. Therefore, contamination of animal feeds with mycotoxins is considered to be a world-wide problem [4, 5].

Several toxic effects of OTA have been described, such as inhibition of protein synthesis, impairment of calcium homeostasis, induction of lipid peroxidation, oxidative stress, and DNA damage, with species and tissue specific differences [6]. Furthermore, this mycotoxin is considered to be nephrotoxic, cytotoxic, carcinogenic, teratogenic and immunosuppressive [7]. It may also induce gene mutation, although the mechanism of genotoxicity is not clear [8]. Oral LD50 values are 20 mg/kg for young rats and 3.6 mg/kg for chicks [9, 10].

The course of acute ochratoksing is very rare, but cases of minor poisoning are very common (without noticeable clinical symptoms), causing growth of body weight, laying, bigger use of fodder and water, deficiency of vitamins A, C and E, and reduced blood coagulability. The toxins attack the digestive and respiratory systems, kidneys, and liver, they damage the circulatory system, destroy digestive enzymes, cause allergies to food, and hamper the synthesis of proteins. Ochratoxins also reduce immunity, which leads to higher susceptibility to colibacillosis and salmonellosis [11, 12].

Biological detoxification of mycotoxins by the use of microorganisms is one of the well-known strategies for the management of mycotoxins in foods and feeds. Among the different potentially decontaminating microorganisms, *Saccharomyces cerevisiae* and lactic acid bacteria represent unique species which are widely used in food fermentation and preservation [13, 14].

The aim of this study was to determine the effeciency of a probiotic preparation composed of defined strains of lactic acid bacteria, yeasts and a yucca extract in reduction of ochratoxin A in chicken feed mixture during incubation *in vitro*.

MATERIALS AND METHODS

Materials. Wheat grain was inoculated with the strain of *Aspergillus ochraceus* NRLL 3174 (Collection of Pure Cultures, Agriculture Research, Peoria, USA) and incubated

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to obtain high concentration of ochratoxin A in accordance with a procedure described by Xiao et al. [15]. Twenty Erlenmeyer flasks (500 mL), each containing 30 g of feed-grade wheat and 30 ml of distilled water, were autoclaved for 30 min at 120°C and then inoculated with *Aspergillus ochraceus* NRLL 3174. The culture was maintained at 30°C in a dark room. The fermentation was terminated on day 12 of incubation, the contaminated wheat was dried for 72 h, ground and the ochratoxin A concentration was determined. The diet was formulated to meet or exceed the requirements of broilers (Table 1), adequate amount of contaminated wheat was substituted for uncontaminated one to obtain the final concentration of 1 or 5 mg of ochratoxin A per kg of diet.

Table 1. Composition of the feed mixture for broiler chickens, g/kg[1]

Component	g/kg
Wheat	330.7
Soyabean meal	380.6
Maize	200.0
Limestone	8.5
Dicalcium phosphate	18.0
NaCl	3.0
Rapeseed oil	50.0
Vitamin-mineral premix	5.0
Wheat starch or probiotic	1.0
L-lysine (78%)	1.0
DL-methionine (98%)	1.2
Feed enzyme	1.0

[1] concentration of ochratoxin A in feed mixture was 1 mg/kg or 5 mg/kg

The probiotic preparation (PP) used contained (per 1 kg): 10^{10} of *Lactobacillus* cells (*L. paracasei* LOCK 0920, *L. brevis* LOCK 0944 and *L. plantarum* LOCK 0945), 10^6 of yeast *Saccharomyces cerevisiae* LOCK 0140 cells and 50 g of *Yucca schidigera* extract. The strains derived from Centre of Industrial Microorganisms (LOCK), Institute of Fermentation Technology and Microbiology, Technical University of Lodz in Poland. The strains were formerly used in broiler trials [29], the preparation possess full probiotic documentation and is licensed [16].

Fermentation. The 1 kg samples of broiler diets with different ochratoxin A concentration were closed tightly in polyethylene bags and sterilized by radiation with doses ranging 25 kGy at ambient temperature. Radiation was carried out by ^{60}Co γ-rays at a dose rate of 0.2 Gy s^{-1}, Fricke dosimetry being employed. Sterilized control samples were kept tightly closed at 37°C and sampled after 6, 12 and 24 h for ochratoxin A analysis.

The one kg samples of each unsterilized diet without supplement (spontaneous fermentation) or with addition of 1 g PP per kg (probiotic fermentation) was mixed with distilled water in a proportion of 1:1.5 (w/w) and incubated at 37°C in an aerobic atmosphere for 24 h. Fermentation was made in triplicate. After 6, 12 and 24 h of fermentation the 10 g of each diet were sampled for microbiological and ochratoxin A analysis.

Analytical Procedures. The concentration of ochratoxin A in wheat grain and in diets was measured with an enzyme linked immunosorbent assay (ELISA direct competitive immunoassay with horseradish peroxidase conjugate) using a commercial ELISA kits (OchraQuant Ochratoxin, Romer Labs Diagnostic, Singapore) according to the procedure described in the Ochra-Quant Assay kit manual. In brief: ochratoxin A was extracted from 10 g of sample with 50 mL methanol:water (70:30 v/v). Independently, 100 µL of conjugate mixture were added to each well containing standards and previously filtered samples (50 µL). After mixing, 50 µL were transferred to wells containing antibodies and incubated for 15 min. Then, the content was eliminated, and the wells were washed five times with deionized water. Any excess of water was discarded and the wells were dried. Substrate (50 µL) was then added to each well, incubated for 5 min, and the reaction was stopped by adding 50 µL stop solution. Optic density was read with an UVM 340 microplate reader (Asys, Austria) using a 450 nm filter. The ochratoxin A concentration was calculated by extrapolating the optic density with the respective calibration curve [17].

Microbiological Analyses. The microbiological analyses were carried out according to the Polish Standard of PN-ISO [18]. The following bacteria species were identified: *Lactobacillus* on MRS agar medium (Merck), using a double-layer technique and anaerobic incubation at 35°C/72 h; *Clostridium* on TSC agar (Merck) and anaerobic incubation at 37°C/18–24 h; *Pseudomonas* on PM5 agar (BTL) and anaerobic incubation at 37°C/24 h; the coli group on VRBL agar (Merck) and aerobic incubation at 35°C/24 h; total number of anaerobic bacteria on Plate Count agar (Merck) and anaerobic incubation at 35°C/24 h, and total number of yeasts on YGC agar (Merck) and aerobic incubation at 20°C/120 h. Anaerobic bacteria were cultured in the anaerobic atmosphere of H:N:CO_2 1:8:1 (Anaerobic Workstation Concept 400, Biotrace Int.). The specific morphology of cells was checked under a microscope (Olympus CX-41). Each determination was done in triplicate. The results are presented as colony forming units (CFU) per gram samples.

Calculations and Statistical Analysis. Data were collected in triplicate subjected to tree-way analysis of variance by the general linear model (GLM) procedure of Statgraphics Centurion XVI. Statistical significance was accepted at $P<0.05$. In case when significant difference was found, post-hoc Tukey HSD difference test was used to compare between mean differences. For bacterial counts the analysis of variance was carried out on data submitted to logarithmic transformation.

RESULTS

Fermentation With a Probiotic Preparation

After 6-hours of fermentation with the probiotic cultures, the amount of ochratoxin A was reduced to 0.45 mg/kg (by 55%) in the case of the sample containing the initial concentration of ochratoxin A equal to 1 mg/kg in the medium, and to 1.36 mg/kg (by 73%) in the sample with a higher dose of the toxin. This reduction was sustained on the same level throughout the following hours of incubation. The results of the analysis of variation in the ANOVA test showed that the probiotic preparation reduced the level of ochratoxin A in a statistically significant way in comparison with the sample subject to spontaneous fermentation (Fig. 1).

Spontaneous Fermentation

During spontaneous fermentation, we observed that after 6-hours of fermentation of feed for broiler chickens containing ochratoxin A (with the initial concentration of 1 or 5 mg/kg) the amount of the toxin was reduced to 0.79 and 3.15 mg/kg, respectively, which translates into 21% and 37% (Figure 1). This tendency was sustained during the following hours of incubation (12 and 24 hours).

Control (Irradiated Feed)

After 6-hours of incubation at the temperature of 37°C of a feed mix that had been previously subject to radiation sterilization, the amount of ochratoxin A was reduced to 0.81 mg/kg (by 19%) in the case of the sample containing the initial concentration of ochratoxin A equal to 1 mg/kg in the medium, and to 4.23 mg/kg (by 15%) in the sample with a higher dose of the toxin. This reduction was sustained on the same level throughout the following stages of incubation (Fig. 1).

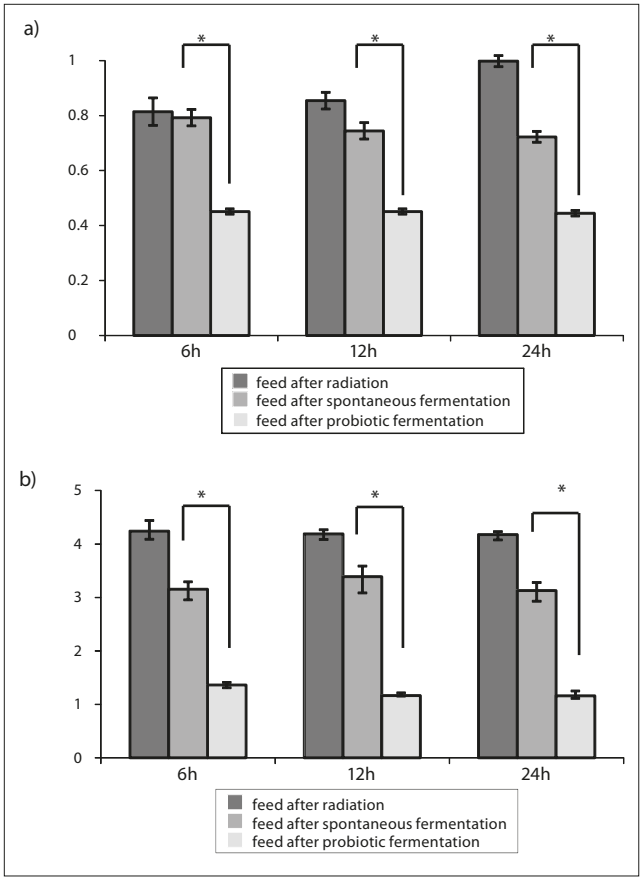

Figure 1. Concentration of ochratoxin A [mg/kg] in the diet (values are means of 3 replicates ± SEM):
a) initial concentration 1 mg/kg
b) initial concentration 5 mg/kg
Asterisks indicate significant differences (P < 0.05).

Change in the Microflora Pattern

Main effects of experimental treatments on bacteria and yeasts number was shown in Table 2. Initial dietary ochratoxin A did not affect bacteria and yeasts counts. *Lactobacillus*, total bacteria and yeasts counts increased (P<0.01) during incubation in diets without and with probiotic preparation. However, the increase in bacterial counts was higher by 2 orders of magnitude, in yests counts by 4 orders of magnitude in the diets supplemented with the probiotic preparation (interactions probiotic x time were highly significant).

The inhibition of viable aerobic spore forming bacteria increased in unsupplemented diets and decreased in the diets supplemented with probiotic (interactions probiotic x time were highly significant). Significant interaction (P<0.05) was found also between initial dietary ochratoxin A level and time of incubation for anaerobic spore forming bacteria, their numbers decreased in the diet containing 1 mg ochratoxin A after 6 h, than slightly increased, while in the diet containing 5 mg ochratoxin A it increased with time of incubation (Tab. 2).

Table 2. Main effects of supplementation with probiotic preparation, dietary ochratoxin A level and time of incubation on bacterial and yeasts counts, log CFU/g diet

Main effect	Total no of bacteria	Anaerobic spore forming bacteria	*Lactobacillus*	Total no of yeasts
Ochratoxin A level (A)				
1 mg/kg	7.62	3.02	6.44	4.92
5 mg/kg	7.55	3.32	6.27	4.82
SEM	0.341	0.418	0.246	0.210
Probiotic (P) supplement				
Without probiotic	7.12	4.10 ^{a}	4.33 ^{A}	2.87 ^{A}
With probiotic	8.05	2.24 ^{b}	8.37 ^{B}	6.87 ^{B}
SEM	0.341	0.418	0.246	0.210
Time of incubation (T)				
0	6.62 ^{A}	3.26	4.93 ^{A}	3.72 ^{A}
6 h	7.35 ^{B}	3.08	6.08 ^{B}	4.52 ^{B}
12 h	7.95 ^{C}	3.13	6.98 ^{C}	5.18 ^{C}
24 h	8.42 ^{D}	3.22	7.42 ^{D}	6.07 ^{D}
SEM	0.095	0.079	0.135	0.100
Interactions				
A x P	Ns	Ns	Ns	Ns
A x T	Ns	0.05	ns	ns
P x T	>0.001	>0.001	>0.001	>0.001

$^{a,b,\ A,B}$ within columns, for each main effect, means with different superscript letters are significantly different at: ^{a,b,} P <0.05; ^{A,B} P<0.01

DISCUSSION

Protection against mycotoxin contamination of plant raw materials processed in the course of biotechnological procedures and designated as feed for animals is primarily focused on preventive actions. These actions include proper conditions of cultivation, harvesting and storing of the crops [19]. If the plant raw material happens to be contaminated with mycotoxins it should be subject to detoxication. In case of feeds, FAO accepts some methods of mycotoxin elimination that use chemical compounds and physical processes, but they have to fulfill numerous requirements. The requirements include a condition saying that the feeds have to preserve their nutritive and sensory values, as well as the physical properties of the product. Moreover, the process of detoxification has to be economically justified [20].

Ochratoxin A detoxification strategies are classified depending on the type of treatment – physical, chemical or microbiological – and their objective is to reduce or eliminate the toxic effects of ochratoxin A by destroying, modifying or absorbing this mycotoxin. The ideal detoxification method would be easy to use and economical, and would not generate toxic compounds or alter other food quality parameters such as nutrient content [21]. Thus, first the effect of particular stages of processing on toxin reduction should be studied [22]. Where this is impossible, other additional ways of treatment (physical, chemical or microbiological) should be considered [23].

Biological detoxification of mycotoxins in food, raw products, mixed protein feeds and also in human and animal organisms is a novel and very promising method. Among the organisms that have been used in scientific research into mycotoxin elimination, one should enumerate: *Acinetobacter calcoaceticus* bacteria, *Aspergillus*, *Alternaria, Botrytis, Cladosporium, Phaffia, Penicillum* and *Rhizopus* moulds [24], lactic acid bacteria of the *Lactobacillus* strains [25] and *Saccharomyces* yeasts [26, 27]. Ŝtyriak et al. [28] examined 10 yeast strains of *Saccharomyces, Kluyveromyces* and *Rhodotorula* in respect of detoxification of ochratoxin A. It was proven that some yeast species of the *Saccharomyces* strains were characterized with the highest capability of ochratoxin biodegradation. Scott et al. [29] showed a decrease in the amount of ochratoxin A of 21% in malt wort during the fermentation of *Saccharomyces cerevisiae* var. *carlsbergensis* yeast. Nevertheless, special interest should be paid to lactic acid bacteria due to their favorable influence on human organisms and the widespread use in the production of fermented food. These bacteria inhibit the growth of moulds as well as their production of mycotoxins [30]. Probiotics also increase the use of feeds by means of producing hydrolytic enzymes [31]. Non-digestible carbohydrates (often described as non-starch polysaccharides) hamper the access of gastric juices to nutrients contained in the cells of plant feeds. They are also characterized by the ability to bind substantial amounts of water. Young animals fed with feeds containing significant amounts of soluble non-digestible carbohydrates are subject to a decrease in their productivity indicators and diarrhea. They also tend to suffer from deficiency of vitamins and mineral elements. Probiotics produce enzymes that decompose carbohydrates. They also increase the activity of the host's enzymes, such as β-galactosidases, saccharase and maltase. Ŝkinjar et al. [32] examined four species of lactic acid bacteria (*Lactobacillus salivarius* subsp. *thermophilus, Lactobacillus delbrueckii* susp. *bulgaricus, Bifidobacterium bifidum*) in respect of their ability to eliminate ochratoxin A from the environment. It was stated that all the examined strains eliminated the toxin in 100% up to the amount of 0.1 μg/ml. However, the higher the toxin concentration was, the lower their ability to eliminate the toxin, and above 1 μg/ml it did not exceed 40%.

In order to investigate the mechanisms which account for the removal of mycotoxins by LAB, the effects of viable and heat inactivated bacteria were compared in a number of studies [33]. Additionally, the bacteria were treated with enzymes (such as pronase E and lipase) or periodate, which cause alterations to the structure of the cell walls [34]. On the basis of the results obtained in these experiments, it was postulated that the removal of aflatoxin B_1 and zearalenone is due to the non-covalent binding of the toxins to the carbohydrate moieties of the cell walls. Also the detoxification of heterocyclic aromatic amines was explained by this mechanism [35]. However, since a decrease in their toxic effects was also observed in the case of cytosolic preparations of LAB, it was hypothesized that other mechanisms (e.g. interactions with short chain fatty acids) might also play a role [36]. As mentioned above, ochratoxin A is removed far more efficiently by viable bacteria, which can be taken as an indication that processes other than binding to the cell walls are involved (*e.g.* metabolic conversion by the release of specific enzymes). However, the mechanisms which account for the detoxification are not yet understood. In this context, it is notable that it has been shown that ochratoxin A is detoxified by the representatives of the ruminal microflora via cleavage of the peptide bond, which leads to release of phenylalanine [37].

On the other hand, the research presented hereby showed that a probiotic preparation containing both lactic acid bacteria (*Lactobacillus paracasei* LOCK 0920, *Lactobacillus brevis* LOCK 0944, *Lactobacillus plantarum* LOCK 0945, in the dose of 10^{10}/1 kg of the preparation) and *Saccharomyces cerevisiae* yeasts LOCK 0140 (10^6/1 kg of the preparation) was conducive to inactivation of ochratoxin A in a typical feed mix for chickens. After 6-hours of fermentation at a low concentration of ochratoxin A (1 mg/kg), the amount of ochratoxin A decreased by 55%. In the case of a high concentration (5 mg/kg), the reduction in ochratoxin A was lower and equaled about 73%. This tendency was sustained also in the subsequent (12 and 24) hours of incubation.

It can be concluded that the probiotic preparation containing bacteria of *Lactobacillus* strains and yeasts *Saccharomyces cerevisiae* used in the study was conducive to detoxification of ochratoxin A added to a feed mixture for chickens. The application of probiotic cultures of bacteria and yeasts also resulted in the reduction of aerobic spore forming bacteria.

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