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Regular paper

Probiotic preparation reduces faecal water genotoxicity and cytotoxicity in chickens fed ochratoxin A contaminated feed (*in vivo* study)*

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The aim of the present study was to assess the genotoxicity and cytotoxicity of the faecal water of chickens fed ochratoxin A (OTA) contaminated feed with and without probiotic preparation. The study was performed on 20 healthy female Ross broiler chickens divided into 4 groups: control chickens — fed with non-supplemented feed; PP chickens — fed feed supplemented with the probiotic preparation; OTA chickens — fed feed contaminated with 1 mg per kg of OTA; OTA + PP chickens — fed feed contaminated with 1 mg per kg of OTA and supplemented with the probiotic preparation. Faecal water samples were collected on the 35th day of life of chickens from each group. Genotoxicity was measured using the comet assay, and cytotoxicity by means of MTT tests. Mean DNA damage, measured as the percentage of DNA in the tails of the comets, was 8.50 ± 1.10 for chickens fed OTA at 1 mg/kg and 6.41 ± 0.67 in the controls. The supplementation of feed with the probiotic preparation decreased the extent of DNA damage to 4.74 ± 0.78. In the control group of chickens the average cytotoxicity was 38.5 ± 0.5 (in MTT), while in the probiotic preparation group (PP group) it was 31.8 ± 0.7 (in MTT). After supplementation of the feed with the probiotic preparation, the genotoxicity and cytotoxicity were decreased in a statistically significant manner.

Key words: ochratoxin A, probiotics, chicken, genotoxicity, cytotoxicity

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INTRODUCTION

Mycotoxins are secondary fungal metabolites and remain the subject of major concern throughout the world. Where present, they usually occur as trace contaminants in agricultural products, in concentrations ranging from nanogram to microgram quantities per gram of material. Intensive research on mycotoxins has been conducted for the past 35 years. The first group of mycotoxins which was isolated and described in 1961, in the wake of several acute animal disease outbreaks in 1960, consisted of aflatoxins (Goldblatt, 1969). After the discovery of aflatoxins, ochratoxins were the next major group of mycotoxins identified (van der Merwe *et al*., 1965). It was typical for mycotoxicoses in general that a seasonal peak in the occurrence of a toxin drew attention to the agents which triggered acute clinical diseases. Such acute intoxications can be dramatic and economically devastating, but they constitute only a small fraction of the biological and economic consequences of the more usual chronic intoxications caused by lower levels of toxin (Rocha *et al*., 2014).

Data published by FAO in 2001 show that 25% of agricultural products are contaminated with mycotoxins, with their type and concentration largely dependent on the climatic zone. In spite of the fact that in Europe we can observe less favorable conditions for the synthesis of mycotoxins than *e.g.* in North America or Asia, the problem of mycotoxins in grains is a very important issue also for many European countries (primarily the Scandinavian countries, the southern parts of Germany as well as Austria and Italy) (FAO/WHO, 2001). Therefore, the contamination of animal feeds with mycotoxins is considered to be a world-wide problem (Siegel and Babusco, 2011).

Ochratoxin A (OTA), a secondary metabolite with teratogenic, hepatotoxic, carcinogenic and nephrotoxic activity in many animal species, including human beings (Höhler, 1998), is mainly produced by *Aspergillus ochraceus* and *Penicillium viridicatum*. Being ubiquitous, these moulds can easily contaminate foodstuffs. OTA is predominantly found in cereal grains, cereal products, legumes, oilseed, coffee beans and animal feed. Moreover, OTA has been identified in the tissue and organs of animals (pigs, chicken) fed contaminated feed. The presence of OTA has also been reported in human milk. The IARC (International Agency for Research in Cancer) has classified OTA as Group 2B, a possible human carcinogen (IARC, 1993). In several areas of Eastern Europe, where chronic exposure to OTA occurs, involvement of this mycotoxin in the aetiology of cancer of the urinary system and in kidney pathologies typical of Balkan Endemic Nephropathy (BEN) has been suspected. Studies on the correlation between OTA and BEN (Puntaric *et al*., 2001) have shown higher OTA contamination levels in cereals from endemic areas with respect to cereals from non-endemic areas (Muscarella *et al*., 2004).

Protection against mycotoxin contamination of raw plant material which is to be biotechnologically processed and used as animal feed focuses on its growth requirements, harvest and storage (Fraga *et al*., 2007).

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Minimal Essential Medium; EDTA, ethylenediaminetetraacetic acid; FAO, Food and Agriculture Organization of the United Nations; MTT test, reduction assay the tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OTA, ochratoxin A; PP, probiotic preparation

However, sometimes it is very difficult to minimise the production of toxins by moulds, especially if a crop is exposed to changeable weather conditions favourable to contamination of cereals with moulds and their toxic products. If plant material is contaminated with mycotoxins, it should be subject to detoxification (Akande *et al.*, 2006). In the case of feed, FAO accepts the methods of eliminating toxins by means of chemical compounds and physical processes which fulfil several requirements, concerning *e.g.* the preservation of the nutritive and sensory value and physical properties of products, as well as the economic justification for the decontamination process (Allameh *et al.*, 2005).

Biological detoxication of mycotoxins in food, raw material and concentrated feed as well as in human and animal organisms is a new and very promising method. Microorganisms used for the elimination of mycotoxins include lactic acid bacteria *Lactobacillus* sp. and yeast *Saccharomyces* sp. (El-Nezami *et al.*, 2000; Shetty & Jespersen, 2006). Particular attention is paid to lactic acid bacteria (LAB), because they can contribute to the inhibition of mould development and production of mycotoxins (Haskard *et al.*, 2001; Slizewska *et al.*, 2010).

The aim of the present study was to assess the influence of the probiotic preparation on the genotoxicity and cytotoxicity of the faecal water of chickens fed OTA contaminated feed with and without probiotic preparation.

MATERIALS AND METHODS

Probiotic preparation. The probiotic preparation consisted of (per 1 kg): 10^{10} Lactobacillus cells (Lb. paracasei LOCK 0920, Lb. brevis LOCK 0944 and Lb. brevis LOCK 0945), 10^6 yeast Saccharomyces cerevisiae LOCK 0140 and 50 g of Yucca Schidigera extract. The strains were from the Collection of Industrial Microorganisms (LOCK), Institute of Fermentation Technology and Microbiology, Technical University of Lodz, Poland. The strains used in the study for the preparation possess full probiotic documentation (Śliżewska *et al.*, 2012) and are licensed (Michalowski *et al.*, 2012).

Treatment of animals. The study was performed on 20 healthy female Ross broiler chickens divided into 4 groups, 5 animals each, kept separately at the Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna, Poland. The groups were: control chickens – fed non-supplemented feed; PP chickens – fed feed supplemented with the probiotic preparation (2 g/kg of feed between 1st and 14th day of life and 1 g/kg until the end of the experiment); OTA chickens – fed feed contaminated with 1 mg per kg of OTA; OTA + PP chickens – fed feed contaminated with 1 mg per kg of OTA and supplemented with the probiotic preparation. All experimental procedures involving animals were conducted according to the Polish legal regulations concerning experiments on animals (following a decision issued by the Local Ethical Committee for Experiments on Animals at the University of Life Sciences in Warsaw).

Faecal water preparation. Faecal water samples were collected on the 35th day of life, for each diet, in plastic bags, sealed and stored at –20°C until analysis. Faecal water samples were extracted from faeces by homogenising the faeces with sterile water (1:5, v/v) for 2 min followed by centrifugation (10 700 × *g*, 40 min, 4°C). The supernatant fractions were distributed to 1.5 ml Eppendorf tubes and stored at –20°C prior to analysis.

Lymphocyte isolation and cell treatment. Blood was obtained from young, healthy, non-smoking donors. Peripheral blood lymphocytes were isolated by centrifugation at a density gradient of Histopaque-1077 (15 min, 182 × *g*) (Sigma). The pellet containing peripheral blood lymphocytes was suspended in RPMI 1640 medium (Sigma). The final concentration of the lymphocytes in each sample was adjusted to 1×10^5 cells/ml. Lymphocytes were incubated with 10% faecal water for 1 h at 37°C. In addition to the dietary treatments, two additional treatments were included in order to validate the assay. These treatments included control cells that only received the RPMI 1640 medium and a treatment in which cells were treated with hydrogen peroxide at 20 μM applied for 10 min at 4°C.

Caco-2 cell culture and treatment. The cells were cultured in Roux flasks as monolayer in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM, Sigma) with the addition of 10% FBS (Gibco), 200 mM L-glutamine (Sigma), 25 mM HEPES (Sigma), 100 IU/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). The cells were incubated in a CO_2 incubator at 37°C in 5% CO_2 for 7–10 days to become fully differentiated. After reaching confluence, the cells were subcultivated every week. The medium was changed every 2–3 days. Caco-2 cells were tripsinised with 1% trypsin-EDTA (Sigma) for 2 min and gently shaken off the plastic flask. The reaction was terminated by addition of 10 ml of DMEM with 10% FBS. For the removal of trypsin, the cell suspension was transferred to a 15 ml Falcon tube, centrifuged (182 × *g*, 5 min), decanted and resuspended in DMEM. After the determination of cell count and viability by trypan blue, the cells were ready to use.

Comet assay. After incubation, the cells were centrifuged (182 × *g*, 15 min, 4°C) and the comet assay was performed in alkaline conditions according to the procedure of Singh et al. (1988) with some modifications (Blasiak and Kowalik, 2000). The cells were suspended in 0.75% LMP agarose and layered onto slides precoated with 0.5% agarose and lysed for 1 h at 4°C in a buffer consisting of 2.5 M NaCl, 1% Triton X-100, 100 mM EDTA and 10 mM Tris. After lysis, the slides were placed in an electrophoresis unit and DNA was allowed to unwind for 20 minutes in an electrophoretic solution containing 300 mM NaOH and 1mM EDTA. Electrophoresis was conducted at 4°C for 20 minutes at an electric field strength of 0.73 V/cm (30 mA). Then, the slides were neutralized with 0.4 mol/l Tris and stained with 1 μg/ml DAPI (4',6-diamidino-2-phenylindole) and covered with cover slips. Comets were observed at 200× magnification with a fluorescence microscope (Nikon, Japan) attached to a video camera and connected to a personal computer-based image analysis system Lucia-Comet v. 4.51 (Laboratory Imaging, Prague, The Czech Republic). Fifty images were selected from each sample, and DNA damage was measured as the percentage of DNA in the tail of the comets. Two parallel tests with aliquots of the same sample were performed for a total of 100 cells and mean DNA damage was calculated. Each experiment was repeated three times. Comet results were analysed using two-way analysis of variance (ANOVA) while a particular mode of interaction × time was used to compare effects evoked by chemicals at this mode of interaction and suitable control. As no statistically significant interaction was found, one-way analysis of variance was applied. Differences between the means were compared using Scheffe's multiple comparison test. Results were presented as mean ± S.E.M.

Table 1. DNA damage measured as a percentage of DNA in the comet tail in an alkaline comet assay in human peripheral blood lymphocytes induced by faecal water of broiler chickens.

Chicken number	Control	PP	OTA	OTA + PP
	DNA [%] in comet tail±S.E.M.			
1	8.19±1.48	6.17±0.01	10.43±0.64	6.79±0.46
2	5.90±1.24	4.93±1.40	9.28±0.86	6.27±0.70
3	6.90±1.07	6.82±1.62	8.13±3.96	3.94±0.46
4	7.49±2.27	4.14±0.51	3.70±0.52	2.60±0.48
5	4.15±0.81	4.99±1.46	7.78±2.35	4.09±0.42
Mean[a] ± S.E.M.	6.41±0.67	5.30*±0.59	8.50±1.10	4.74**±0.78

The number of cells analysed in each treatment was 100. Data are mean values (± S.E.M.) from all chickens in each group for which faecal samples were available. [a] the results displayed are the mean of three independent experiments; *,**statistically different from: *control, **OTA (1 mg/kg), ANOVA ($P < 0.05$).

Cytotoxicity testing. Caco-2 cells were added at 5×10^3 cells/well in 96 well plates and 80 µl of culture medium was added into each well. The cells were then incubated at 37°C overnight in 5% CO_2 to allow them to attach to the plates. Next, a 20 µl aliquot of the appropriate sample of chicken faecal water was added to each well. The cells were incubated in a CO_2 incubator at 37°C in 5% CO_2 for 72 h. After incubation, the cells were washed twice with PBS/EDTA, and 100 µl of MTT (0.5 mg/ml in PBS) was added into each well. The cells were further incubated at 37°C in 5% CO_2 for 3 h. After incubation, the neutral red was carefully taken off and 50 µl of desorbing solution (1% acetic acid, 50% ethanol and 49% distilled water) was added to each well. In the MTT test, the formazan precipitates were solubilized by the addition 50 µl of DMSO (Sigma). The absorbance was measured at 550 nm using a microplate reader (ASYS, Biogenet). The absorbance of the control sample (Caco-2 cells in DMEM) was set at as 100% cell viability.

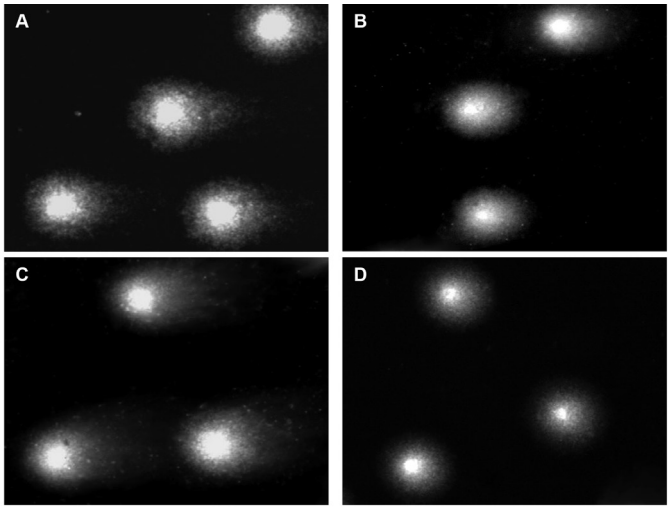

Figure 1. Representative comets of DAPI stained human blood lymphocytes incubated for 1 h at 37°C with faecal water of broiler chickens: (**A**) control groups of chickens; (**B**) chickens fed with fodder supplemented with probiotic preparation; (**C**) chickens fed with fodder contaminated with 1 mg/kg of OTA; (**D**) chickens fed with fodder contaminated with 1 mg/kg of OTA and supplemented with probiotic preparation.

Analysis was performed in three independent experiments, each conducted in triplicate.

% cell viability = (sample O.D./control O.D.) × 100%
% cytotoxicity = 100 – % cell viability

Indicate main effect and interactive terms for the two-way ANOVA.

Results were presented as mean ± S.D.

RESULTS

Genotoxicity

Non-exposed samples (cells in RPMI 1640, the negative control) displayed DNA damage levels of 1.45±0.84. Treatment of the cells with 20 µM hydrogen peroxide (positive control samples) resulted in damage levels of 26.36±1.62 (results from three independent experiments).

Supplementation of the feed with the probiotic preparation only (PP — probiotic group of chickens) decreased the genotoxicity of faecal water compared to the control group of chickens (with no supplementation) (Table 1). In the control group, DNA damage was in the range from 4.15±0.81 to 8.19±1.48 (with the mean value of 6.41±0.67), while in the probiotic preparation group of chickens (PP group) it was from 4.14±0.51 to 6.82±1.62 (with the mean value of 5.30±0.59) (Table 1, Fig. 1A and B). The results were statistically significant as marked in the Table 1.

Incubation of human lymphocytes with the faecal water of the chickens induced considerable DNA damage, yielding comet tails ranging from 3.70±0.52 to 10.43±0.64 (with the mean value of 8.50±1.10) for chickens fed feed contaminated with 1 mg/kg of OTA (OTA group) (Table 1 and Fig. 1C).

After supplementation of the feed with the probiotic preparation, we observed a statistically significant decrease in the level of DNA damage, which was in the range from 2.60±0.48 to 6.79±0.67 (with the mean value of 4.74±0.78) for chickens fed with feed contaminated with 1 mg/kg of OTA (OTA + PP group). Supplementation with the probiotic preparation reduced the genotoxicity of faecal water in chickens fed feed contaminated with 1 mg/kg of OTA (Table 1 and Fig. 1D).

Cytotoxicity

Supplementation of the feed with the probiotic preparation only (PP — probiotic group of chickens) statistically decreased the cytotoxicity of faecal water compared to the control group of chickens (with no supplementation) (Fig. 2). In the control group of chickens, the average cytotoxicity was 38.5±0.5, while in the probiotic preparation group (PP group) it was 31.8±0.7 (Fig. 2).

Incubation of Caco-2 cells with the faecal water of chickens fed feed contaminated with 1 mg/kg OTA (OTA group) induced considerable cytotoxicity with a mean value of 53.9±0.4 for chickens fed with feed contaminated with 1 mg/kg of OTA (OTA group) (Fig. 2).

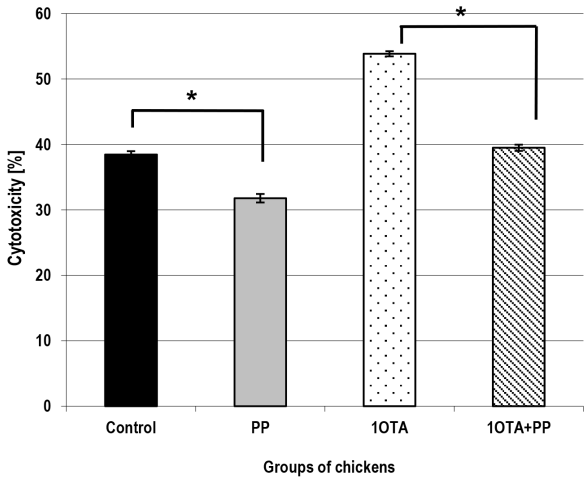

Figure 2. Cytotoxicity of the faecal water of chickens after feeding with probiotic preparation and/or ochratoxin A.
Asterisks indicate significant differences ($P < 0.05$). The results displayed are means (±S.D.) of three independent experiments.

After supplementation of the feed with the probiotic preparation, a statistically significant decrease in the cytotoxicity of the faecal water was observed, which was 39.5±0.5 for chickens fed feed contaminated with 1 mg/kg of OTA (OTA + PP group).

DISCUSSION

Biological detoxification of mycotoxins in food, raw products, mixed protein feeds and also in human and animal organisms is a relatively new and promising method. Several microorganisms have been used to eliminate mycotoxins: *Acinetobacter calcoaceticus*, *Aspergillus niger*, *Bifidobacterium* sp., *Lactobacillus* sp., *Enterococcus faecium*, *Pseudomonas* sp. and *Saccharomyces* sp. strains (Sangare *et al*., 2014; Štyriak *et al*., 2001; Topcu *et al*., 2010; Vinderola and Ritieni, 2015). Special attention should be paid to lactic acid bacteria because of their favourable influence on human organisms (probiotic bacteria) and widespread use in the production of fermented foods. These bacteria inhibit the growth of moulds as well as mycotoxins production (Madrigal-Santillán *et al*., 2006).

The aim of our study was to check if the probiotic preparation (composed of lactic acid bacteria, yeasts and *Yucca schidigera* extract) influenced the genotoxicity and cytotoxicity of the faecal water of chickens fed OTA contaminated feed. We observed that genotoxicity of the faecal water occurred both in the presence or absence of OTA. However, after supplementation of the feed with the probiotic preparation, genotoxicity decreased in a statistically significant manner. The decrease was more pronounced in the group of chickens fed feed contaminated with OTA (OTA+PP). Therefore, supplementation with the probiotic preparation contributes to the reduction of genotoxicity of the faecal water of chickens fed OTA contaminated feed. The mechanism underlying this effect may be associated with the ability of the probiotic preparation to bind OTA, resulting in its decreased concentration in the chicken colon, although the supplementation of feed with the probiotic preparation only (PP) also decreased DNA damage in the control group. After the supplementation of feed with the probiotic preparation, a statistically significant decrease in the cytotoxicity of faecal water was observed in chickens fed feed contaminated with 1 mg/kg of OTA (OTA + PP group).

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 (Haskard *et al*., 2001). Additionally, the bacteria were treated with enzymes (such as pronase E and lipase) or periodate which cause alterations in the structure of the cell walls (Fuchs *et al*., 2008). On the basis of the results obtained in those 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. The detoxification of heterocyclic aromatic amines was also explained by this mechanism (Knasmüller *et al*., 2001). However, since a decrease in their toxic effects was also seen with cytosolic preparations of LAB, it was hypothesized that other mechanisms (e.g. interactions with short chain fatty acids) may also play a role (Stidl *et al*., 2007).

The mechanisms involved in the cellular toxicity of OTA are still unresolved. Distinct mechanisms such as active transport by organic anion transporters and cellular accumulation (Doorten *et al*., 2006), as well as the chelation of OTA with cellular Fe^{2+} molecules resulting in an increased production of ROS, have been proposed to mediate OTA toxicity (Schaaf *et al*., 2002). Cellular DNA damage has been linked in several studies to biotransformation processes, both *in vitro* and *in vivo*, as an increased number of DNA adducts were found in human bronchial epithelial cells expressing CYP2C9 (El Adlouni *et al*., 2000) and kidney microsomes (Obrecht-Pflumio and Dirheimer, 2000) as well as in mice (Obrecht-Pflumio *et al*., 1996), rats and pigs (Faucet *et al*., 2004) treated with OTA. OTA induced mutations on the lacZ' gene in NIH/3T3 cells, transfected with distinct human CYP450 enzymes (de Groene *et al*., 1996). These cells, transfected with a specific human CYP450 enzyme and at the same time with human oxidoreductase were used in the experiments described here, directed to the analysis of single-stranded DNA breaks (SSBs) following single cell electrophoresis (comet assay) (Doorten *et al*., 2006).

Several lines of evidence indicate that the carcinogenic action of OTA is correlated with its genotoxicity, as reflected by DNA adduct formation (Castegnaro *et al*., 1998; Pinelli *et al*., 1999), leading to mutagenic effects (Malaveille *et al*., 1991; de Groene *et al*., 1996). DNA-xenobiotic binding is considered to be a critical step in the initiation of mutagenesis and carcinogenesis (Miller & Miller, 1981). The process of chemical carcinogenesis is initiated by the covalent binding of carcinogens or their reactive metabolites to DNA, thus forming DNA adducts (Pinelli *et al*., 1999). There is a good correlation between DNA adduct formation and the frequency of mutations (for a review see Lutz & Gaylor, 1996). To interact with cellular macromolecules and thus initiate cancer, most chemical carcinogens require metabolic activation (Miller & Miller, 1981), but the metabolic pathways involved in OTA toxicity and genotoxicity remain poorly understood (Pinelli *et al*., 1999).

There are several mechanisms for OTA toxicity at the cellular level. OTA is a competitor of phenylalanine-tRNA ligase, thereby inhibiting protein synthesis. The presence of phenylalanine or aspartame (an analogue of phenylalanine) decreases toxicity by competition with OTA (Creppy *et al*., 2004; Bayman & Baber, 2006). Other enzymes are also affected by exposure to OTA. Other mechanisms include formation of DNA adducts, apoptosis, interference with the cytoskeleton, lipid peroxidation and inhibition of mitochondrial respiration (Bayman & Baker, 2006).

The cytotoxicity of ochratoxin A has been investigated by several authors and EC_{50} values were generally observed to lie within the micromolar range. Differences between the specific EC_{50}s reported can probably be attributed to the use of different cell lines, different endpoints (e.g., MTT reduction, neutral red uptake, cell counting or LDH release) or indeed to the presence or absence of serum in the culture medium (Dietrich *et al.*, 2001; Heussner, 2006).

CONCLUSION

In summary, our results show that the probiotic preparation decreases the genotoxicity of the faecal water of chickens fed feed contaminated with 1 mg/kg ochratoxin A and can be considered as a preventive agent in poultry breeding.

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