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Impact of dietary *Trichosporon mycotoxinivorans* on ochratoxin A induced immunotoxicity; *In vivo* study

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ABSTRACT

Ochratoxin A (OA), the secondary metabolite of certain *Aspergillus* and *Penicillium* species, is one of the potent biological immune-suppressor. The present study was designed to explore the *in-vivo* efficacy of *Trichosporon mycotoxinivorans* (TR); yeast strain isolated from the hindgut of the termite *Mastotermes darwiniensis*, against the immunotoxicity of OA in broiler birds. For this purpose, broiler chicks were offered diet added with TR (0.5, 1.0 or 2.0 g/kg feed) and/or OA (0.15, 0.3 or 1.0 mg/kg feed) for 42 days. Dietary OA at all levels, resulted in significant reduction ($p \leq 0.05$) in the immune response of broiler birds as recorded by vacuolation and darkly stained pyknotic nuclei in bursa of Fabricius and thymus, humoral immune responses to sheep red blood cells (SRBC), *in-vivo* lymphoproliferative response to Phytohemagglutinin-P (PHA-P) and mononuclear phagocytic system function assay. Addition of TR in broiler diet significantly reduced ($p \leq 0.05$) the immunotoxicity of OA at 0.15 and 0.30 mg/kg; however, against higher dietary level of OA (1.0 mg/kg), a partial protection was observed. Feeding TR alone had no immunomodulatory effect at any of tested level. Dietary addition of TR is proposed as an approach to combat the OA mediated immunological damages in broiler birds.

1. Introduction

Ochratoxins (OT), the secondary metabolites of toxigenic fungi, produced by the some *Aspergillus* and *Penicillium* species, frequently contaminate the food and feed (Murugesan et al., 2015; Hassan et al., 2012a). The favorable environmental and/or improper storage conditions at all stages of cultivation and processing, results in toxigenic fungal growth and mycotoxins accumulation to the levels injurious to animal and human health (Zain, 2011). Ochratoxin A (OA), the most toxic and frequently detected as compared to ochratoxin B and ochratoxin C is comprised of a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-R-methylisocoumarin (OTα) moiety linked via amide bond to amino acid L-phenylalanine group at the 12-corboxyl group (Vanhoutte et al., 2016; Kuiper-Goodman and Scott, 1989). The toxic potential of OA is attributed mainly to the isocoumarin moiety and perhaps not to phenylalanine moiety (Xiao et al., 1996). OA, after aflatoxin B1, is the most important mycotoxin concerning the associated economic losses worldwide (Indresh and Umakantha, 2013), and has also been placed in group 2B by IARC (International agency for research on cancer) as a possible human carcinogen. The data on worldwide occurrence of OA from year 2006–2016 suggested that in raw cereal grains the incidence and maximum concentration was 29% and 1164 μg/kg, respectively (Lee and Ryu, 2017).

Ochratoxicosis-the disease produced by dietary exposure to OA is a common condition in avian and mammalian species. Although, OA has been recognized as a specific nephrotoxic, but due to its broad mode of action, has also resulted in hepatotoxic, immunotoxic, mutagenic and teratogenic effects (Ahmad et al., 2012; Pfohl-Leszkowicz and Manderville, 2007). Several studies have shown that OA intoxication negatively influenced the size of central or primary lymphoid organs, including the thymus and bursa of Fabricius (Al-Anati and Petzinger, 2006; Elaroussi et al., 2006) which produce T and B cells, respectively (Davison et al., 2008). The experimental studies have revealed its immunomodulatory effect even at the levels below the toxic threshold limit when the typical nephrotoxic effect could not be observed (Marin and Taranu, 2015). The use of maximum recommended level of OA by the European Commission Recommendation 2006/576/EC (0.1 mg OA/kg) for poultry feeds resulted in the reduction of thymus weight and decreased serum concentrations of total proteins, albumins and alpha-, beta- and gamma-globulin, although the parameters related to birds

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Abbreviation

ANOVA	Analysis of variance
CFU	Colony-forming unit
CPD	Carboxypeptidase
DGS	Directorate of Graduate Studies
DMR	Duncan's multiple range test
GYP	Glucose yeast peptone
H&E	Haemotoxylin and Eosin
HPLC	High Performance Liquid Chromatography
IARC	International agency for research on cancer
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
ME	Mercaptoethanol
OA	Ochratoxin A
OD	Optical density
OT	Ochratoxins
OTα	ochratoxin α
PBS	Phosphate buffer saline
PHA-P	Phytohemagglutinin-P
SRBC	Sheep red blood cells
TR	*Trichosporon mycotoxinivorans*

performance, hematology, liver enzymes and renal functions were not affected (Pozzo et al., 2013). Khan et al. (2018) also reported decreased weight of both thymus and bursa of Fabricius, even after exposure to 0.1 mg OA/kg feed in broiler chicks. OA intoxication in the experimental birds also resulted in significant reduction in the immunoglobulin containing cells in all the lymphoid organs, owing to the cytotoxic and DNA damaging activity of mycotoxin (Hassan et al., 2012b; Liu et al., 2012). The injurious effects of OA to suppress the immune system of the poultry birds have also been documented in several earlier studies conducted by Hassan et al., 2012b and Verma et al. (2004). Considering the level of contamination in feed and feed ingredients, and their subsequent toxic effects; the control measures at various levels including, inhibition of the growth of toxigenic fungi, removal of toxins from contaminated feed or its ingredients or reduction of systemic bioavailability of toxins are in practice.

The non-nutritive clay based entero-sorbents have been used as a strategy to reduce the bioavailability of food or feed born mycotoxins in animals and humans. The low rate of dietary inclusion and easy management led to the introduction of diverse nature of these mycotoxin binders. However, the adsorption ability of these binders is limited to only a few mycotoxins; polar being easily adsorbed compared to non-polar. The adsorption of OA into the clay based binders is limited due to its non-polar nature (Murugesan et al., 2015; Avantaggiato et al., 2005). Therefore, alternative counteracting strategies have been adopted by using the microorganism or their enzymes to detoxify the specific mycotoxins. A viable alternative approach would be the addition of mycotoxin modifiers in the animal feed; can alter the molecular structure of mycotoxin to less toxic or non-toxic metabolites which are excreted from the body (Grenier et al., 2013). A yeast strain, *Trichosporon mycotoxinivorans* (TR), isolated from hindgut contents of lower termites (*Mastotermes darwiniensis*), has been thoroughly investigated to access its mycotoxin degradation potential and has shown efficacy to degrade some mycotoxins including OA and zearalenone (Vekiru et al., 2010; Molnar et al., 2004). An increase in the OTα contents after degradation of OA by TR (Molnar et al., 2004), clearly explained the metabolic end products of OA-TR interaction. However, *in vivo* immunomodulatory role of OTα still need to be explored. The present study was designed to investigate, i) the dietary ratio of TR to OA for efficient mycotoxin degradation, and ii) *in-vivo* modifying ability of different dietary levels of TR upon OA or its metabolites induced immune-toxicological alterations in broiler chicks.

2. Materials and methods

2.1. Production of ochratoxin A

Fresh cultures of *Aspergillus ochraceus* (CECT 2948) were used to inoculate the broken wheat grains for the production of OA in the present experiment. Briefly, broken wheat grains (80 g) were soaked with 200 ml of distilled water for 2 h in flat-bottom Erlenmeyer flask (1000 ml). Prior to inoculation with spore suspension (3 ml) of *A. ochraceus*, each flask contents were autoclaved at 121 °C for 20 min. These flasks were incubated for 14 days in the dark followed by OA extraction by using acetonitrile-water (60:40). Quantification of OA was performed using High Performance Liquid Chromatography (HPLC) equipped with florescent detector as described by Bayman et al. (2002).

2.2. Production of Trichosporon mycotoxinivorans

Pure culture of TR (HB 1175) procured from the culture collection center of University of Natural Resources and Life Sciences Vienna, Austria, were inoculated on glucose yeast peptone broth (GYP), prepared by adding dextrose (20 g), peptic digest of animal tissue (10 g), yeast extract (5 g) in 1 L of distilled water (Molnar et al., 2004). In a total volume of 150 ml of GYP broth in a 1000 ml Erlenmeyer flask, 0.5 ml of the inoculum from 24 h old pre-culture was added, closed with cotton gauze swab and incubated at 25 °C. After 48–72 h of incubation the biomass was collected for mycotoxin degradation experiments by centrifugation at 3750 rpm for 15 min.

2.3. Animal housing and experimental design

Four hundred and eighty (480) 1-day old broiler chicks were obtained from the commercial hatchery. The birds were housed on rice husk bedding material according to standard brooding and growing conditions and were offered *ad libitum* feed and water. A corn and soybean meal based basal broiler feed without inclusion of antibiotics and toxin binder was formulated with 22% total protein and 3100 kcal/kg metabolizable energy. After the acclimatization period, at day 3 of age, birds were randomly allocated in groups to be used in the experiment (Table 1).

The experimental birds were randomly assigned to 16 different groups having 30 birds each, and feeding treatments consists of three dietary concentrations of TR and OA alone and in different combinations. The feed offered to the control group was used as a basal diet for all the experimental groups. In groups O1, O2 and O3 basal broiler diet was contaminated with 0.15, 0.3 and 1.0 mg OA/kg, respectively, while

Table 1
Layout of the experiment.

Sr. No	Groups	No. of birds (in each group)	Treatments OA (mg/kg feed), TR (g/kg feed)
1	Control	30	OA 0, TR 0
2–4	O1, O2, O3	30	OA: 0.15, 0.3, 1.0
5–7	TR1, TR2, TR3	30	TR: 0.5, 1.0, 2.0
8–10	O1TR1,O2TR1,O3TR1	30	OA: 0.15, 0.3, 1.0 TR: 0.5 with each OA level
11–13	O1TR2, O2TR2,O3TR2	30	OA: 0.15, 0.3, 1.0 TR: 1.0 with each OA level
14–16	O1TR3, O2TR3,O3TR3	30	OA: 0.15, 0.3, 1.0 TR: 2.0 with each OA level

birds in groups TR1, TR2 and TR3 were fed basal diet concurrently with 0.5, 1.0 and 2.0 g TR/kg, respectively. The birds in the experimental groups O1TR1, O2TR1 and O3TR1 were offered three dietary OA contamination levels simultaneously with 0.5 g/kg of TR while in groups O1TR2, O2TR2, O3TR2, 1.0 g/kg of TR was fed simultaneously with same dietary OA levels. The groups O1TR3, O2TR3 and O3TR3 were offered three dietary contamination levels of OA amended with 2.0 g/kg of TR.

All the ethical guidelines laid down by national legislation and Directorate of Graduate Studies (DGS), University of Agriculture Faisalabad were followed. Prior to conducting these experiments, the layout of the study was approved by ethical committee and DGS.

2.4. Antibody responses against sheep red blood cells (SRBC)

For this study, 6 experimental birds from each group were randomly selected to access the antibody responses to the SRBC following the method described by Delhanty and Solomon (1966).

Briefly, at day 13 of age the experimental birds were injected with primary dose of 3% suspension of SRBC (1 ml) in phosphate buffer saline (PBS) via wing vein. After 14 days of the primary dose the birds were injected (1 ml) with booster dose of SRBC (3% suspension in PBS). Blood samples were collected at day 7 and 14 of the primary and booster dose via wing vein and serum was separated. The collected serum samples from different groups were heat inactivated (56 °C for 30 min) before being used for the analysis of total antibodies, mercaptoethanol-2 (ME) sensitive (IgM), and ME resistant (IgG) anti-SRBC antibodies.

For this purpose, 50 µL of PBS was dispensed into each well of round-bottomed 96-well microtiter plates before adding heat-inactivated serum samples (50 µl) to the first column. The incubation was performed at 37 °C for 30 min and then two-fold serial dilution was made from column 2 to 11. Subsequently, to each well of the titration plate 50 µl of 2% SRBC suspension was added and were shaken for 1 min prior to incubation at 37 °C for 30 min. The plates were visually examined to determine the endpoint titer for agglutination by recording the well immediately preceding the well with distinct SRBC button formation. To measure anti-SRBC IgG and IgM antibodies titer, 50 µl of ME (0.01 M) solution in PBS was used by similar procedure used for total antibodies. The IgM titer was considered to be equal to the difference between total antibody and IgG titers.

2.5. Mononuclear phagocytic system function assay

In situ phagocytic activity of the circulatory macrophages was accessed by the method of Hassan et al., 2012b. Briefly, the randomly selected 6 experimental birds from each group were injected (1 ml/kg body weight) with colloidal suspension of carbon black (Black India ink, Pelikan- 4001, Sharjah, UAE) via wing vein. Before injection, the ink was centrifuged for 30 min at 3000 × *g* to collect the supernatant fraction to be used in the assay. In total, 200 µl of blood sample from the injected birds was collected before injection (0 min) and at an interval of 3 and 15 min after the ink injection and was rapidly shifted into 4 ml of 1% aqueous sodium citrate solution. The collected blood suspension was centrifuged at 50 × *g* for 4 min, to measure the optical density (OD) of the collected supernatant at 640 nm. The phagocytic index was calculated by using the formula:

Increased absorbance (%) = 100 x [OD at specific time - OD at time 0]/OD at time 0.

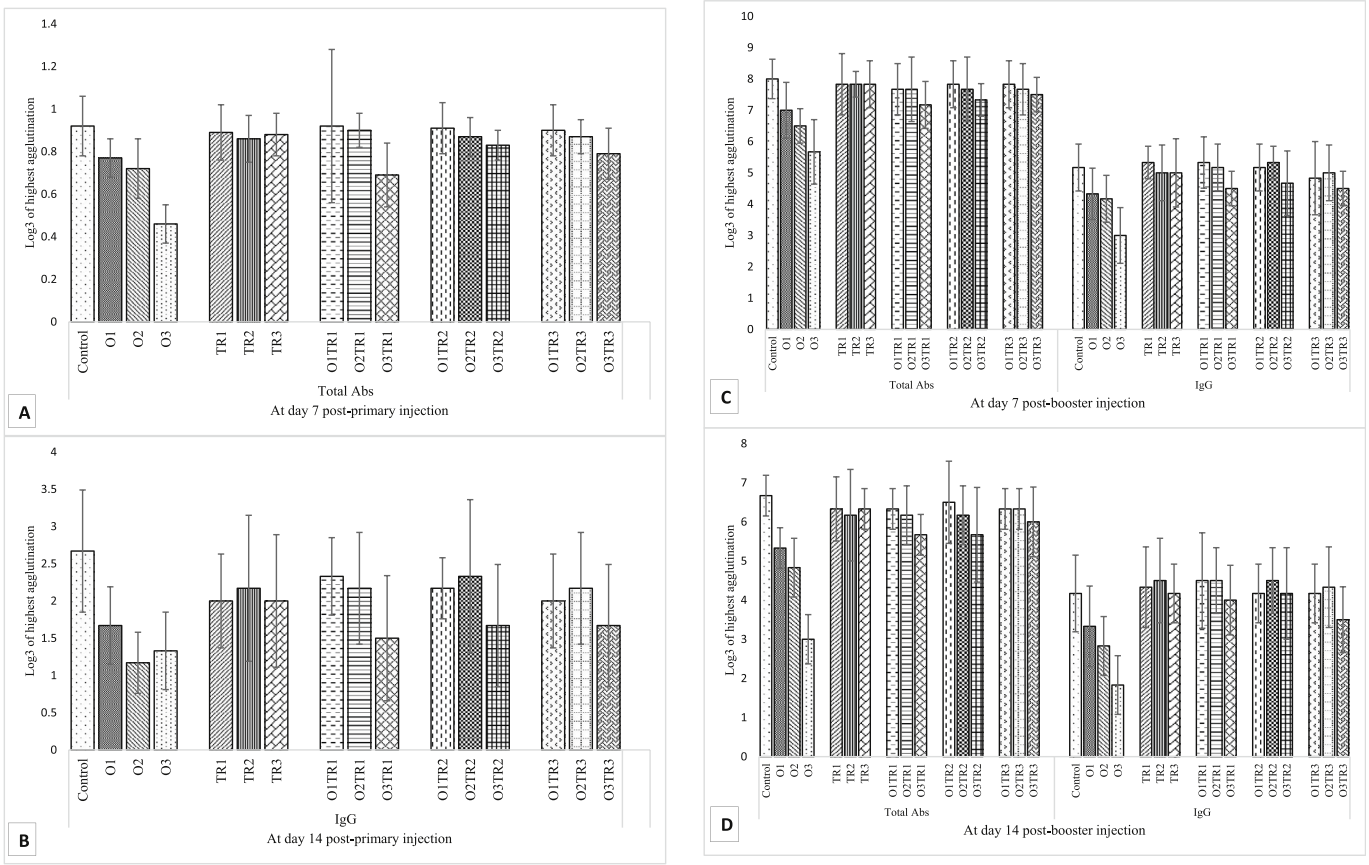

Fig. 1. Antibodies titers in broiler chicken against SRBCs fed graded concentrations of ochratoxin A and *Trichosporon mycotoxinivorans,* alone and/or in combination. Values are shown mean ± SD (n = 6/group) and are significantly different from control at $p \leq 0.05$. **Abbreviations:** O1 = 0.15 mg OA/kg feed, O2 = 0.3 mg OA/kg feed, O3 = 1.0 mg OA/kg feed, TR1 = 0.5 g TR/kg feed, TR2 = 1.0 g TR/kg feed, TR3 = 2.0 g TR/kg feed.

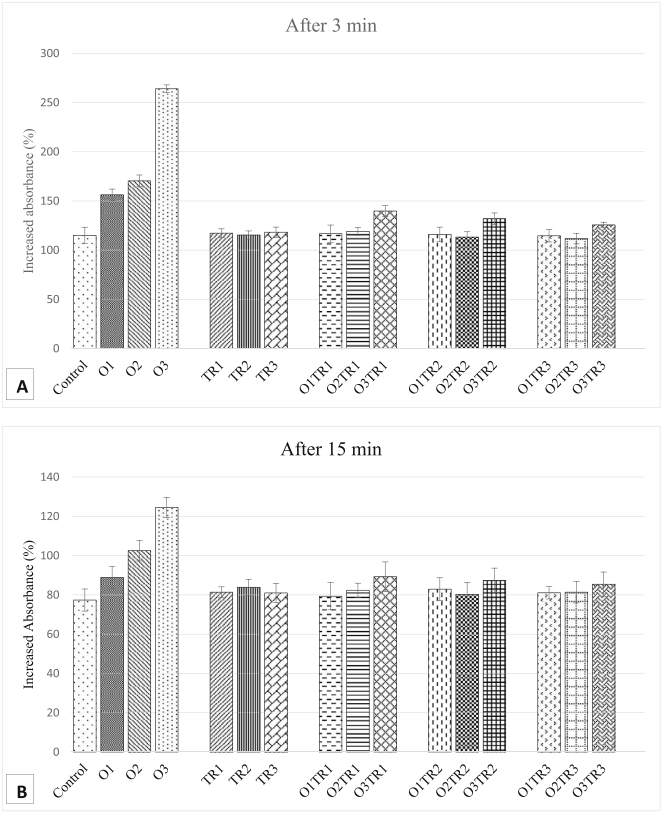

Fig. 2. Mononuclear phagocytic potential of broiler chicken fed graded concentrations of ochratoxin A and *Trichosporon mycotoxinivorans*, alone and/or in combination. Values are shown mean ± SD (n = 6/group) and are significantly different from control at $p \leq 0.05$. Abbreviations are as reported in Fig. 1.

2.6. In-vivo cutaneous basophil response to Phytohemagglutinin-P (PHA-P)

The *in vivo* lymphoproliferation was accessed by injecting PHA-P (Sigma, St. Louis, MO) into the experimental birds at 35 day of age. Briefly, the randomly selected 6 birds from each experimental group received intradermal injection of PHA-P (50 μg/100 μL of PBS) into the toe web of the right foot between the third and fourth digits. The left foot injected intradermally with PBS (100 μL, pH 7.4) alone between third and fourth digit served as a control. The response of different experimental groups was assessed by measuring the thickness of interdigital skin before injection (0 h) and 24, 48, and 72 h after injection, using pressure sensitive micrometer screw gauge (Global Sources, Shanghai, China). The response status at each time point was assessed as: [skin thickness in right foot - skin thickness in left foot], according to the method as described by Corrier (1990).

2.7. Histopathology and morphometric analysis

At 42 day of age, 6 birds in each experimental group were humanely scarified by cervical dislocation for the collection of immune organs (bursa of Fabricius and thymus). The tissues fixed in 10% buffered neutral formalin were processed for the histopathological (bursa of Fabricius and thymus) and morphometric (bursa of Fabricius) evaluation by the method of Bancroft and Gamble (2007). The morphometric analysis of bursa of Faricius was performed by using light microscope fitted with ocular micrometer. Briefly, the ocular scale was calibrated by focusing on the calibrated stage micrometer. After calibration, the ocular scale was used to calculate the follicular diameter and interfollicular connective tissue of bursa of Fabricius (in μm) at low (× 20) and high (× 40) magnification, respectively. The mean value was calculated by measuring at least three different sections from each group at seven different places.

2.8. Statistical analysis

Analysis of variance (ANOVA) of the collected data was performed by MSTATC (Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI) statistical software package. Comparisons between different group means were made by Duncan's multiple range test (DMR). Statistical significance was considered at a p-value ≤ 0.05.

3. Results

3.1. TR counters OA mediated humoral immune suppression

At day 7 of primary injection (Fig. 1-A), in groups O1, O2, O3 and O3TR1 a significant decrease ($p \leq 0.05$) in total antibody titers were observed, whereas difference was non-significant ($p \leq 0.05$) in all other groups from control. However, among all groups a non-significantly ($p \leq 0.05$) different response in terms of IgG and IgM titers was observed from control. At day 14 of primary injection (Fig. 1-B), the difference was non-significant ($p \leq 0.05$) among all the groups in terms of total antibody and IgM titers. However, significant ($p \leq 0.05$) decreased value of IgG titer was observed in experimental birds of groups O2, O3 and O3TR1 from control birds whereas the rest of the groups had non-significant ($p \leq 0.05$) difference from control.

The value of total antibody titer at day 7 and 14 post-booster injection (Fig. 1-C and 1-D), was significantly ($p \leq 0.05$) decreased in experimental birds in groups O2 and O3, while in group O1 the titers were significantly lower ($p \leq 0.05$) only at day 7 of booster injection in comparison to control. The difference was non-significant ($p \leq 0.05$) in experimental birds of all other groups from control birds. In comparison to the control group the IgG titers were not significantly different ($p \leq 0.05$) in all the experimental birds after day 7 of booster injection (Fig. 1-C) except group O3 exhibited a significant lower ($p \leq 0.05$) value of IgG titer from control birds. At day 14 after booster injection (Fig. 1-D), the value of IgG titer in birds of groups O2 and O3 was significantly ($p \leq 0.05$) decreased however, the level was non-significant ($p \leq 0.05$) in remaining experimental birds in comparison to control birds. The titer value of IgM in all experimental groups at day 7 and 14 after booster injection was non-significant ($p \leq 0.05$) from the value in control birds.

3.2. Mononuclear phagocytic system function assay

The experimental birds kept on different dietary levels of OA alone (O1, O2, O3) and 1.0 mg OA/kg in combination with three dietary concentrations of TR (O3TR1, O3TR2, O3TR3) exhibited a significant ($p \leq 0.05$) delay in carbon clearance at 3 and 15 min, in comparison to the clearance rate observed in the control birds (Fig. 2-A and 2-B). The difference in clearance rate was non-significant ($p \leq 0.05$) in all other groups from control.

3.3. In-vivo cutaneous basophil response to Phytohemagglutinin-P (PHA-P)

At 24 h post PHA-P injection the experimental birds in groups O2, O3 and O3TR1 exhibited a significantly ($p \leq 0.05$) lower skin thickness response from control group birds (Table 2). At 48 h post injection, the experimental birds in groups given 1.0 mg/kg dietary contamination of OA alone (O3) and simultaneously with three dietary concentrations of TR (O3TR1, O3TR2, O3TR3) exhibited a significant ($p \leq 0.05$) decreased response to PHA-P in comparison to control group birds. At 72 h, the response was significantly ($p \leq 0.05$) decreased in experimental birds of all the groups than the control birds.

Table 2
Cutaneous basophil response to PHA-P, of the broiler chicken fed graded concentrations of ochratoxin A and *Trichosporon mycotoxinivorans*, alone and/or in combination (Mean ± SD).

Groups	24 h post PHA-P (mm)	48 h post PHA-P (mm)	72 h post PHA-P (mm)
Control	0.93 ± 0.14^{a}	0.75 ± 0.09^{a}	0.58 ± 0.21^{a}
O1	0.78 ± 0.09^{abc}	0.62 ± 0.07^{abc}	0.29 ± 0.14^{bc}
O2	0.73 ± 0.14^{bc}	0.57 ± 0.16^{abc}	0.26 ± 0.11^{bcd}
O3	0.47 ± 0.09^{d}	0.34 ± 0.14^{d}	0.12 ± 0.12^{d}
TR1	0.90 ± 0.13^{ab}	0.68 ± 0.13^{abc}	0.38 ± 0.08^{bc}
TR2	0.87 ± 0.11^{abc}	0.67 ± 0.13abc	0.30 ± 0.07bc
TR3	0.89 ± 0.10^{abc}	0.67 ± 0.10^{abc}	0.26 ± 0.12^{bcd}
O1TR1	0.93 ± 0.36^{a}	0.73 ± 0.25^{ab}	0.41 ± 0.05^{b}
O2TR1	0.91 ± 0.08^{ab}	0.71 ± 0.09^{ab}	0.38 ± 0.09^{bc}
O3TR1	0.70 ± 0.15^{c}	0.50 ± 0.06^{cd}	0.37 ± 0.14^{bc}
O1TR2	0.92 ± 0.12^{ab}	0.70 ± 0.12^{ab}	0.35 ± 0.09^{bc}
O2TR2	0.88 ± 0.09^{abc}	0.68 ± 0.06^{abc}	0.29 ± 0.05^{bc}
O3TR2	0.84 ± 0.07^{abc}	0.54 ± 0.13^{bc}	0.33 ± 0.15^{bc}
O1TR3	0.91 ± 0.12^{ab}	0.70 ± 0.19^{ab}	0.27 ± 0.10^{bc}
O2TR3	0.88 ± 0.08^{abc}	0.67 ± 0.08^{abc}	0.24 ± 0.04^{cd}
O3TR3	0.80 ± 0.12^{abc}	0.49 ± 0.24^{cd}	0.23 ± 0.16^{cd}

In columns, values with different superscript letter are significantly different from each other at $p \leq 0.05$; n = 6/group. Abbreviations are as reported in Fig. 1.

3.4. Histopathology of bursa of Fabricius and thymus

Histologically (H&E stain, X200), control birds revealed a normal appearances of bursa of Fabricius and thymus and those fed graded dietary levels of TR exhibited a well maintained morphology. The experimental birds in groups O1, O2 and O3 exhibited a dose dependent decrease in cellular population in medullary region of bursa of Fabricius. In the medullary region of thymus the darkly stained pyknotic nuclei were accompanied with excessive amount of empty spaces compared to the normal histological pattern of thymus observed in control group birds. The histological pattern of bursa of Fabricius of experimental birds in groups given 0.15 and 0.3 mg OA/kg feed simultaneously with different dietary concentrations of TR exhibited a clear demarcation between cortex and medullary region and normal concentration of inter-follicular connective tissue. However, thymus revealed a highly variable proportion between cortex and medulla along with medullary cavitation at few places in comparison to the birds in control. The experimental birds in groups O3TR1, O3TR2 and O3TR3 exhibited an increased proliferation of interfollicular connective tissue and at some places invagination of intact superficial epithelium of bursa of Fabricius was present in comparison to the histological pattern of control group birds. The histological appearance of thymus revealed an increase in severity of the microscopic changes characterized by variable proportions of cortex and medullary region and a substantial increased amount of empty spaces was observed in the medullary area in comparison to control birds. The histopathological alterations were more severe in the birds of group O3TR1 followed by O3TR2 and were least in experimental birds of group O3TR3 compared to control birds (see Figs. 3 and 4).

3.5. Morphometric study bursa of Fabricius

A significantly increased ($p \leq 0.05$) concentration of inter-follicular connective tissue (Table 3) was observed in experimental birds offered different OA contamination levels alone in feed (O1, O2, O3) and 1.0 mg OA/kg feed along with three dietary concentrations of TR (O3TR1, O3TR2, O3TR3) compared to control birds. The mean follicular diameter in experimental birds offered dietary contamination levels of OA alone revealed a significant decrease ($p \leq 0.05$) from control birds expect group 'O1' given 0.15 mg OA/kg feed differ non-significant ($p \leq 0.05$) from control birds. The experimental birds in groups TR1, TR2 and TR3 exhibited non-significant ($p \leq 0.05$) different mean follicular diameter from control birds. In combination groups the difference was non-significant ($p \leq 0.05$) in birds of groups O1TR1, O1TR2, O1TR3 and O2TR3 from control birds. All other groups exhibited a significant decreased ($p \leq 0.05$) mean follicular diameter in comparison to control.

4. Discussion

The ubiquities nature of OA in food and feed commodities and the resultant deleterious effects on animal and human health is a global concern. The prevention of feeds and food stuff from OA contamination is the viable approach to avoid the deleterious health effects and the associated economic losses, but in fact, the complete decontamination is nearly impossible. Therefore, it is necessary to adopt complementary measures for the detoxification of OA contaminated cereals and grains and to prevent the systemic bioavailability. The addition of mycotoxin adsorbents/modifiers in the feed is presently one of the most accepted methods. In this connection, several microbials have been successfully tested for their potential to absorb, degrade and/or detoxify the target mycotoxins. In the present study, attempt using antagonistic yeast (TR) has been made to evaluate biodegradation of OA in broiler diet and subsequent ameliorations in the OA induced immunotoxicity.

The experimental birds fed OA alone, in the present study, exhibited a significant ($p \leq 0.05$) dose dependent suppression of immunological response to SRBC, *in-vivo* phagocytic potential of circulatory macrophages and lymphoproliferative response to PHA-P. The bursa of Fabricius and thymus exhibited degenerative and necrotic changes in addition to the depletion of the lymphoid cells in these organs. The decreased immunological responses observed in the present study were also previously reported by Hassan et al., 2012b and Verma et al. (2004). The experimental birds offered graded dietary contamination levels of OA (0.4 and 0.5 mg/kg) for the period of 35 days exhibited reduced lymphoproliferative response to intradermal injection of PHA antigen (Elaroussi et al., 2006) and similarly the lowered skin thickness response values, following administration of dinitrochlorbenzene suggested a depressed activity of T cells in broiler birds given OA contaminated diet (Sakhare et al., 2007; Verma et al., 2004). One of the major mechanism of immunosuppression is considered the inhibition of protein synthesis due to the structural resemblance of OA to the amino acid phenylalanine. OA has an inhibitory effect on a number of enzymes that use phenylalanine as a substrate, in particular, phenylalanine-tRNA synthetase, required at the initial step of protein synthesis (Heussner and Bingle, 2015; Rhouati et al., 2013). OA exposure also inhibited the production of IL-2 and IL-2 receptor expression of activated T lymphocytes and response of B lymphocytes to polyclonal activators. Thus, OA induced suppression of humoral immune response is not only due to the inhibition of T helper cell function but also due to the interference with the essential processes in cell metabolism irrespective of lymphocyte population or subpopulation (Lea et al., 1989).

In the present study, TR was used as a feed additive as the name of the strain refers to its ability to detoxify mycotoxins. The non-significant differences ($p \leq 0.05$) among the immunological status of the birds fed dietary concentrations of TR alone (0.5, 1.0 and 2.0 g/kg) and those in the control group suggested that TR at all the tested levels did not exert any deleterious effects on immunological aspects of the experimental birds. Previously, Hanif et al. (2012) and Politis et al. (2005) also reported that dietary addition of TR (1–2 g/kg and 10^4–10^6 CFU/g, respectively) in the broilers diet did not exert any harmful effect on the broiler birds health status. Khalel et al. (2012) and Khaliel et al. (2011) reported that oral administration of 0.250 ml of TR (HB 1230) in mice increases the overall weight gain and decreases the mortality rate without any negative impact on the blood constituents and also did not alter the normal histological appearance and enzymatic concentrations of the liver.

The *in vitro* efficacy evaluation methods have been developed as a

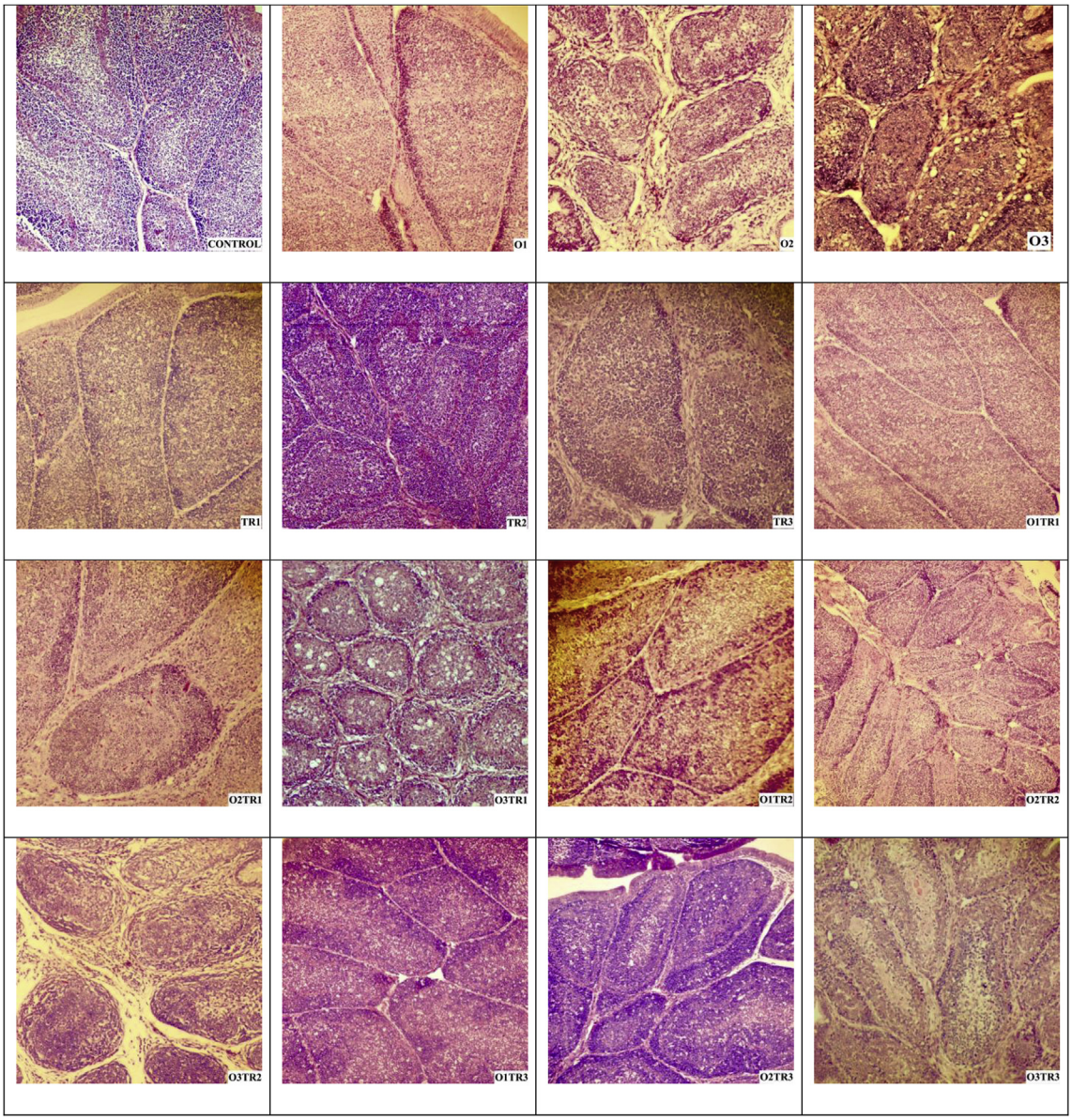

Fig. 3. Histological alteration in the bursa of Fabricius of broiler chicken fed graded concentrations of ochratoxin A and *Trichosporon mycotoxinivorans*, alone and/or in combination. Normal histological architecture of the bursa of chick given basal diet (CONTROL), low level of OA alone (O1), or in combination with TR (O1TR1, O1TR2, O1TR3) and TR at all the test levels (TR1, TR2, TR3). Moderate to high depletion in the bursal follicles along with significant proliferation of inter-follicular connective tissues are seen in the groups give medium (O2) to higher (O3) levels of OA. Pyknotic nuclei along with cellular vacuolation in OA fed birds (O3) was seen at some places. A significant protective effects of TR are seen in the groups given OA in combination with higher levels of TR (O2TR2, O2TR3, O3TR2, O3TR3) as compared to groups given OA alone (O2, 03). All the tissue sections were stained with H&E (X200).

way to effectively pre-screen mycotoxin modifier/binder before its use in the animal feed (Lemke et al., 2001). However, *in vitro* studies do not always predict *in vivo* results because during *in vitro* studies the physiological parameters and composition of feed are rarely accounted. The *in vitro* studies conducted previously have effectively demonstrated the degrading potential of TR against OA and zearalenone. Schatzmayr et al. (2006) reported that after 6 h of co-incubation using pieces of pig intestines, TR degraded approximately 90% of OA (400 ng/mL). TR also exhibited neutralizing effect on the estrogenic activity of zearalenone and effectively degrade zearalenone by opening the macrocyclic ring at ketone group and the resulting metabolite (ZOM-1) did not show any estrogenic effect even at 1000 folds higher concentration than that of parent zearalenone (Vekiru et al., 2010). Competitive binding between zearalenone and TR in cell culture also prevent the estrogenic effect of mycotoxin (Schatzmayr et al., 2004). During an *in vitro* study, after 2.5 h TR degraded all the added OA (200 μg/L) into OTα, however for zearalenone (1 μg/ml) the complete detoxification occurs after 24 h of incubation (Schatzmayr et al., 2006), which questioned its practical *in-vivo* application against zearalenone, because the detoxification should occur in $^{<}$ 8 h after ingestion.

On the basis of *in vitro* biodegradation efficacy of TR, it has been assumed that dietary inclusion of TR during *in vivo* experiment might significantly decrease the immunosuppressive effects of OA. In the present *in vivo* study in broiler birds, the groups given OA and TR together, the enhanced antibody response to SRBC, phagocytic index of circulatory macrophages and cutaneous basophilic sensitivity, suggested that the dietary addition of TR at all levels was equally effective against low doses of OA (0.15 and 0.3 mg/kg), however the biodegradation response was not significant ($p \leq 0.05$) against higher dose of OA (1.0 mg/kg). Similarly, the dietary incorporation of TR exhibited a protective effect on the OA induced histopathological and morphometric changes in bursa of Fabricius, however a partial protection was

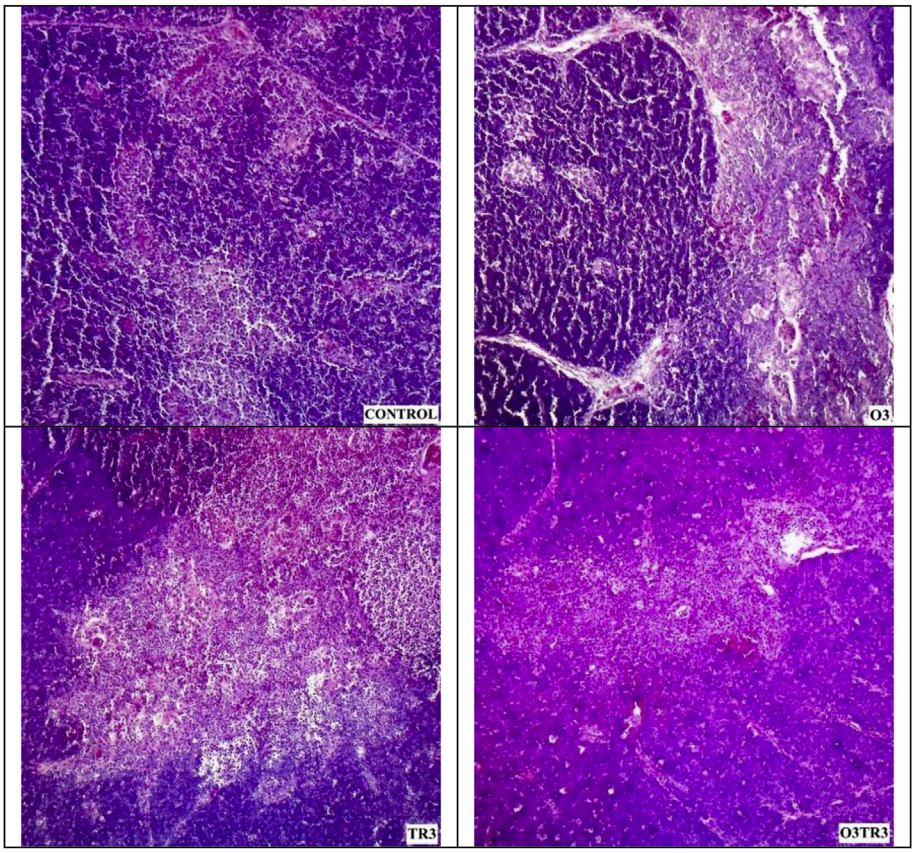

Fig. 4. Histological alteration in the thymus of broiler chicken fed graded concentrations of ochratoxin A and *Trichosporon mycotoxinivorans*, alone and/or in combination. Normal histological architecture of the thymus of chick given basal diet (CONTROL), higher level of TR alone (TR3) or in combination with higher level of OA (O3). The severity of degenerative changes characterized by variable proportions of cortex and medullary region and increased empty spaces in chicks given O3 alone. All the tissue sections were stained with H&E (X200).

Table 3
Morphometric evaluation of bursa of Fabricius of the broiler chicken fed graded concentrations of ochratoxin A and *Trichosporon mycotoxinivorans*, alone and/or in combination (Mean ± SD).

Groups	Bursa of Fabricius	
	Interfollicular connective tissue (μm)	Diameter of follicle (μm)
Control	5.14 ± 1.66^{e}	334.63 ± 54.14^{a}
O1	12.00 ± 2.40^{c}	293.49 ± 33.65^{ab}
O2	14.06 ± 1.66^{b}	235.89 ± 39.91^{c}
O3	17.14 ± 2.16^{a}	148.11 ± 28.72^{d}
TR1	5.49 ± 1.17^{e}	297.60 ± 46.04^{ab}
TR2	5.14 ± 0.91^{e}	316.80 ± 35.49^{ab}
TR3	6.17 ± 1.28^{e}	305.83 ± 38.17^{ab}
O1TR1	5.49 ± 1.17^{e}	297.60 ± 53.74^{ab}
O2TR1	5.83 ± 1.28^{e}	286.63 ± 42.00^{b}
O3TR1	10.29 ± 1.17^{d}	138.51 ± 24.07^{d}
O1TR2	5.83 ± 1.28^{e}	294.86 ± 34.49^{ab}
O2TR2	5.49 ± 1.17^{e}	286.63 ± 32.52^{b}
O3TR2	9.94 ± 2.57^{d}	126.17 ± 16.09^{d}
O1TR3	5.49 ± 1.17^{e}	303.09 ± 34.10^{ab}
O2TR3	6.17 ± 1.28^{e}	293.49 ± 38.74^{ab}
O3TR3	9.25 ± 1.66^{d}	126.17 ± 19.54^{d}

In columns, values with different superscript letter are significantly different from each other at $p \leq 0.05$; n = 6/group. Abbreviations are as reported in Fig. 1.

observed in the birds fed OA at dose rate of 1.0 mg/kg by all the three dietary concentrations of TR. These partial protective response of TR against higher dietary OA might be due to intensive protein losses nephropathies induced by mycotoxin (Elaroussi et al., 2008). In the present study the phagocytic index of macrophages in different combination groups indicated that the dietary incorporation of TR reversed the OA induced impaired phagocytic potential of circulatory macrophages. Macrophages being part of non-specific first line of defense play a vital role by acting as secretory cells other than their phagocytic activity. They secrete lysosomal enzymes and produce nitric oxide and cytokines that play a vital role in regulating the immune system activities (Awaad et al., 2011). Politis et al. (2005) also reported a complete protection of TR (10^{4}–10^{6} CFU/g) against the deleterious effects of 0.5 mg OA/kg feed on the immune responses of broiler birds. The birds also exhibited an improved weight gain, and decrease residual concentration of OA in the plasma as compared to the birds offered OA contaminated diet alone. Hanif et al. (2012, 2008) reported that the dietary inclusion of TR (1 and 2 g/kg) inhibited the OA (0.5 and 1 mg/kg feed) induced histopathological changes in bursa of Fabricius and also decreased the residual concentration of OA in serum, liver and kidney of, however, contrary to present study they have used a commercial product Mycofix® Plus having TR as a component of binder mixture. Schatzmayr et al. (2004) investigated TR for its OA degrading ability and reported that the dietary incorporation of TR (10^{5} CFU/g) against 1.0 mg OA/kg feed improved the average weight gain (61 g) of broiler birds compared to the birds fed OA contaminated diet alone, and also suggested that the combined use of TR with Eubacterium BBSH 797 strain can be a useful approach to prevent swine from mycotoxicosis caused by OA, aflatoxins, trichothecenes and zearalenone.

Structurally, the amide bond present between the isocoumarin moiety and phenylalanine in OA molecule mimics a peptide bond, is therefore susceptible to the hydrolyzation action of proteases produced by different bio-transforming microorganisms. The biodegradation mechanism of OA might involves the two pathways, i) by the proteolytic hydrolysis of the amide bond that links OTα moiety to L-β -phenylalanine molecule and, ii) by the hydrolysis of the lactone ring (Karlovsky, 1999). In the first case the resulting metabolites OTα and L-β-phenylalanine are virtually non-toxic, however, when the opened lactone form was administered to rats they exhibited the toxicological properties comparable to that of OA (Li et al., 1997; Xiao et al., 1996). Although several enzymes including lipases, amidases, deoxygenases

and several commercial proteases might be responsible for the degradation of OA, but only few of them have been purified and characterized. The first one in this category was carboxypeptidase (CPD), isolated from bovine pancreas (De Bellis et al., 2015). Two classes of CPD, namely CPD-A and CPD-Y are considered the major classes associated with biodegradation process of OA (Chang et al., 2015; Dridi et al., 2015; Stander et al., 2001). Although depending on the enzyme involved in OA degradation, the intermediate product might be different but the end product is always OTα. Molnar et al. (2004) reported an increase in the concentration of OTα after chromatographic analysis of biodegradation product of OA by TR during an *in vitro* study. The studies conducted in mice and rats demonstrated that OTα did not suppress the immune system and had rapid elimination half-life compared to OA (Abrunhosa et al., 2010). The biodegradation is one of most promising method used for the decontamination of various feeds and food stuffs from mycotoxins contamination, since other conventional physical and chemical methods are likely to alter the nutritive value and organoleptic properties of feed.

5. Conclusions

The results of the present study suggested that dietary contamination of OA suppressed the immunological status of the broilers birds. The supplementation of TR ensure amelioration against OA (at low doses) mediated immune-toxicological effects. However, to counter the immunotoxicity of higher levels of OA in avian diets, more stronger approaches will be needed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110696.

References

Abrunhosa, L., Paterson, R.R.M., Venancio, A., 2010. Biodegradation of ochratoxin a for food and feed decontamination. Toxins 2, 1078–1099.
Ahmad, M.F.U.D., Saleemi, M.K., Khan, M.Z., Muhammad, F., Hassan, Z.U., Khatoon, A., Bhatti, S.A., Abbas, A.Z., Rizvi, F., Ahmed, I., 2012. Effects of ochratoxin A feeding in white leghorn cockerels on hematological and serum biochemical parameters and its amelioration with silymarin and vitamin. E. Pak. Vet. J. 32, 520–524.
Al-Anati, L., Petzinger, E., 2006. Immunotoxic activity of ochratoxin A. J. Vet. Pharmacol. Ther. 29, 79–90.
Avantaggiato, G., Solfrizzo, M., Visconti, A., 2005. Recent advances on the use of adsorbent materials for detoxification of Fusarium mycotoxins. Food Addit. Contam. A. 22, 379–388.
Awaad, M.H.H., Atta, A.M., El-Ghany, W.A.A., Elmenawey, M., Ahmed, K., Hassan, A.A., Nada, A.A., Abdelaleem, G.A., 2011. Effect of a specific combination of mannan-oligosaccharides and β-glucans extracted from yeast cell wall on the health status and growth performance of ochratoxicated broiler chickens. J. Am. Sci. 7, 82–96.
Bancroft, J.D., Gamble, A., 2007. Theory and Practice of Histological Technique, sixth ed. Churchill Livingstone, pp. 179.
Bayman, P., Baker, J., Doster, M., Michailides, T., Mahoney, N., 2002. Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. Appl. Environ. Microbiol. 68, 2326–2329.
Chang, X., Wu, Z., Wu, S., Dai, Y., Sun, C., 2015. Degradation of ochratoxin a by Bacillus amyloliquefaciens ASAG1. Food Addit. Contam. Part A- Chem. Anal. Control Expos. Risk Assess. 32, 564–571.
Corrier, D., 1990. Comparison of phytohemagglutinin-induced cutaneous hypersensitivity reactions in the interdigital skin of broiler and layer chicks. Avian Dis. 34, 369–373.
Davison, F., Magor, K.E., Kaspers, B., 2008. Structure and evolution of avian immunoglobulins. In: Davison, F., Kaspers, B., Schat, K.A. (Eds.), Avian Immunology, first ed. Elsevier, San Diego, CA, USA, 978-0-12-370634-8, pp. 107–127.
De Bellis, P., Tristezza, M., Haidukowski, M., Fanelli, F., Sisto, A., Mule, G., Grieco, F., 2015. Biodegradation of ochratoxin a by bacterial strains isolated from vineyard soils. Toxins 7, 5079–5093.
Delhanty, J., Solomon, J., 1966. The nature of antibodies to goat erythrocytes in the developing chicken. Immunology 11, 10–113.
Dridi, F., Marrakchi, M., Gargouri, M., Saulnier, J., Jaffrezic-Renault, N., Lagarde, F., 2015. Comparison of carboxypeptidaseY and thermolysin for Ochratoxin A electrochemical biosensing. Anal. Method. 7, 8954–8960.
Elaroussi, M., Mohamed, F., El Barkouky, E., Atta, A., Abdou, A., Hatab, M., 2006. Experimental ochratoxicosis in broiler chickens. Avian Pathol. 35, 263–269.
Elaroussi, M.A., Mohamed, F.R., Elgendy, M.S., El Barkouky, M., Abdou, A.M., Hatab, M.H., 2008. Ochratoxicosis in broiler chickens: functional and histological changes in target organs. Int. J. Poult. Sci. 7, 414–422.
Grenier, B., Bracarense, A., Schwartz, H., Lucioli, J., Cossalter, A., Moll, W., Schatzmayr, G., Oswald, I., 2013. Biotransformation approaches to alleviate the effects induced by fusarium mycotoxins in swine. J. Agric. Food Chem. 61, 6711–6719.
Hanif, N., Muhammad, G., Muhammad, K., Tahira, I., Raja, G., 2012. Reduction of ochratoxin A in broiler serum and tissues by *Trichosporon mycotoxinivorans*. Res. Vet. Sci. 93, 795–797.
Hanif, N., Muhammad, G., Siddique, M., Khanum, A., Ahmed, T., Gadahai, J., Kaukab, G., 2008. Clinico-pathomorphological, serum biochemical and histological studies in broilers fed ochratoxin A and a toxin deactivator (Mycofix Plus). Br. Poult. Sci. 49, 632–642.
Hassan, Z.U., Khan, M.Z., Khan, A., Javed, I., Sadique, U., Hameed, M.R., 2012a. Effect of Ochratoxin A (OTA)-contaminated feed on several health and economic parameters in white leghorn cockerels. Pak. Vet. J. 32, 35–40.
Hassan, Z.U., Khan, M.Z., Saleemi, M.K., Khan, A., Javed, I., Noreen, M., 2012b. Immunological responses of male White Leghorn chicks kept on ochratoxin A (OTA)-contaminated feed. J. Immunotoxicol. 9, 56–63.
Heussner, H.H., Bingle, L.E.H., 2015. Comparative ochratoxin toxicity: a review of the available data. Toxins 7, 4253–4282.
Indresh, H.C., Umakantha, B., 2013. Effects of ochratoxin and T-2 toxin combination on performance, biochemical and immune status of commercial broilers. Vet. World 6, 945–949.
Karlovsky, P., 1999. Biological detoxification of fungal toxins and its use in plant breeding, feed and food production. Nat. Toxins 7, 1–23.
Khalel, A.S., Khaled, J.M., Kandeal, S.A., 2012. Enzymatic activity and some molecular properties of Trichosporon mycotoxinivorans yeast and their effects on liver function in mice. Afr. J. Microbiol. Res. 6, 2567–2573.
Khaliel, A.S., Khaled, J.M., Kandeal, S.A., 2011. Ultra-structure of the yeast *Trichosporn mycotoxinivorans* and its effect on some biological parameters in mice. Afr. J. Microbiol. Res. 5, 5427–5433.
Khan, S.A., Venancio, E.J., Fernandes, E.V., Hirooka, E.Y., Oba, A., Flaiban, K.K.M.C., Itano, E.N., 2018. Low doses of ochratoxin-A decrease IgY and IgA production in broiler chicks. Toxins 10, 316.
Kuiper-Goodman, T., Scott, P.M., 1989. Risk assessment of the mycotoxin ochratoxin. A. Biomed. Environ. Sci. 2, 179–248.
Lea, T., Steien, K., Stormer, F.C., 1989. Mechanism of ochratoxin A-induced immunosuppression. Mycopathologia 107, 153–159.
Lee, H.J., Ryu, D., 2017. Worldwide occurrence of mycotoxins in cereals and cereal-derived food products: public health perspectives of their Co-occurrence. J. Agric. Food Chem. 65, 7034–7051.
Lemke, S.L., Ottinger, S.L., Mayura, K., Ake, C.L., Pimpukdee, K., Wang, N., Phillips, T.D., 2001. Development of a multi-tiered approach to the in vitro prescreening of clay-based enterosorbents. Anim. Feed Sci. Technol. 93, 17–29.
Li, S., Marquardt, R.R., Frohlich, A.A., Vitti, T.G., Crow, G., 1997. Pharmacokinetics of ochratoxin A and its metabolites in rats. Toxicol. Appl. Pharmacol. 145, 82–90.
Liu, J., Wang, Y., Cui, J., Xing, L., Shen, H., Wu, S., Liana, H., Wang, J., Yan, X., Zhang, X., 2012. Ochratoxin A induces oxidative DNA damage and G1 phase arrest in human peripheral blood mononuclear cells in vitro. Toxicol. Lett. 211, 164–171.
Marin, D.E., Taranu, I., 2015. Ochratoxin A and its effects on immunity. Toxin Rev. 34, 11–20. https://doi.org/10.3109/15569543.2014.958757.
Molnar, O., Schatzmayr, G., Fuchs, E., Prillinger, H., 2004. *Trichosporon mycotoxinivorans* sp. nov., a new yeast species useful in biological detoxification of various mycotoxins. Syst. Appl. Microbiol. 27, 661–671.
Murugesan, G.R., Ledoux, D.R., Naehrer, K., Berthiller, F., Applegate, T.J., Grenier, B., Phillips, T.D., Schatzmayr, G., 2015. Prevalence and effects of mycotoxins on poultry health and performance, and recent development in mycotoxin counteracting strategies. Poult. Sci. 94, 1298–1315.
Pfohl-Leszkowicz, A., Manderville, R.A., 2007. Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. Mol. Nutr. Food Res. 51, 61–99.
Politis, I., Fegeros, K., Nitsch, S., Schatzmayr, G., Kantas, D., 2005. Use of *Trichosporon mycotoxinivorans* to suppress the effects of ochratoxicosis on the immune system of broiler chicks. Br. Poult. Sci. 46, 58–65.
Pozzo, L., Salamano, G., Mellia, E., Gennero, M.S., Doglione, L., Cavallarin, L., Tarantola, M., Forneris, G., Schiavone, A., 2013. Feeding a diet contaminated with ochratoxin A for chickens at the maximum level recommended by the EU for poultry feeds (0.1 mg/kg). 1. Effects on growth and slaughter performance, haematological and serum traits. J. Anim. Physiol. Anim. Nutr. 1, 13–22.
Rhouati, A., Yang, C., Hayat, A., Marty, J., 2013. Aptamers: a promosing tool for ochratoxin a detection in food analysis. Toxins 5, 1988–2008.
Sakhare, P., Harne, S., Kalorey, D., Warke, S., Bhandarkar, A., Kurkure, N., 2007. Effect of Toxiroak® polyherbal feed supplement during induced aflatoxicosis, ochratoxicosis and combined mycotoxicoses in broilers. Vet. Arh. 77, 126–146.
Schatzmayr, D., Nitsch, S., Binder, E.M., Taubel, M., Loibner, A., Schatzmayr, G., 2004. Detoxification of mycotoxins. World Nutrition Forum 2004, 128–129 (Erber).
Schatzmayr, G., Zehner, F., Taubel, M., Schatzmayr, D., Kilmitsch, A., Loiber, A., Binder, E., 2006. Microbiologicals for deactivating mycotoxins. Mol. Nutr. Food Res. 50, 543–551.
Stander, M.A., Steyn, P.S., Van Der Westhuizen, F.H., Payne, B.E., 2001. A kinetic study in

to the hydrolysis of the ochratoxins and analogues by carboxypeptidase A. Chem. Res. Toxicol. 14, 302–304.
Vanhoutte, I., Audenaert, K., De Gelder, L., 2016. Biodegradation of mycotoxins: tales from known and unexplored worlds. Front. Microbiol. 7, 561.
Vekiru, E., Hametner, C., Mitterbauer, R., Rechthaler, J., Adam, G., Schatzmayr, G., Krska, R., Schuhmacher, R., 2010. Cleavage of zearalenone by *Trichosporon mycotoxinivorans* to a novel nonestrogenic metabolite. Appl. Environ. Microbiol. 76, 2353–2359.
Verma, J., Johri, T., Swain, B., Ameena, S., 2004. Effect of graded levels of aflatoxin, ochratoxin and their combinations on the performance and immune response of broilers. Br. Poult. Sci. 45, 512–518.
Xiao, H., Madhyastha, S., Marquardt, R.R., Li, S., Vodela, J.K., Frohlich, A.A., Kemppainen, B.W., 1996. Toxicity of Ochratoxin A, its opened lactone form and several of its analogs: structure-activity relationships. Toxicol. Appl. Pharmacol. 137, 182–192.
Zain, M.E., 2011. Impact of mycotoxins on humans and animals. J. Saudi Chem. Soc. 15, 129–144.