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Dietary *Trichosporon mycotoxinivoron* modulates ochratoxin-A induced altered performance, hepatic and renal antioxidant capacity and tissue injury in broiler chickens

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ABSTRACT

Ochratoxin A (OTA), an important fungal metabolite in foods and feeds has been shown to induce oxidative stress and cellular injuries to human and animal subjects. This study was designed to investigate the mode of action of a biological modifier *Trichosporon mycotoxinivorans* (TM), against OTA-mediated oxidative stress and tissue toxicity on broiler chickens. The birds were offered diets supplemented with OTA (0.15 and 0.3 mg/kg feed) and/or TM (0.5, 1.0 g/kg) for 42 days of age, and blood and tissue samples were collected to examine the oxidative stress, biochemical and histopathological parameters. Dietary OTA at all the tested levels induced the hepatic and renal tissue injury as indicated by significant decreased total antioxidant capacity in these organs along with significant decreased ($p \leq 0.05$) serum concentrations of total proteins and albumin. The serum concentrations of alanine aminotransferase (ALT) and urea were significantly increased, and these observations were further supported by degenerative changes and increased relative weights of liver and kidneys. The dietary supplementation of TM at both tested levels relieved the detrimental impact of 0.15 and 0.3 mg OTA/kg on the studied parameters. The results of the study demonstrated that dietary TM significantly protects broiler chickens by reducing OTA-induced oxidative damage and tissue injury.

1. Introduction

Mycotoxins are low molecular weight secondary metabolites of toxigenic filamentous fungi found as contaminants of foods and feeds throughout the world [1]. Environmental temperature and humidity and/or the inappropriate storage conditions favor the proliferation of these toxigenic fungi on agricultural crops at all stages of cultivation and processing [2]. Recent data surveys suggested a much higher prevalence of mycotoxin contamination in the world's crops than the widely cited 25 % estimate by Food and Agriculture Organization [3]. Although, presently over 400 mycotoxins of diverse chemical structures have been identified but the ochratoxin A (OTA), after aflatoxin B1, has been identified as the most potent mycotoxin in terms of economic losses as well as range and severity of the toxic effects [4]. Among three subgroups of ochratoxins (A, B and C), OTA has been extensively studied compared to less toxic and less common OTB and OTC. OTA is known to be produced by some species of *Aspergillus* and *Penicillium* and its post-harvest contamination can appear in wide range of agricultural products particularly in cereals and their byproducts [5]. According to the data from the year 2006–2016, the maximum concentration of 1164 μg OTA/kg with 29 % incidence has been reported in raw cereal grains [6]. Being ubiquitous in nature, the resultant OTA contamination of the feeds and feed ingredients not only represents a potential threat to animal health due to its multiple toxic effects but the carry over mechanism of the toxin through food chain to the humans also raises food safety concerns [7].

OTA contaminated feeds and feed ingredients have their major impact on the poultry birds and monogastric animals and in general, the OTA exposure reduces the growth rates and animal production [8]. Besides the acute nephrotoxicity and hepatotoxicity, OTA has been found to induce immunotoxic effects and based on sufficient carcinogenic evidences in experimental animals, International Agency for Research on Cancer (IARC) has placed OTA in group 2 B; possibly

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Abbrevations

OTA	Ochratoxin A
TM	*Trichosporon mycotoxinivorans*
OTB	Ochratoxin B
ALT	Alanine aminotransferase
OTC	Ochratoxin C
OTα	Ochratoxin alpha
FCR	Feed conversion ratio
TAC	Total antioxidant capacity
H&E	Hematoxylin and eosin
ANOVA	Analysis of variance
SD	Standard deviation
DMRT	Duncan's multiple range test
CFU	Colony-forming unit
IARC	International Agency for Research on Cancer
HPLC-FLD	High Performance Liquid Chromatography equipped with fluorescence detector

carcinogenic to humans [9]. In experimental studies with poultry birds, feeding OTA contaminated diets (0.5–5 mg/kg), significantly affected the performance parameters including the poor growth rate and feed conversion efficiency along with altered serum biochemical parameters [10]. Liver and kidney being the main organs associated with several metabolic, detoxifying and excretion processes of toxic compounds, are severely affected by OTA intoxication [11]. Among the others, oxidative stress is proposed effect of OTA toxicity both in the liver and kidney, resulting in subsequent peroxidation of polyunsaturated fatty acids [12]. Several *in vitro* and *in vivo* studies have also revealed OTA as a potent inhibitor of protein synthesis by interfering with the activity of phenylalanine t-RNA synthase and also has a strong negative impact on the production of cellular energy [5]. Based on European Commission Recommendation 2006/576/EC, maximum permissible limit of 0.1 mg OTA/kg has been established in the poultry feed [13].

Considering the OTA-contamination levels and associated health effects, prevention from pre-harvest fungal contamination of crops is therefore the best solution to reduce the crops mycotoxin burden before harvest. However, the complete elimination at pre-harvest stage is rather an irrational goal and the major part of contamination threats is also present at post-harvest stage [14]. In practice, physical and chemical methods for mycotoxin decontamination of agricultural commodities have very restricted use due to associated safety issues, possible losses in the nutritional quality together with cost implications and limited efficacy [15]. Considering the facts, significant post-harvest efforts have been made; i) to remove the toxins from contaminated feed or its ingredients, and ii) to reduce the systemic bioavailability of mycotoxins when ingested. Although, the integration of nutritionally inert entero-sorbents into contaminated feeds to decrease the mycotoxin bioavailability from the gastrointestinal tract (GIT) resulted in the introduction of diverse nature of these binding agent but their binding capacity is dependent on physiochemical properties of both adsorbent and the target mycotoxins [16]. OTA being non-polar in nature is difficult to adsorb by these binding agents compared to other polar mycotoxins [17]. Therefore, alternate counteractive strategies have been employed to alter the molecular structure of the mycotoxins by using mycotoxin modifiers; converting them virtually non-toxic or less toxic metabolites to be excreted from the body. *Trichosporon mycotoxinivorans* (TM), a basidiomycetous yeast isolated from hindgut of *Mastotermes darwiniensis,* has been proposed as a microbial feed additive with degrading potential against some mycotoxins including OTA and zearalenone. After the isolation of this novel yeast strain, its degrading capability against OTA was revealed in an *in vitro* study, that after 2.5 h of incubation, TM deactivated the added OTA (400 μg/L) in mineral solution to OTα; accessed by the chromatographic analysis of biodegradation product [18]. Similarly, the results of *in vitro* degradation experiments using pieces of pig intestines and buffer solution demonstrated that approximately 90–100 % of OTA (400 ng/mL) was decomposed to OTα after 6 and 5 h of incubation with TM, respectively. However, at a concentration of 200 μg/L, TM degraded all the added OTA after 2.5 h of incubation in buffer solution [19]. *In vitro* studies using zearalenone (1 mg/L) as a target mycotoxin, the complete degradation occurred after 24 h of incubation; by opening the macrocyclic ring at ketone group (C-6) to produce ZOM-1, resulting in complete loss of estrogenic activity even at a 1000 times higher concentration of ZOM-1 than the parent zearalenone [18–20]. Considering the fact, that *in vitro* studies have been developed to effectively pre-screen mycotoxin modifier/binder but these studies cannot predict *in vivo* results as the composition of feed and physiological parameters are rarely accounted during *in vitro* analysis [21]. Therefore, it was reasonable to design a study to investigate the *in vivo* application of antagonistic yeast TM against OTA in broiler chickens. In depth focus was made on the exploration of any modulation in health and economic parameters and their association with oxidative status of broiler chickens offered dietary OTA and TM alone and in combination.

2. Materials and methods

2.1. Cultivation of Trichosporon mycotoxinivorans

Trichosporon mycotoxinivorans (TM) was produced in 1.0 L flat bottom Erlenmeyer flasks by inoculating 150 mL of glucose yeast peptone broth (dextrose, 20 g; peptic digest of animal tissue 10 g, yeast extract 5.0 g in 1 L of distilled water) with 0.5 mL of TM pre-culture [18]. TM (HB 1175) was obtained from culture collection center University of Natural Resources and Life Sciences Vienna, Austria. After inoculation, the flasks were closed by using sterile cotton-gauze plugs and incubated for 48–72 h at 25 °C prior to harvest the biomass by centrifugation at 3750 rpm for 15 min.

2.2. Production of ochratoxin A

Aspergillus ochraceus strain CECT: 2948, procured from culture collection center, University of Valencia, Spain, was used for OTA production on broken wheat grains. Briefly, wheat grains placed in flat bottom Erlenmeyer flask (1.0 L) were soaked with distilled water (200 mL). After 2 h, the contents of the flask were autoclaved (20 min at 121 °C) and inoculated with *A. ochraceus*; 3 mL spore suspension. The flasks were incubated in dark for 14 days at 28 °C and the produced OTA was extracted using 60:40 acetonitrile and water. The extracted OTA was quantified by using High Performance Liquid Chromatography equipped with fluorescence detector (HPLC-FLD) [22].

2.3. Animals, housing and experimental diets

A total of 270 1-day-old broiler chicks (Hubbard) were obtained from a local commercial hatchery and were kept on saw dust litter material. The birds were provided with *ad lib* food and water during the whole length of the experiment. Corn-soybean meal based basal broiler feed (total protein 22 %, metabolizable energy 3100 kcal/kg) was formulated without inclusion of toxin binder and antibiotics. After initial acclimatization for 2 days, the birds were randomly allocated to 09 experimental groups (30 birds/group) to be used for different feeding treatments (Table 1). The experimental diets consisted of basal broiler feed (control) or the basal diet supplemented with different dietary concentrations of OTA and TM alone and/or in combination. The experimental groups O1 and O2 were kept on basal feed spiked with 0.15 and 0.3 mg OTA/kg, respectively, whereas the groups T1 and T2 served as positive controls for 0.5 and 1.0 g TM/kg feed, respectively. In combination groups, the experimental birds in groups O1T1 and O2T1

Table 1
Layout of Experimental design.

Sr. No.	Groups	Number of Birds (n) in each group	Treatment OTA (mg/kg feed), TM (g/kg feed)
1	Control	30	[a]OTA 0,[b] TM 0
2–3	O1, O2	30	OTA: 0.15, 0.3
4–5	T1, T2	30	TM: 0.5, 1.0
6–7	O1T1, O2T1	30	OTA: 0.15, 0.3 TM: 0.5 g/kg with each OTA level
8–9	O1T2, O2T2	30	OTA: 0.15, 0.3 TM: 1.0 g/kg with each OTA level

[a] Ochratoxin A.
[b] *Trichosporon mycotoxinivorans*.

were fed the tested dietary contamination levels of OTA simultaneously with 0.5 g TM/kg feed, whereas in groups O1T2 and O2T2, 1.0 g TM/kg feed was used against both the tested levels of OTA.

Before the preparation of experimental rations, mycotoxin analysis of each batch of basal feed was performed to ensure that the concentrations of OTA, aflatoxin B1 and zearalenone were <1 ng/g. The experimental feeds were prepared by incorporating the known quantity of OTA in the basal feed [23]. Briefly, the fermented wheat grains were soaked overnight in three fold quantity of chloroform ($CHCl_3$). The mixture was filtered through cotton cheese cloth to separate the $CHCl_3$ and the resulting residue was re-suspended in $CHCl_3$ to ensure the complete recovery of OTA. The $CHCl_3$ was evaporated to dryness on a rotary evaporator (Rotavapor® R-210 A, Buchi, Switzerland) and concentrated OTA was re-suspended in polyethylene glycol (PEG). The resulting suspension was then evenly mixed initially with 2–3 Kg of the basal feed to prepare the mycotoxin feed stock. The desired final OTA concentration in different experimental groups was obtained by further diluting the feed stock with basal feed. The representative sample from each experimental diet was analyzed by HPLC prior to being used for feeding.

2.4. Performance and relative organ weights

At day 42 of the experiment, birds in each group were weighed to access their body weight and from each experimental group, six birds were humanly sacrificed by cervical dislocation and examined for gross lesions on liver and kidney. The weights of the liver and kidney were recorded to estimate relative internal organ weights i.e. percent of the total body weight. Feed intake was recorded daily from day 3–42 of age for the calculation of feed conversion ratio (FCR) by dividing the total amount of feed consumed with total live body weight at the end of trial.

2.5. Serum biochemical parameters

At day 41 of the experiment, blood samples were collected form the wing vein of six birds from each group. Biuret and bromocresol green dye-binding methods were used to analyze the serum samples for total protein and albumin concentrations, respectively [24]. Serum concentration of globulin was assessed as: [concentration of serum total proteins – concentration of serum albumin]. Commercially available kits were used for the analysis of collected serum samples to determine the concentrations of alanine aminotransferase (ALT) [Merck, France, Catalog # 5.17531.0001], urea [Merck, France, Catalog # 5.17611.0001] and creatinine [Merck, France, Catalog #5.17551.0001].

2.6. Total antioxidant capacity of hepatic and renal tissue

At day 42 of the experiment, tissue samples (liver and kidney) were collected from six birds of each experimental group to access the total antioxidant capacity (TAC) [25]. Immediately after collection the samples were kept at – 80 °C until further analysis. Briefly for analysis, the blank solution was prepared by mixing the 5 μL of sample with 200 μL of acetate buffer (reagent I). The absorbance of the blank solution was recorded at 660 nm before the addition of 20 μL 2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonate (reagent II) to blank solution. Prior to determine the second absorbance, the solution mixture was incubated at 37 °C for 5 min. The TAC of different experimental groups was calculated from the delta absorbance of each sample by using the standard curve plotted against the different concentrations of standard.

2.7. Histopathological evaluation

The histopathological evaluation was performed according to the standard protocol [26]. Tissue samples (liver and kidney) of six birds/group collected at necropsy, were fixed in neutral buffer formalin (10 %), embedded in paraffin, sectioned (4–5 μm) and stained with hematoxylin and eosin (H&E) and studied under a light microscope (three slides/treatment).

2.8. Statistical analysis

MSTAT-C statistical software was used for the analysis of the collected experimental data. The data was subjected to analysis of variance (ANOVA) and the values represents the average of the each measurement conducted and expressed as mean ± standard deviation of the mean (SD). Duncan's multiple range test (DMRT) was used as *post hoc* analysis to estimate the differences between group means of different experimental groups. The P value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Performance and relative organ weights

At day 42 of the experiment, the body weight of the experimental birds in groups O1 and O2 exhibited a dose dependent significant decrease compared to the birds in control group. A non-significant difference from control was observed in the body weight of the birds fed TM alone (T1 and T2) and also in combination groups offered tested levels of OTA (O1 and O2) simultaneously with dietary concentrations of TM (O1T1, O2T1, O1T2 and O2T2) (Fig. 1). The FCR values (Fig. 2) of the groups offered two tested levels of OTA alone (O1 and O2) were higher compared to the control group. The groups offered TM alone (T1 and T2) and in combination with tested levels of OTA showed improved FCR values compared to the birds in corresponding control group. The

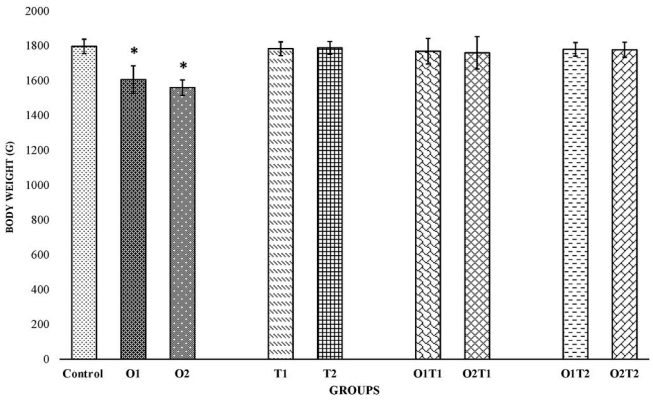

Fig. 1. Body weight of the broiler birds at day 42 of age, offered basal diet supplemented with dietary concentrations of OTA and TM, alone and in combination (*$p < 0.05$). O1: 0.15 mg ochratoxin A/kg, O2: 0.3 mg ochratoxin A/kg, T1: 0.5 g *Trichosporon mycotoxinivorans*/kg, T2: 1.0 g *Trichosporon mycotoxinivorans*/kg.

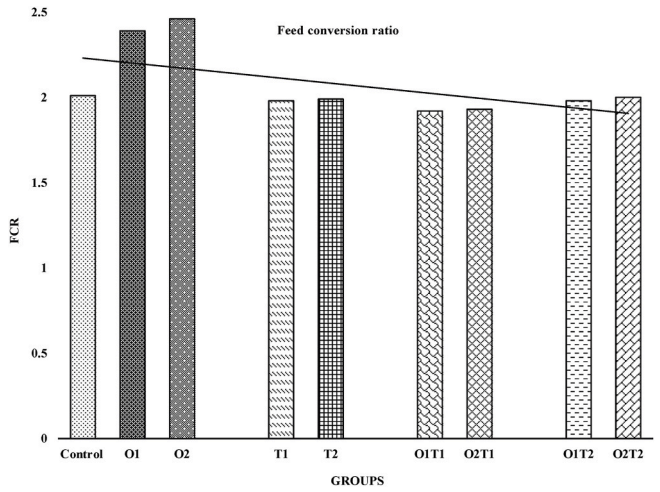

Fig. 2. Feed conversion ratio of the broiler birds at day 42 of age, offered basal diet supplemented with dietary concentrations of OTA and TM, alone and in combination. Abbreviations are same as in Fig. 1.

results demonstrated a significant increased relative liver weight of the broiler chickens in experimental groups offered OTA contaminated feed alone (O1 and O2). However, the groups served as positive control for TM and in combination groups the difference from the control group was non-significant (Fig. 3A). A significant higher relative kidney weight was observed in the experimental birds of all the groups compared with the birds in control (Fig. 3B). During the experiment no mortality was observed in groups offered TM alone or in combination with O1 and O2 except that group O2T1 which had 3.34% mortality. Highest mortality was observed in group O2 (10.00 %) whereas group O1 had the lowest mortality (3.34 %).

3.2. Serum biochemical parameters

The serum biochemical parameters (Table 2) analyzed in the present study revealed that the serum concentrations of total protein and albumin differed non-significantly from control in experimental birds offered dietary levels of TM alone (T1 and T2), except group T2 (TM @ 1.0 g/kg feed), which exhibited significantly lower serum albumin concentration compared with the birds in control group. In all other groups served as positive control for OTA and in combination groups the experimental birds showed significant lower serum proteins concentration from control. Serum globulin concentration of the experimental

Table 2
Serum biochemical parameters of the broiler birds offered basal diet amended with OTA and TM, alone and in combination. (Mean ± SD).

Group	Total protein (g/100 mL)	Albumin (g/100 mL)	Globulin (g/100 mL)	ALT (IU/ µL)	Urea (mg/ 100 mL)	Creatinine (mg/100 mL)
Control	5.34 ± 0.04^{a}	3.88 ± 0.06^{a}	1.45 ± 0.07^{a}	17.76 ± 3.18^{g}	12.69 ± 1.94^{f}	0.32 ± 0.02
O1	3.81 ± 0.05^{h}	3.15 ± 0.03^{g}	0.65 ± 0.06^{d}	31.43 ± 3.78^{c}	17.81 ± 1.47^{bc}	0.35 ± 0.01
O2	3.45 ± 0.18^{i}	3.01 ± 0.05^{h}	0.44 ± 0.22^{e}	35.48 ± 3.48^{b}	19.49 ± 3.08^{b}	0.39 ± 0.01
T1	5.26 ± 0.07^{a}	3.84 ± 0.06^{ab}	1.41 ± 0.08^{a}	21.40 ± 2.20^{efg}	14.19 ± 1.78^{ef}	0.33 ± 0.01
T2	5.27 ± 0.04^{a}	3.82 ± 0.03^{b}	1.44 ± 0.05^{a}	20.90 ± 3.19^{efg}	14.55 ± 2.63^{def}	0.34 ± 0.01
O1T1	4.81 ± 0.10^{d}	3.41 ± 0.03^{e}	1.38 ± 0.11^{a}	21.17 ± 2.19^{efg}	14.99 ± 1.86^{def}	0.32 ± 0.01
O2T1	4.44 ± 0.04^{f}	3.27 ± 0.03^{f}	1.16 ± 0.04^{b}	23.65 ± 3.78^{de}	13.11 ± 1.05^{f}	0.32 ± 0.01
O1T2	4.84 ± 0.08^{d}	3.58 ± 0.04^{d}	1.25 ± 0.06^{b}	18.86 ± 2.71^{fg}	13.46 ± 1.77^{f}	0.32 ± 0.01
O2T2	4.70 ± 0.04^{e}	3.31 ± 0.04^{f}	1.38 ± 0.03^{a}	21.82 ± 2.58^{ef}	14.98 ± 1.98^{def}	0.31 ± 0.01

In the same column different superscripts indicate the significant differences between means of different experimental groups ($p \leq 0.05$). O1: 0.15 mg ochratoxin A/kg, O2: 0.3 mg ochratoxin A/kg, T1: 0.5 g *Trichosporon mycotoxinivorans*/kg, T2: 1.0 g *Trichosporon mycotoxinivorans*/kg.

birds in groups T1, T2, O1T1 and O2T2 exhibited non-significant difference from control whereas all other groups had significant reduced concentration compared with the birds in control. Serum ALT concentration of the experimental birds in groups T1, T2, O1T1 and O1T2 differ non-significantly from control whereas a significant higher concentration was exhibited by all other groups from control birds. The difference of serum urea concentration was non-significant from control in birds of experimental groups offered dietary concentrations of TM alone (T1 and T2) and simultaneously with two dietary tested levels of OTA (O1 and O2).

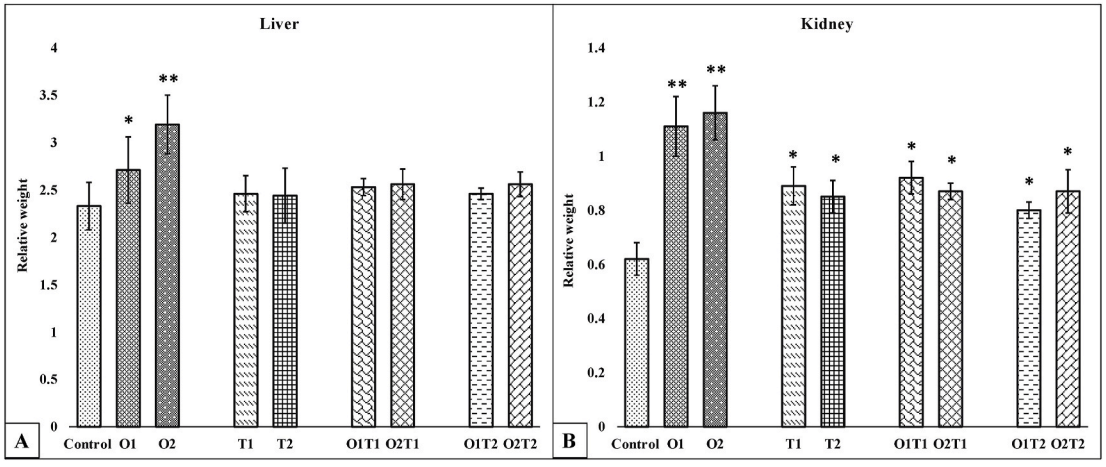

Fig. 3. Relative internal organ weights of liver (A) and kidney (B) of the broiler birds offered basal diet amended with OTA and TM, alone and in combination (*$p <$ 0.05; **$p < 0.01$). Abbreviations are same as reported in Fig. 1.

3.3. Total antioxidant capacity (TAC) of hepatic and renal tissue

The results showed a dose dependent significant decrease in hepatic TAC of the birds in experimental groups fed OTA alone, at all the tested dietary contamination levels (O1 and O2), compared with the birds in control. Dietary supplementation of TM alone at all tested levels (T1 and T2) did not significantly affected the hepatic TAC. In combination groups (O1T1, O1T2 and O2T2) the hepatic TAC was non-significantly different from control, except group O2T1, which exhibited a significantly lower TAC than control group (Fig. 4A). Meanwhile, the TAC of renal tissue exhibited a significant decrease, in birds fed OTA contaminated feed alone (O2 > O1), while the groups offered dietary TM alone exhibited non-significant different TAC from control. In combination groups, significant lower renal tissue TAC was observed in groups O2T1 and O2T2 compared with the control group while all other groups were non-significantly different from the birds in control group (Fig. 4B).

3.4. Macroscopic examination

The macroscopic examination of livers (Fig. 5A–D) of the birds in control group showed normal size, color, and consistency with sharp edges. These morphological characteristics were similar to the birds as observed in groups T1 and T2. However, the birds offered OTA contaminated feed alone (O1 and O2) the liver exhibited variable degree of enlargement, pale to yellowish discoloration, friable consistency and hemorrhages on the surface in a dose related manner, being maximum in O2 group. In combination groups, the birds offered 0.15 mg OTA/kg simultaneously with TM (O1T1 and O1T2), the morphological characteristics were similar to control group. However, in groups offered 0.3 mg OTA/kg along with TM (O2T1 and O2T2), the severity of the observed gross lesions were less compared to the birds of group O2.

The kidneys (Fig. 5E–H) of the birds in control group did not exhibit any deviation from the normal morphological pattern. The lobes were confined into the bony sockets and color and consistency was normal. The groups offered dietary TM alone, showed similar gross morphology compared to that of control group, except an increase in the relative weights. In groups O1 and O2 the slightly enlarged and hemorrhagic kidneys were protruding out of the renal sacs. However, the intensity of the gross lesions was much lower in combination groups offered dietary TM simultaneously with graded contamination levels of OTA alone (O1 and O2).

3.5. Histopathological evaluation

The microscopic evaluation of hepatic and renal parenchyma (Fig. 6) of the experimental birds offered basal diet alone (control) and in groups fed basal diet amended with two dietary tested levels of TM exhibited the normal histological appearance. The hepatocytes had intact centrally placed nuclei and cells were arranged in cords with prominent sinusoids around. In the renal parenchyma, thin rim of epithelial cells had surrounded the glomerulus with clear Bowman's spaces and the cells of tubular epithelium had intact nuclei. The experimental birds fed OTA contaminated diet alone (O1 and O2) demonstrated a dose dependent increased severity, as exhibited by degenerative and necrotic changes in hepatic and renal parenchyma. Pyknotic nuclei of the hepatocytes and diminished sinusoids around were suggestive of individual hepatic cell necrosis and acute cell swelling, respectively. The hepatic parenchyma at places also exhibited varying degree of congestion. The renal parenchyma was congested, and tubular epithelium had pyknotic nuclei and at places detachment of tubular cells from basement membrane was observed. The order of intensity of these changes was O2 > O1. In combination groups, the experimental birds offered 0.15 mg OTA/kg (O1) simultaneously with two tested levels of TM (O1T1 and O1T2), the histological structure of hepatic and renal parenchyma was comparable to that of control birds. The cellular accumulation around hepatic triad was absent, however, at few places in hepatic and renal parenchyma mild congestion was observed. In groups O2T1 and O2T2, hepatic parenchyma showed mild congestion along with individual hepatic cell necrosis. In the renal parenchyma, at some places, areas of mild congestion along with pyknotic degeneration of tubular epithelial cells was observed. The severity of histopathological alterations were least in group O1T2 followed by O1T1, O2T2 and O2T1.

4. Discussion

Ochratoxin A (OTA), among the other prevalent mycotoxins has attracted the increased attention to safeguard the food and feed products. Intensive studies have been made to understand the potential toxic effects of OTA in animals and humans and have also reported to produce severe economic losses associated with OTA contamination [8]. OTA intoxication results in damaging effects on the liver and kidney; being the main organs related to several metabolic, detoxifying and excretion functions processes of toxic compounds [11]. Recent studies have proved the induction of oxidative stress as one of the major mechanisms involved with OTA-induced toxic effects [27]. The widespread OTA contamination and associated health effects resulted in adoption of various complementary measures, mainly by the addition of entero-sorbents/modifiers in contaminated feeds to prevent the systemic bioavailability or to convert mycotoxins to less toxic or non-toxic metabolites by altering the molecular structure; subsequently reduced

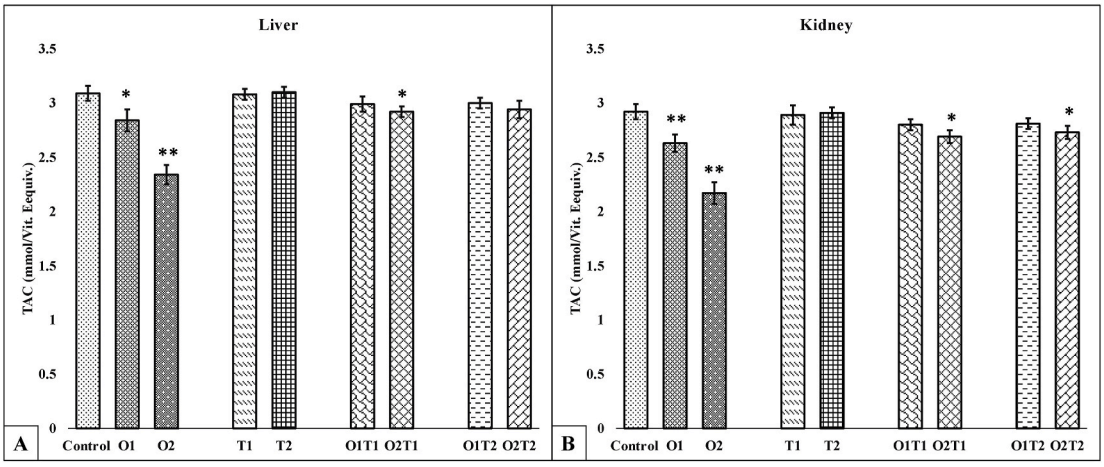

Fig. 4. Total Antioxidant Capacity (TAC) in the liver (A) and kidney (B) of the broiler birds offered basal diet supplemented with dietary concentrations of OTA and TM, alone and in combination (*$p < 0.05$; **$p < 0.01$). Abbreviations are same as in Fig. 1.

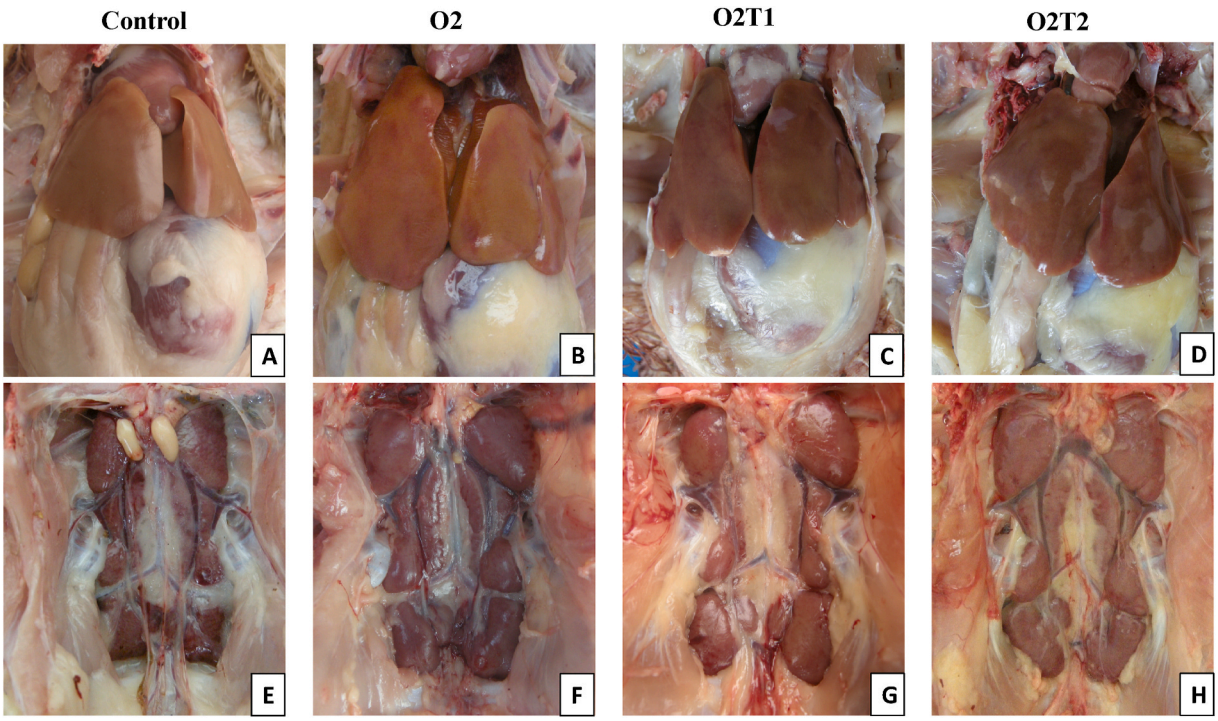

Fig. 5. Photographs of liver (A–D) and kidney (E–H) of the birds from the selected groups kept on experimental diet till 42 days of age. A; liver of the bird from control group showing normal size, texture and color. B; liver showing size enlargement and hemorrhagic spots on surface. C and D; intensity of OTA-medicated changes showed a decrease with increasing the TM level in feed. E; kidney of the bird fed basal diet showing normal lobes within the bony sockets. F; lobes of kidneys showing bulging out of the sockets in the bird fed 0.3 mg OTA/kg feed. G; moderate enlargement in kidneys of the birds from O2T1 group. H; gross changes are mild and size is almost similar to that of control in the kidney of the bird from group O2T2.

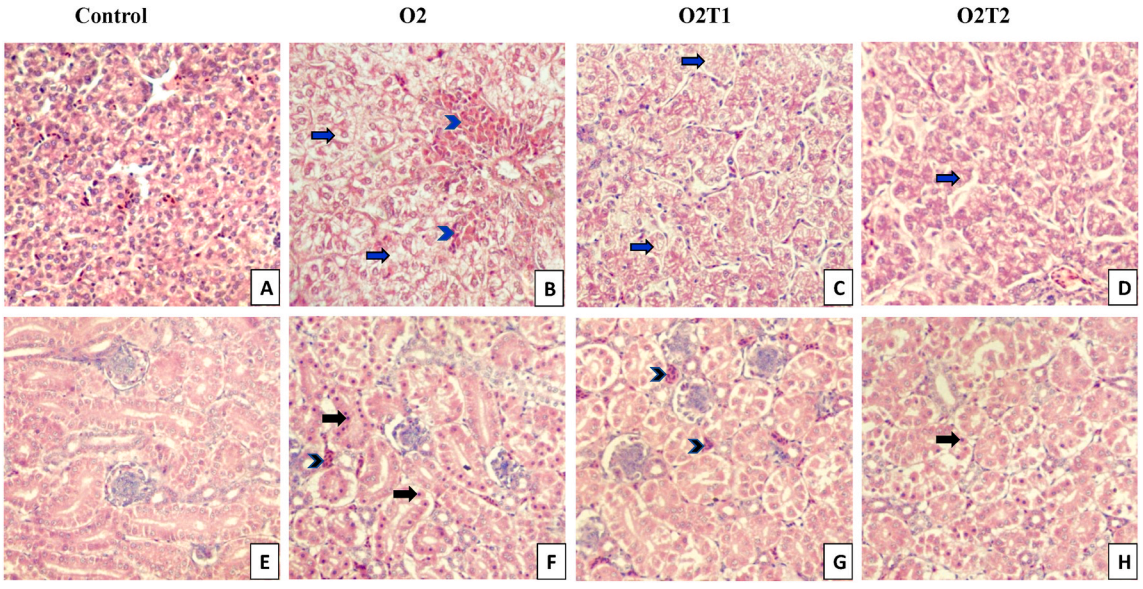

Fig. 6. Histomorphographs of liver (A–D) and kidney (E–H) of the birds from the selected groups kept on experimental diet for 42 days. A; liver of the bird from control group showing normal histological structure. B; hepatocytes showing extensive vacuolation (blue arrows) and accumulation of RBCs around the central vein (blue arrowheads) due to decrease sinusoidal spaces. C and D; intensity of OTA-medicated histological changes showed a decrease with increasing the TM level in feed. E; kidney of the bird fed basal diet showing normal glomeruli and tubules. F; intensive tubular degeneration with pyknotic nuclei (black arrow) and congestion (black arrowheads) in the bird fed 0.3 mg OTA/kg feed. G; moderate pyknosis and tubular degeneration in the birds from O2T1 group. H; degenerative changes are mild in the kidney of bird from O2T2.

the mycotoxin induced deleterious effects. Therefore, in the present study efforts were made to investigate the protective potential of antagonistic yeast TM against OTA in broiler diet and consequent modulation of OTA-induced oxidative stress response and tissue toxicity.

In the present study, birds exposed to dietary OTA contamination demonstrated significant dose dependent reduction in TAC of hepatic and renal tissues. The observed significant decreased serum

concentrations of total protein and albumin as well as significant increased ALT and urea concentrations in this study, have been described as valuable indicators of OTA-induced hepatic and renal tissue injury [28]. The degenerative changes observed on macroscopic and histopathological evaluation of liver and kidney suggested that OTA at both levels was toxic to the broiler chickens. These findings were further supported by the evidences that dietary OTA contamination at both the tested levels significantly increased the relative liver and kidney weights in dose related manner; an indicator of ingested mycotoxin toxicity [29]. The reproduced OTA-induced pathological alterations observed in this study were consistent with several previously reported findings by using dietary OTA contamination range of 0.13–6.4 mg/kg in broiler chickens [28–32]. OTA exposure has a strong negative impact on the synthesis of proteins, disrupt the calcium hemostasis as well as the mitochondrial function, promotes the membrane peroxidation and DNA adducts formation [33]. Kidney being the primary target organ identified for OTA-toxicity in poultry birds and other species, but the well-known mechanism of protein inhibition and free radicals generation showed that liver may be a possible target organ for OTA as well [34].

TM has been tested as a feed additive in the present study to counteract the toxic effects induced by OTA in broiler chickens. The dietary addition of TM (0.5 and 1.0 g/kg) alone to the basal diet did not negatively influence the health status of the experimental birds in groups T1 and T2, as accessed by the non-significant differences of the studied parameters from the birds in control group. In previous reports, the dietary inclusion of TM (1–2 g/kg and 10^4–10^6 CFU/g) did not exert any toxic effect in broiler birds [35,36]. Even in mice, 0.250 mL oral administration of TM (HB 1230) did not negatively influence the blood constituents, liver enzymes and histology; moreover, it resulted in an overall increased weight gain and decreased mortality rate [37,38].

In the combination groups, feeding OTA simultaneously with tested levels of TM, the TAC of the hepatic tissue reveled the protective efficacy of two dietary tested levels of TM against 0.15 mg OTA/kg feed. Similarly, in renal tissue this protective response was also observed against both 0.15 and 0.3 mg OTA/kg feed. The non-significant differences of serum biochemical parameters from control showed the protective potential of all tested levels of TM against dietary contamination levels of OTA (0.15 and 0.3 mg/kg). Meanwhile, the gross and histological assessments of liver and kidney along with their relative weights also demonstrated the protective efficacy of TM against OTA. Compared to the results of the current study, previously, a feeding trial with broiler chicks showed that supplementation of TM significantly counteract the negative impact of OTA (0.2 mg/kg) on weight gain, FCR and mortality percentage [39]. The analysis of plasma OTA concentration confirmed that OTA could be deactivated by the TM when compared to the plasma level of birds fed OTA alone. Similarly, an average increase of 61 g final weight was reported in broiler birds fed OTA (1.0 mg/kg) simultaneously with 10^5 CFU/g of TM, compared to the group served as positive for OTA [40]. The dietary combination of TM and *Eubacterium* BBSH 797 strain could also prevent the swine mycotoxicosis caused by OTA, aflatoxins, zearalenone and trichothecenes [40]. Inclusion of TM (10^4–10^6 CFU/g) in broiler diet completely blocked the injurious impact of 0.5 mg OTA/kg on immunological responses, enhanced feed/gain ratio and lowered the residual plasma concentration of OTA [36]. Using commercial toxin deactivator Mycofix® Plus (1 and 2 g/kg) containing TM (6.0×10^8 cells/g), significantly lowered the residual concentrations of dietary OTA (0.5 and 1.0 mg/kg) in serum, kidney and liver of the broiler birds and also attenuated the OTA-induced adverse effect on health performance parameters [35,41]. Similarly, the use of mycotoxin deactivator product Biomin® MTV (2.0 g/kg) significantly reversed the negative impact of 1.0 and 2.0 mg OTA/kg, as accessed by performance parameters, blood concentration of ALT and total protein and also significantly reduced the bioaccumulation of OTA in liver and kidney of the broiler birds [42]. To the best of our knowledge, this is a first study reporting the efficacy of TM (in non-constituted form) against OTA-induced oxidative damage in liver and kidney of broiler chickens in relation to the serum biochemical changes and histopathological alterations in these organs.

Based on various studies on chicken, mice and rats, it is interesting to note that OTA-induced toxicity might be closely associated to its metabolic process and subsequently its relationship with the ability to induce oxidative stress, cell apoptosis, cell autophagy and inhibition of protein synthesis [43]. Based on various *in vitro* and *in vivo* studies, several mechanisms have been proposed for OTA-induced oxidative stress, including the i) hydroxyl free radicals generation via Fenton reaction, ii) by activation of flavoprotein NADPH-cytochrome P450 and iii) by the inhibition of Nrf2 activation and gene transcription. The major pathways related to the metabolic process of OTA are hydrolysis, hydroxylation, lactone opening and conjugation [44]. The protective mechanism of TM might involve the biodegradation of OTA through amide bond hydrolysis resulting in the formation of L-β-phenylalanine and OTα or by lactone ring opening. Notably, using TM as a biological modifier in an *in vitro* study, resulted in an increased OTα concentration as assessed through chromatographic analysis of biodegradation product [18]. The resulting virtually non-toxic metabolite (OTα) did not affect the activity of macrophages even at concentration of 20 μg/mL, as demonstrated by macrophage activation test. However, in case of lactone ring hydrolysis the resulting metabolite exhibited toxicological properties similar to that of OTA when administered to rats [45–47].

OTA, the major unavoidable contaminant of the agricultural commodities, pose a serious concern to animal and human health throughout the world. Based on the results of the present study it can be concluded that OTA-contamination at both the tested levels (0.15 and 0.3 mg/kg feed) negatively influence the health status of the broiler chickens in dose related manner. TM supplementation (0.5 and 1.0 g/kg feed) counter the OTA-induced significant decrease in total antioxidant capacity, altered serum biochemical and degenerative changes in broiler chickens.

Ethical statement

The experiment was conducted following the approved guidelines regarding protection of animal welfare and all experimental protocols were approved by Directorate of Graduate Studies (DGS), University of Agriculture, Faisalabad.

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CRediT authorship contribution statement

Sheraz Ahmed Bhatti: Conceptualization, Methodology, Data curation, Writing – original draft. **Muhammad Zargham Khan:** Conceptualization, Methodology, Investigation, Supervision. **Muhammad Kashif Saleemi:** &, Data curation, Writing – original draft. **Zahoor Ul Hassan:** Data curation, Writing – original draft, Writing – review &amp editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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