DR. MEHRAN MEHRI (Orcid ID : 0000-0001-8202-8565)

DR. ALI MAGHSOUDI (Orcid ID : 0000-0002-1192-3682)

Article type : Original Article

Running head:

DETOXIFICATION OF AFLATOXIN B_1 BY TRYPTOPHAN

Excess dietary tryptophan mitigates aflatoxicosis in growing quails

Sousan Khanipour*, Mehran Mehri[1,*], Farzad Bagherzadeh-Kasmani*, Ali Maghsoudi*, and Elham Assadi Soumeh†

*Department of Animal Sciences, Faculty of Agriculture, University of Zabol; Department of Special Domestic Animals, Research Institute at the University of Zabol (RIUOZ), IRAN 98661-5538; †School of Agriculture and Food Science, Faculty of Science, The University of Queensland, Gatton, Queensland 4343, Australia

Abbreviation:

AFB_1: aflatoxin B_1

ALP: alkaline phosphatase

[1] Corresponding author at: Department of Animal Science, University of Zabol, Zabol, IRAN 98661-5538. Tel: +98-915-541-6605; E-mail addresses: mehri@uoz.ac.ir (M. Mehri)

ALT: alanine aminotransferase
AST: aspartate aminotransferase
CFU: colony forming unit
CYP: cytochrome
DNCB: 2,4-Dinitro 1-Chlorobenzene
MDA: malondialdehyde
MH-TRP: moderate high tryptophan diet
NDV: Newcastle disease virus
PCA: principal component analysis
PRO: probiotics
SRBC: sheep red blood cells
TBA: thiobarbituric acid
TDO: 2,3-dioxygenase
TMC: total microbial count
TP: total protein
TRP: tryptophan
UA: uric acid

SUMMARY

A biological assay was carried out to evaluate the impact of dietary tryptophan (TRP) in aflatoxin B_1-contaminated diets (AFB_1-D) on performance, blood parameters, immunity, meat quality, and microbial populations of intestine in Japanese quails. Six experimental diets were formulated to include 2 levels of dietary TRP; 2.9 (moderate high: MH-TRP) and 4.9 g/kg (excess: Ex-TRP); and 3 levels of AFB_1 (0.0, 2.5, and 5.0 mg/kg). Each experimental diet was fed to the one of the six groups of birds from 7 to 35 d of age in a completely randomized design with 2 × 3 factorial arrangement. Decrease in feed intake, body weight gain, and gain:feed in birds fed 5.0 mg/kg AFB_1-D was restored to the control level by 4.9 g TRP/kg of the diet. The hepatic enzymes in blood were elevated in quails fed on AFB_1-D but attenuated by 4.9 g TRP/kg of the diet (Ex-TRP; $P \leq 0.01$). High serum uric acid in birds challenged with AFB_1 significantly

decreased by Ex-TRP ($P \leq 0.01$). The skin thickness to 2,4-dinitro-1-chlorobenzene challenge suppressed by AFB_1 but increased by Ex-TRP diet ($P \leq 0.02$). The AFB_1 increased the malondialdehyde in meat whereas TRP efficiently diminished malondialdehyde production ($P \leq 0.01$). The greatest drip loss and pH in meat were observed in the birds fed 5.0 mg/kg AFB_1-D but Ex-TRP augmented the adverse effects of AFB_1 ($P \leq 0.01$). The Ex-TRP reduced the total microbial and *Escherichia coli* counts ($P \leq 0.01$). The adverse effect of AFB_1 on ileal *Lactic acid* bacteria was completely prevented by Ex-TRP ($P \leq 0.03$). This study showed that tryptophan supplementation could be considered as a powerful nutritional tool to ameliorate the adverse effects of AFB_1 in growing quails.

Key words: Aflatoxicosis; antioxidant; immunology; L-tryptophan; malondialdehyde

INTRODUCTION

Aflatoxins are the toxic secondary metabolites that have been recognized in the early 1960s. Although we are trying to remove aflatoxins from the grain before consumption, the symbiosis of grains and fungi allowed for production aflatoxins to be toxic when consumed by man and animals (Richard 2007). This phenomena may be considered as a natural security system against environmental threats to be alive during evolution. The structure of many mycotoxins has been unfold by scientists and harmful consequences of mycotoxin residues in agricultural commodities have been well documented during the last decade that encouraged researchers to find an efficient and safe procedure to produce agricultural products prior to marketing and entering into the animal and human food chains (Pier 1981; Gregory and Edds 1984; Diekman and Green 1992; Zamani-Zadeh and Khoursandi 1995; Jones et al. 1996; Binder et al. 2007; Zain 2011; Bagherzadeh-Kasmani et al. 2012a; Bagherzadeh-Kasmani et al. 2012b; Li et al. 2014; Bagherzadeh-Kasmani and Mehri 2015; Bagherzadeh-Kasmani et al. 2015; Mohammadi et al. 2015; Murugesan et al. 2015; Aftabi et al. 2016). The number of countries with specific mycotoxin regulations increased from 33 in 1981 to 100 in 2003 to protect consumers from 13 different mycotoxins through food safety policies (Van Egmond et al. 2007).

Among mycotoxins, aflatoxin B_1 (AFB_1) with $C_{17}H_{12}O_6$ molecular formula and melting point of 268 to 269°C, mainly synthesized by *Aspergillus flavus and Aspergillus parasiticus* (Fink-Grernmels 1999; Feddern et al. 2013), is the most harmful substrate for the liver

microsomes in oxidation process. In fact, AFB_1 is the most potent hepatotoxic that may cause immunosuppressive, carcinogenic, teratogenic and mutagenic diseases (Richard 2007). Our group has recently showed that 2.5 mg AFB_1/kg of diet significantly decreased the performance and immunological responses of growing Japanese quails as well as increased the hepatic malfunction. There are different methods to detoxify AFB_1 in poultry species including physical (Rustom 1997), chemical (Piva et al. 1995), and biological procedures (Bagherzadeh-Kasmani and Mehri 2015). Among the different methodologies, the biological methods theoretically warrant more safety and nutritional benefits in animal production rather than physical (costly) and chemical (harmful) techniques. The use of probiotics in the diet could mitigate the adverse effects of AFB_1 as a biological technique for detoxification (Bagherzadeh-Kasmani and Mehri 2015). Aflatoxin binding with the probiotics in the body is a reversible reaction (Zoghi et al. 2014) and its stability depends on a variety of uncontrolled factors that should be considered as a drawback of probiotics implementation. Diaz et al. (2010) demonstrated that cytochrome P450 (CYP) enzymes are involved in the metabolic reaction of producing 8,9- epoxide, which is the toxic form of AFB_1. The CYP2A and CYP1A are the most important isoforms in quail and chicken. Therefore any reaction to reduce oxidation process of AFB_1 could be helpful.

Tryptophan as the precursor of serotonin could be catalyzed by tryptophan hydroxylase to produce 5-Hydroxytryptophan (5-HTP) as the intermediate metabolite in the production pathway of serotonin.. Kot et al. (2012) used the free-tryptophan diet to survey the relationship of brain serotoin and liver cytochrome P450 (CYP) deficiency. They suggested that the dietary tryptophan (TRP) level is correlated with the biotransformation of AFB_1 to its toxic metabolite, 8,9- epoxide.

The role of TRP in the production of biological antioxidants and serotonin and relationship of this essential amino acids with oxidation reactions in the liver CYP encouraged us to desin an experiment to study the nutritional manipulation of dietary TRP to diminish the adverse effects of AFB_1.

MATERIALS AND METHODS

Bird management

The experimental procedures for animal trials were approved by the Animal Ethics Committee of the University of Zabol, Iran prior to the commencement of the trial. A total of

240 one-day old quail chicks (*Coturnix coturnix Japonica*) were provided from the meat-type Quail Genetic Stock Centre at the Research Center of the University of Zabol (RCUOZ, Sistan, Iran) and fed standard starter diet to meet or exceed the nutrient recommendations by NRC (1994) from hatch to d 6. On d 7, quail chicks with approximately similar average weight (26.8 g ± 1.34) were randomly distributed across 24 floor pens, 6 treatments and 4 replicates, and 10 birds per pen. The temperature of experimental house was set at 29 and 26^0 C for the second and third weeks of age, respectively, with relative humidity of 55%. The lighting schedule of 23h light and 1h dark was applied throughout the study.

Chemical analysis of feed ingredients

Feed ingredients were analyzed for crude protein (CP) (method 990.03, AOAC 2006) and amino acids contents (method 982.30, AOAC 2006). For amino acid analysis, all samples were hydrolyzed for 24 h at 110°C in 6 N hydrochloric acid under an atmosphere of nitrogen. For Met and Cys, performic acid oxidation was done before acid hydrolysis. Samples were hydrolyzed using barium hydroxide for TRP content (AOAC 2006). Chromatographic separations of amino acids were performed with a Waters HPLC system (Waters, Milford, MA). It consisted of a 1525 Binary HPLC pump, a 2487 Dual λ absorbance detector operating at 254 nm, Breeze chromatography software and a Rheodyne 7725 injection valve (Cotati, CA, USA) which equipped with a 20 µl sample loop. The column was Pico tag (3.9×150 mm I.D.; particle size 5 µm).

Preparation of AFB_1

The AFB_1 was produced by PTCC-5286 strain of *Aspergillus parasiticus* grown on rice grain and fermented under constant stirring and controlled temperature (Dashkevicz and Feighner 1989). Then after, the concentration of AFB_1 in contaminated rice samples was determined using an ELISA kit (Ridascreen Aflatoxin B_1 Art. No. 1211, R-Biopharm, Darmstadt, Germany). Contaminated rice was incorporated into the basal diet to provide the calculated amounts of 2.5 and 5.0 mg AFB_1/kg of feed.

Experimental diets

Six experimental diets were formulated to include 2 levels of dietary TRP; 2.9 (moderate high: MH-TRP) and 4.9 g/kg (excess: Ex-TRP); and 3 levels of AFB_1 (0.0, 2.5, and 5.0 mg/kg). Each

144 experimental diet was fed to the one of the six groups of birds from 7 to 35 d of age in a
145 completely randomized design with 2 × 3 factorial arrangement.

146 **Growth performance and dressing**

147 Feed intake (FI) and body weight gain (BW gain) were recorded weekly on a pen basis and
148 mortality was recorded as it occurred. Gain efficiency (G:F) was calculated from the BW gain
149 and FI data. On d 35, three birds per replicate were killed by cervical dislocation and internal
150 organs including liver, spleen, heart, and bursa of Fabricius were removed and weighed
151 immediately. Relative weights of breast meat, leg meat, spleen, and dressing to live weight were
152 also calculated.

153 **Serum biochemical analysis**

154 Blood samples were collected from 48 birds (2 birds per each replicate) by jugular vein puncture
155 into 10 ml heparinized tubes on d 35. Serum parameters including total protein (TP), alkaline
156 phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and uric
157 acid (UA) were determined by spectrophotometric method using commercially available kits
158 (Parsazmun, Tehran, Iran).

159 **Immunological responses**

160 At d 15 and 25, 2 birds per replicate were randomly selected and challenged with sheep red
161 blood cell (SRBC) antigens and antibody production against SRBC antigen was measured at d
162 35. Two birds per replicate were wing banned and vaccinated against Newcastle disease virus
163 (NDV) at d 14 and 21 using the lyophilized vaccine (Live B_1 strain; Vetrina; Zagreb, Croatia). At
164 d 35, the antibody production against NDV antigen was assessed by a hemagglutination
165 inhibition test in the serum samples according to Cheema et al. (2003). The 2,4-Dinitro 1-
166 chlorobenzene (DNCB; Merck; Darmstadt, Germany) was dissolved in a mixture of acetone and
167 olive oil (4:1 vol/vol) to a final concentration of 1 mg/mL. At d 33, the skin of 2 birds in each
168 replicate was anointed with 0.1 mL of DNCB solution. An area of approximately 4 cm^2 on the
169 left lateral abdomen without feathers was chosen for the challenge with DNCB. The same area
170 on the right side was treated with the solvent alone. The skin thickness (on both sides) before and
171 12, 24, and 48 h after the challenge was measured to assess reactions. Differences before and
172 after the challenge were calculated to determine the mean increase in skin thickness in each bird
173 (Verma et al. 2004).

Meat quality assessment

At the end of the trial, 2 birds per replicate were randomly selected and sacrificed by cervical dislocation and deboned meat of leg was ground with a blender and stored at -20°C for 30 d to determine the oxidation stability in meat samples as described by Mehri et al. (2015), In brief, 1 g of ground meat sample was homogenized (Polytron homogenizer, PCU, Switzerland) with 4 ml of 5% aqueous trichloroacetic acid (TCA) and 2.5 ml of 0.8% butylated hydroxytoluene, and then centrifuged at 3000 × *g* for 3 min. The supernatant (hexane layer) was discarded and the remaining was filtered and made to 5 mL volume with 5% TCA, then placed into a screw-capped tube containing 3 mL of 0.8% aqueous 2-thiobarbituric acid (TBA). Eventually, tubes were heated in 70°C water bath for 30 min, then immediately cooled under tap water and injected into a spectrophotometry (UNIKON 933, Kontron Co. Ltd., Milan, Italy). The height of the third-order derivative peak that appeared at 521.5 nm was used for calculation of the malondialdehyde (MDA) concentration in the samples. Tetraethoxypropane (1, 1, 3, 3- Tetraethoxy propane, T9889, 97%, Sigma, USA.) was used as a MDA precursor in the standard curve. The concentration of MDA was expressed as milligrams per kilogram of meat samples.

The pH of the meat samples was measured by homogenizing 5 g of raw meat with 25 mL of distilled water. The homogenates were filtered, and pH of each sample was measured with a pH meter at room temperature in triplicates (Mehdipour et al. 2013). A 20 g of meat sample was taken 24 h post mortem, placed in a plastic bag, and kept at 4°C. After 24 h, the sample was removed from the bag, dried on absorbent paper, and reweighed. Amount of drip at 48 h post mortem was expressed as a percentage:

Drip loss (%) = [(initial weight-final weight)/initial weight] × 100

Bacterial populations of ileal content

The ileal contents of 3 birds per replicate were separately collected into the sterile tubes for serial dilution as described by Ghazaghi et al. (2014). In brief, 1 g of ileal digesta was added into the test tube containing 9 mL of sterilized phosphate buffered saline, and buffered solutions were transferred to the microbial laboratory of our institute. Microbial populations were determined by serial dilution (10^{-4} to 10^{-6}) of ileal samples before inoculation onto Petri dishes. Plates for lactic acid bacteria (LAB; grown in deMan, Rogosa and Sharpe, MRS agar) and coliform bacteria (grown in Mac Conkey agar) were incubated at 37°C in anaerobic and aerobic media,

respectively. Plate count agar was used for total count of bacteria. Finally, plates were counted between 24 and 48 h after inoculation. All agar media were obtained from the Merck Company, Germany. Colony forming units were defined as distinct colonies measuring at least one mm in diameter.

Statistical analysis

Statistical analyses were performed with the GraphPad Prism (GraphPad Prism Software Inc., San Diego, CA). Two-way ANOVA test was used to detect differences and Tukey test was used to compare means of each experimental group. All data are given as means ± SD. Levels of significance were set at $P < 0.05$. Principal component analysis (PCA) was performed to simply visualize any relationship between variables using Minitab software (Minitab 2015).

RESULTS

Performance

The effects of experimental diets on growth performance of quail chicks are shown in Figure 1. Although the main effect of dietary TRP on FI and BWG was not significant, dietary concentrations of TRP and AFB_1 interactively influenced quail growth performance, where the impaired FI, BWG, and G:F caused by AFB_1 in a dose-dependent manner, were restored by increasing dietary TRP level (AFB_1 by TRP interaction; $P \leq 0.01$). However, an increase in FI (about 7.0%) and BW gain (about 1.7%) were observed in birds receiving 2.5 mg AFB_1/kg and Ex-TRP diet compared to control group.

Carcass attributes

Increased dietary TRP improved leg meat yield ($P = 0.03$), and tended to improve breast meat yield ($P = 0.08$) and dressing ($P = 0.15$). The ABF_1-D resulted in the reduction of breast meat yield (BMY; $P \leq 0.03$) and leg meat yield (LMY; $P < 0.05$), and decreased carcass percentage ($P < 0.01$). No interactions of TRP and ABF_1 was observed on BMY, LMY, and dressing ($P > 0.05$). (Figure 2). Although the main effect of TRP and interaction of TRP × AFB_1 were not significant on dressing percent, however, dressed carcass was increased by 3.0% in birds received 2.5 mg AFB_1/kg in Ex-TRP diet compared to control.

Blood markers

As depicted in Figure 3, dietary TRP and AFB_1 interactively influenced the blood markers of hepatic cell damage including ALP ($P \leq 0.02$), ALT ($P \leq 0.05$), and AST ($P \leq 0.01$). The increase in blood levels of ALP, ALT, and AST in birds fed AFB_1-D was reduced by feeding Ex-TRP diets (AFB_1 by TRP interaction; $P \leq 0.05$). However, increasing dietary TRP in toxin-free group increased the blood concentrations of ALT ($P \leq 0.09$) and AST ($P \leq 0.01$). Serum total protein was decreased in birds fed AFB_1-D but increasing dietary level of TRP elevated total serum protein in those birds (AFB_1 by TRP interaction; $P \leq 0.05$). Although the blood concentration of uric acid was increased in birds fed AFB_1-D, feeding Ex-TRP diets decreased the serum level of uric acid in toxin-treated quails (AFB_1 by TRP interaction; $P \leq 0.01$). However, increasing dietary TRP in toxin-free treatment increased the blood concentration of uric acid ($P \leq 0.02$).

Immune responses

As shown in Figure 4, dietary TRP and AFB_1 had an interaction effect on skin thickness response to DNCB challenge (AFB_1 by TRP interaction; $P \leq 0.02$) and relative weight of spleen (AFB_1 by TRP interaction; $P \leq 0.01$) of quails fed AFB_1-D. The weak response to DNCB challenge and high relative weight of spleen in toxin-treated birds were reversed by feeding Ex-TRP diet. Although the main effect of AFB_1 resulted in low production of antibody against SRBC-antigen and hemagglutination test, no interaction of TRP × AFB_1 was observed in toxin-treated groups (AFB_1 by TRP interaction; $P > 0.50$).

Meat quality

Neither main effect of TRP nor TRP and AFB_1 interaction were significant on drip loss but AFB_1 increased drip loss in meat samples of the birds received 5.0 mg AFB_1/kg diet ($P \leq 0.01$). The interaction of TRP and AFB_1 increased pH in fresh meat samples (AFB_1 by TRP interaction; $P \leq 0.01$). The production of MDA was increased in meat samples of quails fed on AFB_1-D but increasing dietary TRP resulted in dose-dependent reduction of MDA concentration (AFB_1 by TRP interaction; $P \leq 0.01$).

Microbial populations

AFB_1 did not affect total microbial count (TMC) and *E. coli* numbers in small intestine of quails but dietary TRP decreased both TMC and *E. coli* counts ($P \leq 0.01$). Although AFB_1-D decreased

lactic acid CFU in small intestine of birds, increasing dietary TRP remarkably increased *lactic acid* counts in toxin-treated birds (AFB_1 by TRP interaction; $P \leq 0.01$). The AFB_1 did not affect total microbial count (TMC) and *E. coli* numbers in small intestine of quails but dietary TRP decreased both TMC and *E. coli* numbers ($P \leq 0.01$).

DISCUSSION

Decreased feed consumption and growth rate in poultry species fed on AFB_1-D are the first indications of aflatoxicosis as observed in the present study and other reports (Denli et al. 2009; Bagherzadeh-Kasmani and Mehri 2015; Aftabi et al. 2016). In fact, AFB_1 toxicity arise from the generation of intracellular hydroxyl radical, superoxide anion, and hydrogen peroxide during the conversion of AFB_1 to aflatoxin 8,9- epoxide. Although growth performance was impaired by AFB_1, Ex-TRP (almost 2 times concentration of the recommended levels by NRC, 1994: 2.2 g/kg of diet) could restored G:F, FI (about 7.0%), BW gain (about 1.7%), and dressing (about 3.0%) in quails received 2.5 mg AFB_1/kg of diet. da Silva et al. (2017) suggested that an increase in the cholinergic and adenosinergic system of intoxicated quails by AFB_1 might be the reason for the inflammatory process and tissue damage, especially in the liver and gut tissues. Liver is the main detoxifying organ which could be affected by the harmful toxins entering the body through feed, and the liver damage could be diagnosed by high concentrations of the hepatic enzymes in blood (Balachandran and Ramarkrishnan 1988).

Elevated plasma levels of hepatic enzymes such as AST and ALT in the poultry species is a sign of hepatic cell damage caused by a variety of stressors or harmful agents (Valdivia et al. 2001; Miazzo et al. 2005; Yarru et al. 2009; Bagherzadeh-Kasmani and Mehri 2015). On the other hand, protective action of excess dietary TRP in stressful conditions has been reported in pig (Sève et al. 1991; Mao et al. 2014), duck (Liu et al. 2015), broiler chicks (Corzo et al. 2005), and laying hens (Dong et al. 2012). A part of beneficial effects of excess dietary TRP may originate from *de novo* synthesis of its metabolites such as e. g. kynurenine, 5-hydroxytryptophan and melatonin (Le Floc'h et al. 2011). In the present study, excess dietary TRP interactively improved the disturbed oxidative stability and immunity of intoxicated quails.

Since avian immune system relies on B-cells, T-cells, and spleen functionality, any destruction in bursa of Fabricius, thymus, and spleen could be associated with impaired immunological responses (Girish and Smith 2008). Histopathological observation of the spleen showed that

elevated relative weight of spleen caused by aflatoxins could be due to the congestion of red pulp in the spleen (Peng et al. 2014). Reversing the negative effects of AFB_1 on immune organs by Ex-TRP diet may be governed either by kynurenine production or anti-oxidant properties of TRP derivatives such as 5-hydroxytryptophan and melatonin, resulting in the healing of congestion of red pulp in the spleen. The birds fed AFB-contaminated diets suffer from physiological lesion due to reactive oxygen species (ROS) and defense systems in the body would be counteract the dietary toxin. Since the molecule of AFB is not carcinogenic, decreasing oxidation in liver microsomes to inhibit the synthesis of the carcinogenic 8, 9- epoxide might be one of the mechanisms against aflatoxicosis. The most important P450 isoforms in quail and chicken are CYP1A and CYP2A that are functional to conversion of AFB to 8,9-epoxide (Diaz et al. 2010). The TRP and serotonin deficiency could alter the activity of P450, and increase the activity of CYP2A and CYP1A (Kot et al. 2012). As one of the defense mechanisms of the challenged bird with AFB is decreasing the P450 (i.e., CYP2A and CYP1A), excess dietary TRP in AFB-contaminated diets might be the important strategy to control the ROS. Kot et al. (2012) showed that omitting TRP from the diet, resulted in an increase in the activity of hepatic CYP isoforms, especially CYP2A and CYP1A. The lesions in the gastrointestinal track and the decrease in peripheral serotonin down-regulate the gene expression of P450 (Ruggiero et al. 2012).

Presumably, the use of a portion of dietary TRP to detoxify AFB_1 in the body may provide low-doses of intact toxin. This mechanism could stimulate performance through *hormesis* mechanism in AFB_1-treated birds. In this case, Calabrese and Baldwin (2003) suggested that low concentrations of toxin may cause the U-shape response to the chemical/agent stimulators, a concept called “dose-response revolution”, namely *hormesis*. The *hormesis* mechanism could be regulated by the level of dietary TRP, a key amino acid in the regulation of FI in pig (Le Floc'h and Seve 2007), possibly through the rate-limiting synthesis of serotonin (Leathwood 1987). In addition, Zhang et al. (2007) demonstrated that some nutrients in diet such as TRP were more important factors affecting FI than fasting. They showed that TRP could significantly increase the ghrelin secretion in the gastrointestinal tract (i.e., stomach and duodenum) of pig through increasing its mRNA expression. Ghrelin, a 28-amino acid peptide produced mainly by the stomach, is the most important gastric hormone regulating FI in monogastric animals (Le Floc'h and Seve 2007).

Kaiya et al. (2002) indicated that ghrelin in the chicken is composed of 26 amino acids, which is mainly synthesized in the proventriculus and directly act on pituitary gland via the systemic circulation to control of feed consumption. Although we did not measure ghrelin secretion in quail chicks, we speculated that it is likely a part of stimulating effects of TRP on FI in AFB_1-contaminated birds may be related to the upregulated ghrelin mRNA expression in the gut.

In toxin-free groups, increasing the dietary TRP did not exert stimulatory effect on FI but in AFB_1-contaminated birds, the Ex-TRP diet had a positive impacts on FI. Chen et al. (2016) reported that impaired gut function in the AFB_1-contaminated broilers could be completely eliminated by increasing dietary protein, a potential remedy for tissue destructions in the gut that might be governed by bioactive amino acids such as TRP in high protein diets.

A second role of excess dietary TRP on changing blood metabolites and lipid peroxidation index of meat (i.e., MDA concentration) has been shown in this study. In toxin-free group, the Ex-TRP diet increased the AST and uric acid in sera as well as the MDA concentration in the meat samples. However, immunity response and oxidation stability were augmented in toxin-contaminated birds received high levels of dietary TRP. The effect of TRP on meat quality was promising where the increasing TRP in diet considerably decreased the MDA content of meat samples implying on the increased oxidation stability. Some of the TRP derivatives and tryptophan-metabolizing enzymes such as TDO, which is an antioxidant enzyme, inhibit lipid peroxidation in the liver of rats. The TDO is a rate-limiting enzyme catalyzing the oxygenation reaction of TRP by consuming superoxide anion as an oxidative co-factor (Dairam et al. 2006). The AFB_1 upregulates the gene expression of cytochrome P450, especially CYP2A6 in quail, and TDO. Excess dietary TRP not only supplies TRP in the blood, supporting growth rate, but also provides sufficient substrate for TDO (Mao et al. 2014) resulting in the production of the TRP metabolites such as 5-hydroxytryptophan (i.e., a metabolic intermediate in the biosynthesis of the neurotransmitter serotonin), 3- hydroxyanthranilic acid and 3-hydroxykynurenine (i.e., tryptophan metabolites of the kynurenine pathway) with antioxidant capacity (Christen et al. 1990).

Next enzyme involved in TRP metabolism is indoleamine 2,3- dioxygenase (IDO) catalyzing TRP into kynurenine which may explain the positive effects of the Ex-TRP diet in challenged birds to exhibit better immune response than control group.

In terms of intestinal microflora, the Ex-TRP diet changed the gut microbiota profile and *E. coli* and *lactic acid* counts. Decreased the *E. coli* and increased *lactic acid* counts in response to the Ex-TRP diet indicates that the property of the cell membrane of gram negative and gram positive bacteria may determine the efficacy of TRP on microbial survival. This modulatory effect of dietary TRP on the gut microbial diversity may impact the 5-hydroxytryptophan synthesis by the host (Yano et al. 2015). Although this mechanism has not been elucidated in quails, the beneficial modulation of dietary TRP through decreasing *E. coli* and increasing *lactic acid* CFU in the present study, indicates the nutritional modulation of the intestinal microflora.

Based on the principle component analyses (Figure 7), the most important variables positively associated with dietary AFB_1 were AST, ALT, UA, ALP, MDA, drip loss, relative weight of spleen, and *E coli* CFU. In PCA, dimensions of original variables (e.g. bird responses) without a loss of information are reduced and correlation between variables are shown in biplot visualization. By definition, the correlation between the PCs is zero; that is, the variation explained in PC1 is independent of that explained in PC2 and so on (Mehri et al. 2015). As depicted in the biplot, the MH-TRP diet could not reverse the negative effects of AFB_1, however, the Ex-TRP diet neutralized the AFB_1 toxicity without improvement in bird immune response.The positive loadings of DNCB, HI, SRBC, TP, *lactic acid* CFU, and growth performance in response to the high dietary level of TRP revealed the beneficial impact of the TRP in either toxin-free or intoxicated quails with 2.5 mg AFB_1/kg of diet. In addition, the biplot loading shows the higher efficacy of the Ex-TRP in AFB_1-D than the MH-TRP. Figure 7 shows that the DNCB challenge is more related to the dietary TRP compared to SRBC challenge as humoral immune response. The Ex-TRP is more associated with preferable gut environment and cell-mediated immunity and the MH-TRP diet warrants a better growth performance in toxin-free diet.

In conclusion, this research provides evidences that excess dietary TRP may react with AFB_1 in the body, regulating the detoxification pathway of AFB_1. However, more details on the cellular reaction of TRP is needed to elucidate the possible detoxification mechanism.

Acknowledgements

The authors would like to thank Dr. E. Assadi Soumeh for the review and helpful comments. This project was supported by the University of Zabol (grant numbers UOZ-GR-9517-9).

Conflict of interest: The authors declare no conflict of interest.

References

Aftabi, M.; Bagherzadeh Kasmani, F.; Jalilvand, G.; Mehri, M.; Karimi Torshizi, M.A., 2016: Effect of Protexin probiotics supplementation to aflatoxin contaminated diet on performance of Japanese quail. *J. Anim. Prod.* **17**, 131-140.

AOAC, 2006: *Official methods of analysis*. AOAC International, Arlington, VA.

Bagherzadeh-Kasmani, F.; Karimi-Torshizi, M.; Allameh, A.; Shariatmadari, F., 2012a: A novel aflatoxin-binding Bacillus probiotic: performance, serum biochemistry, and immunological parameters in Japanese quail. *Poult. Sci.* **91**, 1846-1853.

Bagherzadeh-Kasmani, F.; Karimi-Torshizi, M.A.; Allameh, A.A.; Shariatmadari, F., 2012b: Aflatoxin detoxification potential of lactic acid bacteria isolated from Iranian poultry. *Iran. J. Vet. Res.* **13**, 152-155.

Bagherzadeh-Kasmani, F.; Mehri, M., 2015: Effects of a multi-strain probiotics against aflatoxicosis in growing Japanese quails. *Livest. Sci.* **177**, 110-116.

Bagherzadeh-Kasmani, F.; Omidikia, S.; Mirzaie, H.R.; Mehri, M., 2015: Effects of Salvia mirzayanii leaf powder on performance and cecal microbial population of broilers. *J. Anim. Prod.* **16**, 103-111.

Balachandran, C.; Ramarkrishnan, R., 1988: Influence of dietary aflatoxin on certain serum enzyme levels in broiler chickens. *Mycopathologia* **101**, 65-67.

Binder, E.; Tan, L.; Chin, L.; Handl, J.; Richard, J., 2007: Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Anim. Feed Sci. Technol.* **137**, 265-282.

Calabrese, E.J.; Baldwin, L.A., 2003: Hormesis: the dose-response revolution. *Annu. Rev. Pharmacol. Toxicol.* **43**, 175-197.

Cheema, M.A.; Qureshi, M.A.; Havenstein, G.B., 2003: A comparison of the immune response of a 2001 commercial broiler with a 1957 randombred broiler strain when fed representative 1957 and 2001 broiler diets. *Poult. Sci.* **82**, 1519-1529.

Chen, X.; Naehrer, K.; Applegate, T., 2016: Interactive effects of dietary protein concentration and aflatoxin B1 on performance, nutrient digestibility, and gut health in broiler chicks. *Poult. Sci.* **95**, 1312-1325.

Christen, S.; Peterhans, E.; Stocker, R., 1990: Antioxidant activities of some tryptophan metabolites: possible implication for inflammatory diseases. *Proceedings of the National Academy of Sciences* **87**, 2506-2510.

Corzo, A.; Kidd, M.; Thaxton, J.; Kerr, B., 2005: Dietary tryptophan effects on growth and stress responses of male broiler chicks. *Br. Poult. Sci.* **46**, 478-484.

da Silva, A.S.; Santurio, J.M.; Roza, L.F.; Bottari, N.B.; Galli, G.M.; Morsch, V.M.; Schetinger, M.R.C.; Baldissera, M.D.; Stefani, L.M.; Radavelli, W.M., 2017: Aflatoxins produced by Aspergillus parasiticus present in the diet of quails increase the activities of cholinesterase and adenosine deaminase. *Microb. Pathog.*, 10.1016/j.micpath.2017.1003.1041.

Dairam, A.; Antunes, E.M.; Saravanan, K.; Daya, S., 2006: Non-steroidal anti-inflammatory agents, tolmetin and sulindac, inhibit liver tryptophan 2, 3-dioxygenase activity and alter brain neurotransmitter levels. *Life Sci.* **79**, 2269-2274.

Dashkevicz, M.P.; Feighner, S.D., 1989: Development of a differential medium for bile salt hydrolase-active Lactobacillus spp. *Appl. Environ. Microbiol.* **55**, 11-16.

Denli, M.; Blandon, J.C.; Guynot, M.E.; Salado, S.; Perez, J.F., 2009: Effects of dietary AflaDetox on performance, serum biochemistry, histopathological changes, and aflatoxin residues in broilers exposed to aflatoxin B1. *Poult. Sci.* **88**, 1444-1451.

Diaz, G.; Murcia, H.; Cepeda, S., 2010: Cytochrome P450 enzymes involved in the metabolism of aflatoxin B1 in chickens and quail. *Poult. Sci.* **89**, 2461-2469.

Diekman, M.A.; Green, M.L., 1992: Mycotoxins and reproduction in domestic livestock. *J. Anim. Sci.* **70**, 1615-1627.

Dong, X.; Azzam, M.; Rao, W.; Yu, D.; Zou, X., 2012: Evaluating the impact of excess dietary tryptophan on laying performance and immune function of laying hens reared under hot and humid summer conditions. *Br. Poult. Sci.* **53**, 491-496.

Feddern, V.; Dors, G.C.; Tavernari, F.d.C.; Mazzuco, H.; Cunha, A.; Krabbe, E.L.; Scheuermann, G.N., 2013: Aflatoxins importance on animal nutrition, Aflatoxins-Recent Advances and Future Prospects. InTech.

438 Fink-Grernmels, J., 1999: Mycotoxins: their implications for human and animal health. *Vet. Q.*
439 **21**, 115-120.
440 Ghazaghi, M.; Mehri, M.; Bagherzadeh-Kasmani, F., 2014: Effects of dietary Mentha spicata on
441 performance, blood metabolites, meat quality and microbial ecosystem of small intestine in
442 growing Japanese quail. *Anim. Feed Sci. Technol.* **194**, 89-98.
443 Girish, C.; Smith, T., 2008: Impact of feed-borne mycotoxins on avian cell-mediated and
444 humoral immune responses. *World Mycotoxin J.* **1**, 105-121.
445 Gregory, J.F., 3rd; Edds, G.T., 1984: Effect of dietary selenium on the metabolism of aflatoxin
446 B1 in turkeys. *Food Chem. Toxicol.* **22**, 637-642.
447 Jones, F.T.; Wineland, M.J.; Parsons, J.T.; Hagler, W.M., Jr., 1996: Degradation of aflatoxin by
448 poultry litter. *Poult. Sci.* **75**, 52-58.
449 Kaiya, H.; Van Der Geyten, S.; Kojima, M.; Hosoda, H.; Kitajima, Y.; Matsumoto, M.;
450 Geelissen, S.; Darras, V.M.; Kangawa, K., 2002: Chicken ghrelin: purification, cDNA cloning,
451 and biological activity. *Endocrinology* **143**, 3454-3463.
452 Kot, M.; Pilc, A.; Daniel, W.A., 2012: Simultaneous alterations of brain and plasma serotonin
453 concentrations and liver cytochrome P450 in rats fed on a tryptophan-free diet. *Pharmacol. Res.*
454 **66**, 292-299.
455 Le Floc'h, N.; Seve, B., 2007: Biological roles of tryptophan and its metabolism: Potential
456 implications for pig feeding. *Livest. Sci.* **112**, 23-32.
457 Le Floc'h, N.; Otten, W.; Merlot, E., 2011: Tryptophan metabolism, from nutrition to potential
458 therapeutic applications. *Amino Acids* **41**, 1195-1205.
459 Leathwood, P.D., 1987: Tryptophan availability and serotonin synthesis. *Proc. Nutr. Soc.* **46**,
460 143-156.
461 Li, X.; Zhao, L.; Fan, Y.; Jia, Y.; Sun, L.; Ma, S.; Ji, C.; Ma, Q.; Zhang, J., 2014: Occurrence of
462 mycotoxins in feed ingredients and complete feeds obtained from the Beijing region of China. *J.*
463 *Anim. Sci. Biotech.* **5**, 37.
464 Liu, Y.; Yuan, J.; Zhang, L.; Zhang, Y.; Cai, S.; Yu, J.; Xia, Z., 2015: Effects of tryptophan
465 supplementation on growth performance, antioxidative activity, and meat quality of ducks under
466 high stocking density. *Poult. Sci.* **94**, 1894-1901.

Mao, X.; Lv, M.; Yu, B.; He, J.; Zheng, P.; Yu, J.; Wang, Q.; Chen, D., 2014: The effect of dietary tryptophan levels on oxidative stress of liver induced by diquat in weaned piglets. *J. Anim. Sci. Biotech.* **5**, 49.

Mehdipour, Z.; Afsharmanesh, M.; Sami, M., 2013: Effects of dietary synbiotic and cinnamon (< i> Cinnamomum verum</i>) supplementation on growth performance and meat quality in Japanese quail. *Livestock Science* **154**, 152-157.

Mehri, M.; Sabaghi, V.; Bagherzadeh-Kasmani, F., 2015: Mentha piperita (peppermint) in growing Japanese quails' diet: Serum biochemistry, meat quality, humoral immunity. *Anim. Feed Sci. Technol.* **206**, 57-66.

Miazzo, R.; Peralta, M.F.; Magnoli, C.; Salvano, M.; Ferrero, S.; Chiacchiera, S.M.; Carvalho, E.C.; Rosa, C.A.; Dalcero, A., 2005: Efficacy of sodium bentonite as a detoxifier of broiler feed contaminated with aflatoxin and fumonisin. *Poult. Sci.* **84**, 1-8.

Minitab, 2015: Minitab Statistical Software. Release 17.2.1. for Windows. Minitab Inc., State College, PA.

Mohammadi, F.; Bagherzadeh-Kasmani, F.; Shojaian, K.; Mehri, M.; Karimi-Torshizi, M.A., 2015: Effect of Hibiscus sabdariffa on performance of broilers fed aflatoxin contaminated diet. *J. Anim. Prod.* **17**, 301-309.

Murugesan, G.; Ledoux, D.; Naehrer, K.; Berthiller, F.; Applegate, T.; Grenier, B.; Phillips, T.; 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.

NRC, 1994: *Nutrient Requirements for Poultry. 9th rev. ed.* National Academy Press, Washington, DC.

Peng, X.; Zhang, K.; Bai, S.; Ding, X.; Zeng, Q.; Yang, J.; Fang, J.; Chen, K., 2014: Histological lesions, cell cycle arrest, apoptosis and T cell subsets changes of spleen in chicken fed aflatoxin-contaminated corn. *Int. J. Environ. Res. Public Health* **11**, 8567-8580.

Pier, A., 1981: Mycotoxins and animal health. *Adv. Vet. Sci. Comp. Med.* **25**, 185-243.

Piva, G.; Galvano, F.; Pietri, A.; Piva, A., 1995: Detoxification methods of aflatoxins. A review. *Nutr. Res.* **15**, 767-776.

Richard, J.L., 2007: Some major mycotoxins and their mycotoxicoses—An overview. *Int. J. Food Microbiol.* **119**, 3-10.

Ruggiero, A.; Cefalo, M.G.; Coccia, P.; Mastrangelo, S.; Maurizi, P.; Riccardi, R., 2012: The role of diet on the clinical pharmacology of oral antineoplastic agents. *Eur. J. Clin. Pharmacol.* **68**, 115-122.

Rustom, I.Y., 1997: Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. *Food Chem.* **59**, 57-67.

Sève, B.; Meunier-Salaün, M.; Monnier, M.; Colléaux, Y.; Henry, Y., 1991: Impact of dietary tryptophan and behavioral type on growth performance and plasma amino acids of young pigs. *J. Anim. Sci.* **69**, 3679-3688.

Valdivia, A.G.; Martinez, A.; Damian, F.J.; Quezada, T.; Ortiz, R.; Martinez, C.; Llamas, J.; Rodriguez, M.L.; Yamamoto, L.; Jaramillo, F.; Loarca-Pina, M.G.; Reyes, J.L., 2001: Efficacy of N-acetylcysteine to reduce the effects of aflatoxin B1 intoxication in broiler chickens. *Poult. Sci.* **80**, 727-734.

Van Egmond, H.P.; Schothorst, R.C.; Jonker, M.A., 2007: Regulations relating to mycotoxins in food. *Anal. Bioanal. Chem.* **389**, 147-157.

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.

Yano, J.M.; Yu, K.; Donaldson, G.P.; Shastri, G.G.; Ann, P.; Ma, L.; Nagler, C.R.; Ismagilov, R.F.; Mazmanian, S.K.; Hsiao, E.Y., 2015: Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell* **161**, 264-276.

Yarru, L.P.; Settivari, R.S.; Gowda, N.K.; Antoniou, E.; Ledoux, D.R.; Rottinghaus, G.E., 2009: Effects of turmeric (Curcuma longa) on the expression of hepatic genes associated with biotransformation, antioxidant, and immune systems in broiler chicks fed aflatoxin. *Poult. Sci.* **88**, 2620-2627.

Zain, M.E., 2011: Impact of mycotoxins on humans and animals. *J. Saudi Chem. Soc.* **15**, 129-144.

Zamani-Zadeh, H.; Khoursandi, H., 1995: Occurrence of Fusarium species and their mycotoxins in wheat in Mazandaran province. *Iran. J. Plant Path.* **28**, 39.

Zhang, H.; Yin, J.; Li, D.; Zhou, X.; Li, X., 2007: Tryptophan enhances ghrelin expression and secretion associated with increased food intake and weight gain in weanling pigs. *Domest. Anim. Endocrinol.* **33**, 47-61.

528 Zoghi, A.; Khosravi-Darani, K.; Sohrabvandi, S., 2014: Surface binding of toxins and heavy
529 metals by probiotics. *Mini Rev. Med. Chem.* **14**, 84-98.
530

531

532 **Table 1.** Composition of experimental diets

Ingredient (g/kg, as is)	2.9 g TRP/kg			4.9 g TRP/kg		
	0.0 mg AFB_1/kg	2.5 mg AFB_1/kg	5.0 mg AFB_1/kg	0.0 mg AFB_1/kg	2.5 mg AFB_1/kg	5.0 mg AFB_1/kg
Corn	340.6	340.6	340.6	340.6	340.6	340.6
Soybean meal	386.8	386.8	386.8	386.8	386.8	386.8
Corn gluten meal	81.90	81.90	81.90	81.90	81.90	81.90
Soybean oil	46.10	46.10	46.10	46.10	46.10	46.10
L-tryptophan	-	-	-	2.02	2.02	2.02
Rice	-	38.93	77.87	-	38.93	77.87
Sand	77.87	38.99	-	77.87	38.99	-
Cornstarch	30.07	30.07	30.13	28.04	28.05	28.08
Oyster	14.20	14.20	14.20	14.20	14.20	14.20
Di- calcium phosphate	6.50	6.50	6.50	6.50	6.50	6.50
DL-methionine	3.50	3.50	3.50	3.50	3.50	3.50
L-lysine	2.10	2.10	2.10	2.10	2.10	2.10
L- threonine	1.00	1.00	1.00	1.00	1.00	1.00
$NaHCO_3$	2.60	2.60	2.60	2.60	2.60	2.60
NaCl	1.70	1.70	1.70	1.70	1.70	1.70
Mineral premix[1]	2.50	2.50	2.50	2.50	2.50	2.50

Vitamin premix[2]	2.50	2.50	2.50	2.50	2.50	2.50
Nutrient content						
AME (Mj/kg)	12.13	12.13	12.13	12.13	12.13	12.13
Crude protein (g/kg)	260.0	260.0	260.0	260.0	260.0	260.0
Tryptophan (g/kg)	2.90	2.90	2.90	4.90	4.90	4.90
Ca (g/kg)	8.00	8.00	8.00	8.00	8.00	8.00
Available P	3.00	3.00	3.00	3.00	3.00	3.00
DEB (mEq/kg)[3]	250	250	250	250	250	250

1- Mineral premix provided per kilogram of diet: Mn (from MnSO4·H2O), 65 mg; Zn (from ZnO), 55 mg; Fe (from FeSO4·7H2O), 50 mg; Cu (from CuSO4·5H2O), 8 mg; I [from Ca (IO3)2·H2O], 1.8 mg; Se, 0.30 mg; Co (from Co2O3), 0.20 mg; Mo, 0.16 mg

2- Vitamin premix provided per kilogram of diet: vitamin A (from vitamin A acetate), 11,500 IU; cholecalciferol, 2,100 IU; vitamin E (from dl-α-tocopheryl acetate), 22 IU; vitamin B12, 0.60 mg; riboflavin, 4.4 mg; nicotinamide, 40 mg; calcium pantothenate, 35 mg; menadione (from menadione dimethyl-pyrimidinol), 1.50 mg; folic acid, 0.80 mg; thiamine, 3 mg; pyridoxine, 10 mg; biotin, 1 mg; choline chloride, 560 mg; ethoxyquin, 125 mg

3- Dietary Electrolyte Balance: represents dietary Na + K – Cl in mEq/kg of diet.

FIGURE LEGEND

Figure 1. Growth performance of growing Japanese quail in response to dietary tryptophan (TRP) in aflatoxin B_1-contaminated (AFB; ● without AFB, ■ 2.5 mg.kg^{-1} AFB, ▲ 5.0 mg.kg^{-1} AFB) diets; λ: hormesis signal.

Figure 2. Dressing and carcass attributes of growing quails (3 birds per replicate) in response to excess dietary tryptophan (TRP) in aflatoxin B_1-contaminated (AFB; ● without AFB, ■ 2.5 $mg.kg^{-1}$ AFB, ▲ 5.0 $mg.kg^{-1}$ AFB) diets; λ: hormesis signal.

Figure 3. Blood parameters of growing quails (2 birds per replicate) in response to dietary tryptophan (TRP) at different levels of aflatoxin B_1 (AFB; ● without AFB, ■ 2.5 $mg.kg^{-1}$ AFB, ▲ 5.0 $mg.kg^{-1}$ AFB).

Figure 4. Immunity responses of growing quails (2 birds per replicate) to dietary tryptophan (TRP) at different levels of aflatoxin B_1 (AFB; ● without AFB, ■ 2.5 $mg.kg^{-1}$ AFB, ▲ 5.0 $mg.kg^{-1}$ AFB).

Figure 5. Meat quality indices of growing quails (2 birds per replicate) in response to dietary tryptophan (TRP) at different levels of aflatoxin B_1 (AFB; ● without AFB, ■ 2.5 mg/kg AFB, ▲ 5.0 mg/kg AFB).

Figure 6. Intestinal microbiome of growing quails (3 birds per replicate) in response to dietary tryptophan (TRP) at different levels of aflatoxin B_1 (AFB; ● without AFB, ■ 2.5 mg/kg AFB, ▲ 5.0 mg/kg AFB).

Figure 7. Biplot of a principal component analysis performed on the interaction between factors [including TRP: tryptophan; AFB: aflatoxin B_1; FI: feed intake; G: body weight gain; G:F, gain/feed; SRBC: sheep red blood cell; HI: hemagglutination inhibition; DNCB: 2,4-dinitrochorobenzene; TP: total protein; LAB: *Lactobacillus*; ECOLI: *Escherichia coli*; MDA: malondialdehyde; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; UA: uric acid; SPLN: spleen; DRIP: drip loss] and treatments [2 levels TRP × 3 levels AFB].

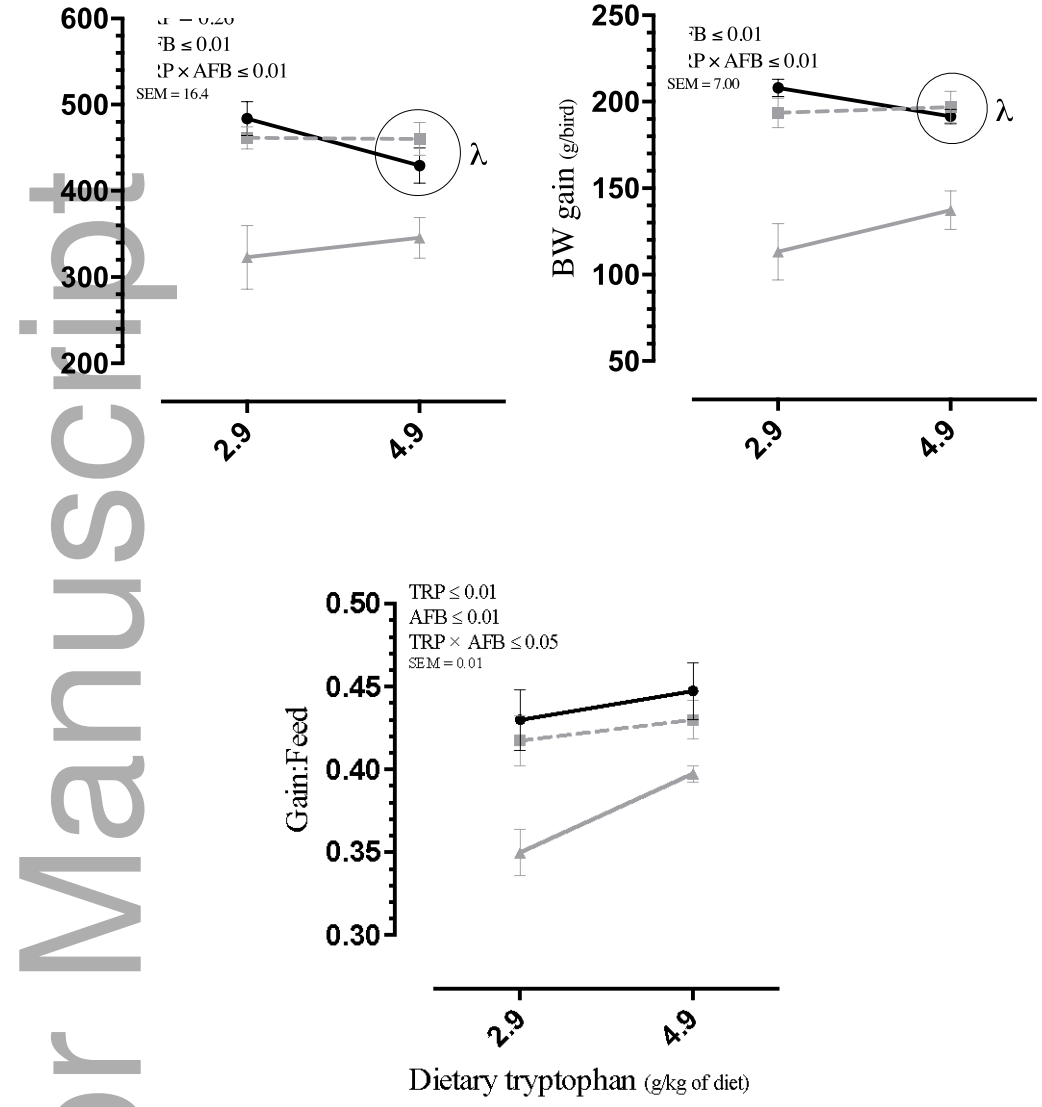

Figure 1

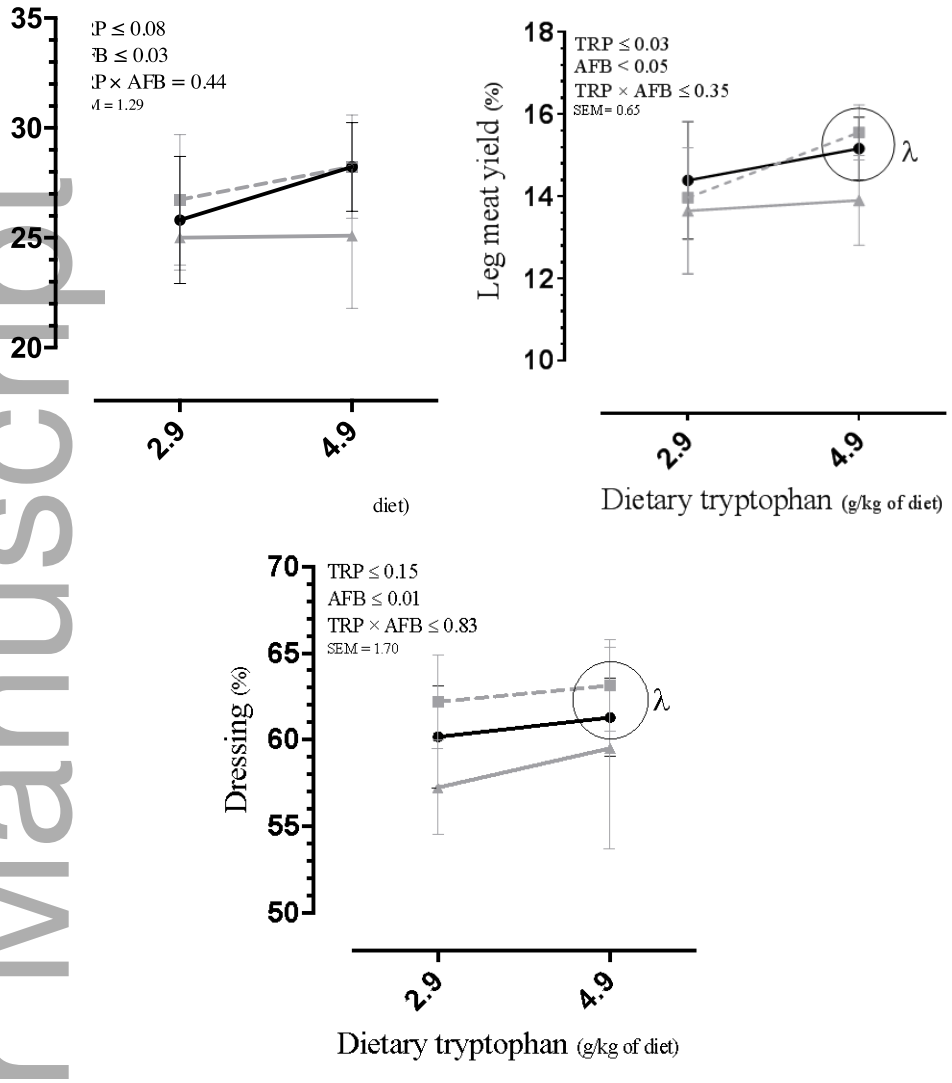

Figure 2

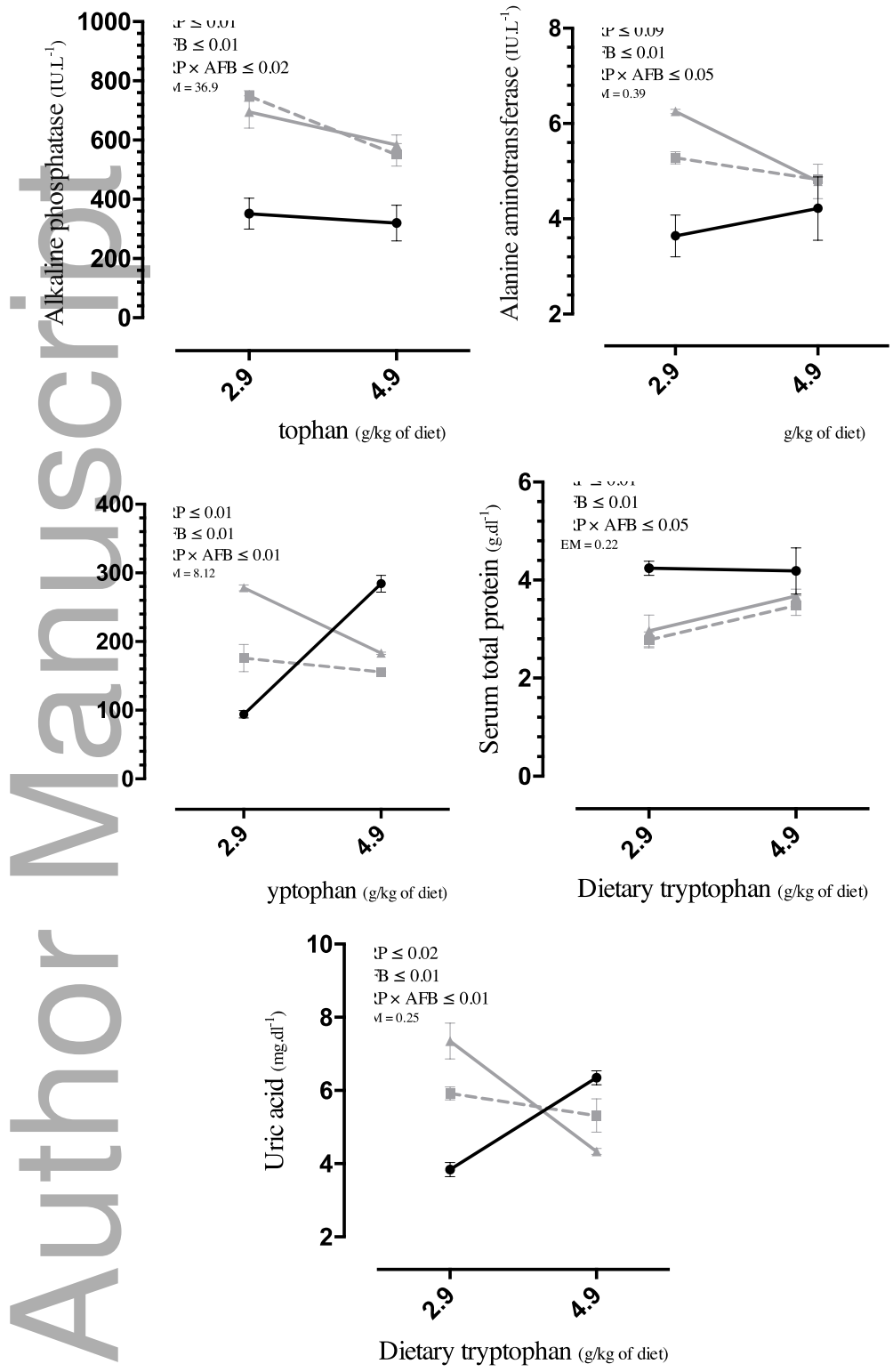

Figure 3

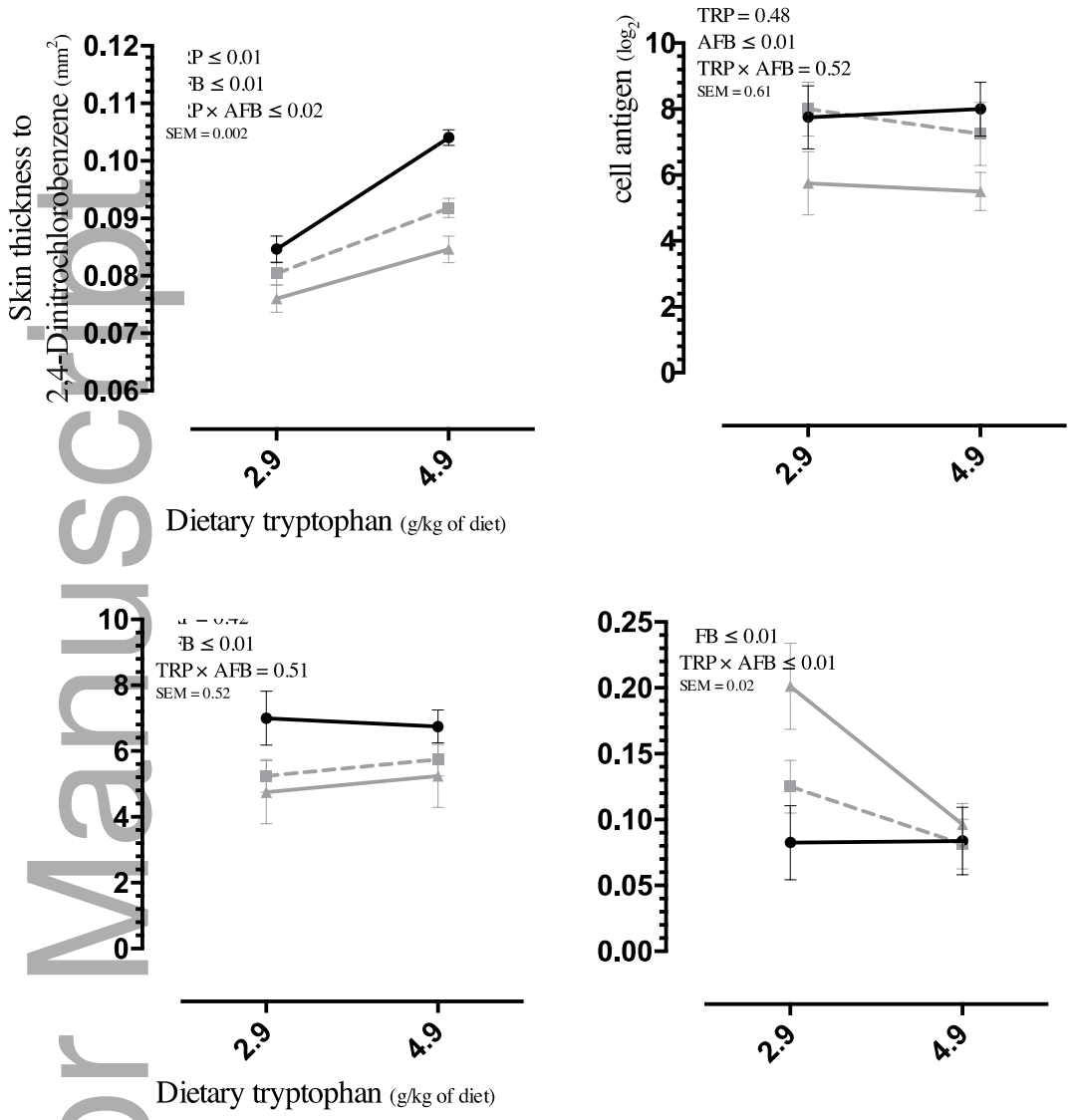

Figure 4

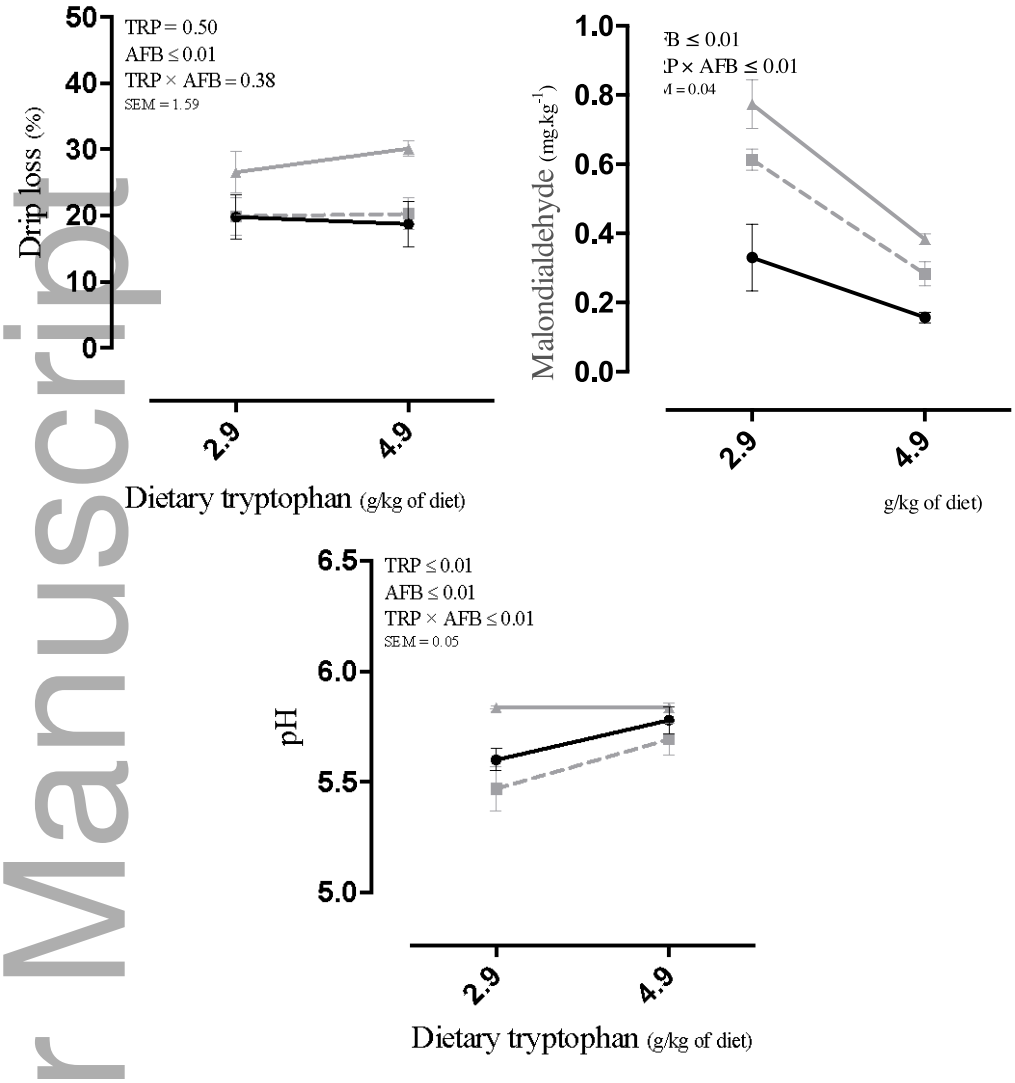

Figure 5

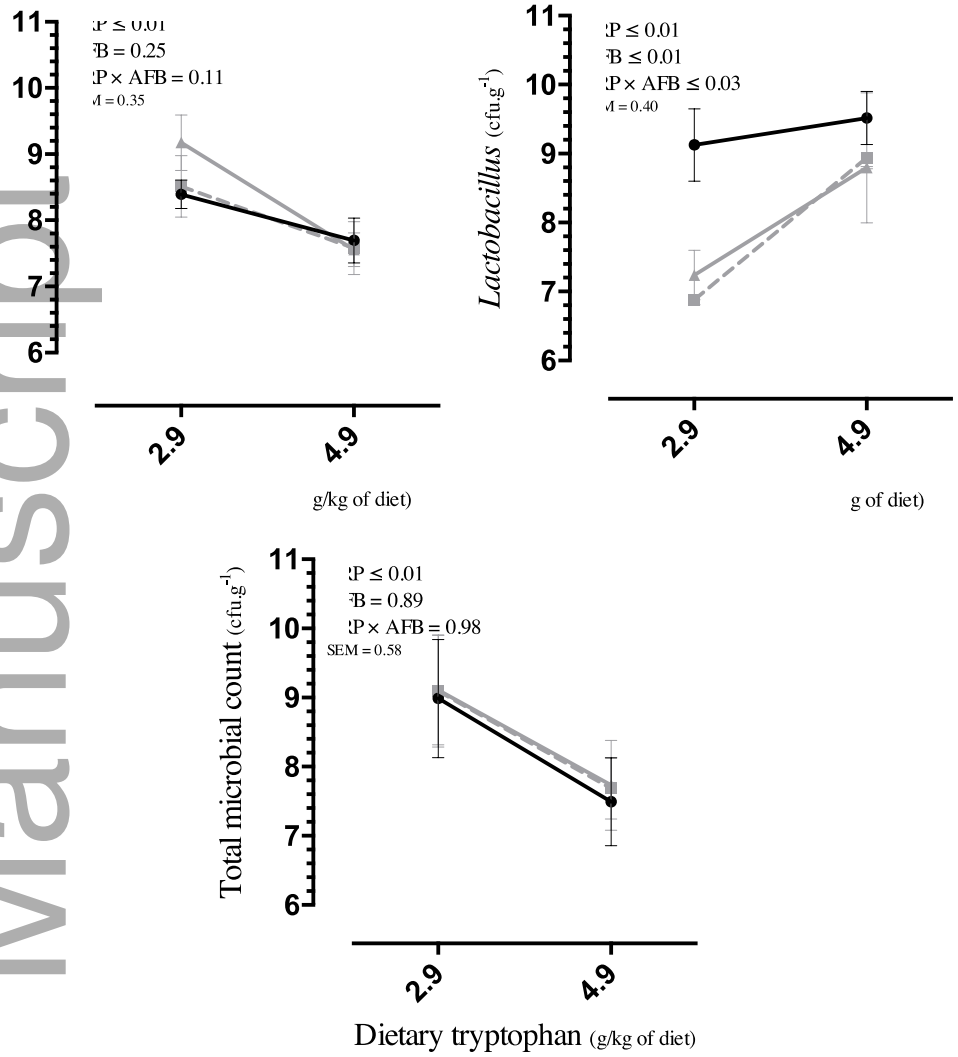

Figure 6

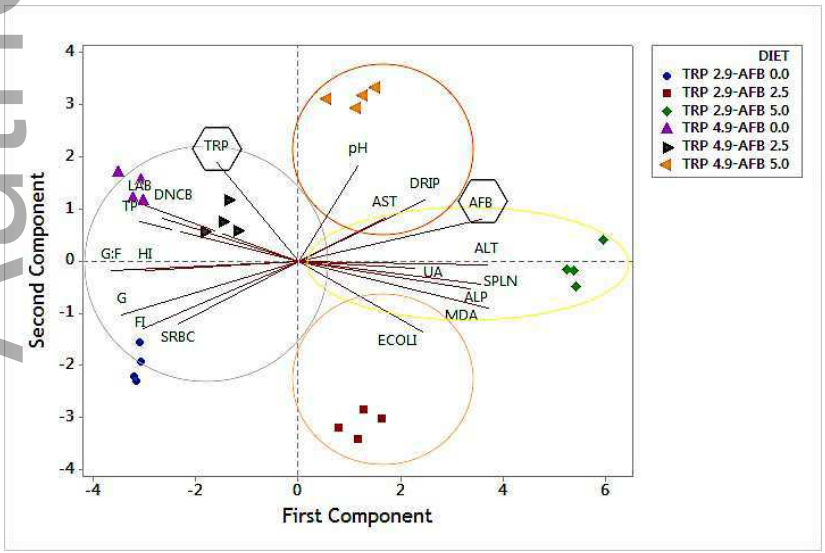

Figure 7