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Full Paper

Protective effects of probiotics against tannin-induced immunosuppression in broiler chickens

Amany RAMAH[1, 2, 4], Masahiro YASUDA[1, 2*], Yuki OHASHI[1], Shoichiro IMATAKE[1], Noriko IMAIZUMI[2], Tetsuo KIDA[2], Tenya YANAGITA[2], Ryoko UEMURA[3], Mahmoud BAAKHTARI[1, 5], Hatem H. BAKRY[4], Nabila M. ABDELALEEM[4] and Elham A. EL-SHEWY[4]

[1]Graduate School of Medicine and Veterinary Medicine, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan
[2]Laboratory of Veterinary Anatomy, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan
[3]Laboratory of Animal Health, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan
[4]Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Benha University, Qalyubia 13518, Egypt
[5]Faculty of Veterinary Science, Balkh University, Balkh 1702, Afghanistan

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Tannins (TAs) are an anti-nutritional substance commonly used as a natural feed additive for livestock. However, our previous study described the dose-dependent adverse effects of TA on immune responses and growth in chickens. In this study, we evaluated the protective effects of a probiotic preparation (BT) consisting of three different bacteria (*Bacillus mesenteric, Clostridium butyricum,* and *Streptococcus faecalis*) against TA-induced immunosuppression in chickens. Forty chicks were divided into 4 groups as follows: the CON group (basal diet), BT group supplemented with 3 g BT/kg diet, tannic acid (TA) group supplemented with 30 g TA/kg diet, and BT+TA group supplemented with 3 g BT/kg diet + 30 g TA/kg diet. The feeding trial lasted for 35 days. Lymphocyte subset, macrophage phagocytosis, cytokine mRNA expression, and primary and secondary IgY immune responses were evaluated. BT supplementation significantly improved TA-induced reductions in final body weight, body weight gain, feed intake, and relative weights of lymphoid organs compared with the TA group. Furthermore, in the spleen and cecal tonsil (CT), the relative populations of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ cells in the BT+TA group were significantly ameliorated compared with the TA group. Additionally, comparison with the TA group showed that the chickens in the BT+TA group had an improved relative population of B cells in the CT and that macrophage phagocytosis in the spleen was significantly increased. Chickens in the BT+TA group showed significant increases in IFN-γ and IL-4 mRNA expression in the spleen compared with the TA group. The primary and secondary IgY responses were significantly improved. These results revealed that supplementation with BT protects against TA-induced immunosuppression in chickens.

Key words: chicken, growth performance, immunity, probiotics, tannin

INTRODUCTION

Tannins are the fourth most abundant group of secondary metabolites and anti-nutritional toxic substances and are classified as hydrolyzable and condensed tannins based on chemical structure [1–4]. Poultry require a large proportion of dietary grains to provide sufficient crude protein and metabolizable energy [5]. Tannic acid (TA) is used as a viable alternative feed additive in the diet of poultry and is derived from plant-based agriculture and industry by-products [6], and it is widely distributed in grains, such as sorghum and millet, and most of the plant kingdom [7, 8].

However, TA toxicity has been reported to cause low feed intake, decrease nutrient digestibility, and inhibit the growth rate in monogastric animals [9–11]. Dietary intake of TA leads to impairment of digestive enzymes and decreases in essential amino acids and minerals, potentially damaging the gut and organs such as the liver, kidney, and lymphoid organs [11–13]. On the other hand, several studies have documented that dietary intake of low concentrations of TA improved the health condition and immune status of animals [11, 14, 15]. Therefore, TA is considered to have a double-edged impact on animal growth and immunity.

*Corresponding author. Masahiro Yasuda (E-mail: yasudaja@cc.miyazaki-u.ac.jp)

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Probiotics are defined as single or mixed cultures of live nonpathogenic microbes that have beneficial effects when administered in adequate numbers as a feed supplement [16, 17]. The enhancement of immunity and health status in poultry and other animals with probiotics has been previously studied [18–21]. Hence, utilization of probiotics are economic and efficient opportunity for use of poor-quality feed such as anti-nutritional substances [22, 23]. Previous *in vitro* studies demonstrated that *Bacillus mesenteric*, *Streptococcus faecalis*, and *Clostridium butyricum* can detoxify agro-industrial by-products into beneficial metabolites [24–26]. Moreover, an *in vitro* study demonstrated that lactic acid bacteria, such as *Lactiplantibacillus plantarum*, possess intracellular enzymes capable of degrading complex hydrolyzable gallotannins, which is achieved by depolymerization of high molecular weight TA [27–31]. TA-degrading microbes, has been reported to increase the nutritional status of TA (a standard of hydrolyzable tannin), thus increase the productivity of livestock in earlier studies [8, 24, 30–33].

However, the protective effect of probiotics against TA-induced immunosuppression has not yet been studied. Therefore, the purpose of this study was to evaluate the protective effects of probiotics on TA-induced immunosuppression in chickens by analyzing lymphocyte subsets, macrophage phagocytosis, cytokine mRNA expression, and primary and secondary IgY immune responses.

MATERIALS AND METHODS

The experimental procedures were approved by the Animal Care and Use Committee at the University of Miyazaki (2018-039-2).

Chemicals

Tannic acid (a representative hydrolyzable tannin) was purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan; a commercial tannin). The probiotic preparation Bio-Three (BT), which consists of three different bacteria (*B. mesenteric, C. butyricum*, and *S. faecalis*) at cell concentrations of 3×10^5, 2×10^5, and 7×10^6, respectively, was provided by Toa Pharmaceutical Co., Ltd. (Tokyo, Japan).

Animals and diets

Forty newly hatched male broiler chickens (ROSS 308) were purchased from a local hatchery (Miyazaki, Japan). The body weights of chickens ranged between 40–50 g. The temperature was 33–34°C on day 1 and then steadily dropped to 24°C on day 35 and remained constant. The lighting program provided 24 hr of continuous light per day until day 2 of the experiment and 23 hr thereafter, in line with local practices. All birds were immunized against Marek's disease, Newcastle disease, and infectious bronchitis disease.

All chickens were fed commercial diets formulated to meet the nutritional requirements of broiler chickens (Hayashikane Sangyo Co., Ltd., Shimonoseki, Japan). The basal diet contained crude protein (>21–18%), crude fat (>3–2%), crude fiber (<6%), crude ash (<13–8%), calcium (>3.1%), phosphorus (>0.45%), and metabolizable energy (>3,100–2,850 Cal/kg). Mortality records were maintained, and birds were not replaced during the experiment.

Experimental design

After a 7-day acclimatization period with *ad libitum* feeding and access to drinking water, the chickens were randomly assigned to 4 experimental groups (10 chicks per group) as follows: the control (CON), BT, TA, and BT+TA groups. The (CON) group was fed the basal diet. The BT group was fed the basal diet supplemented with BT in powder form at a dose of 3 g/kg according to the manufacture's recommendation. The TA group was fed the basal diet supplemented with 30 g TA/kg diet [11, 12]. Finally, the BT+TA group was fed the basal diet supplemented with 3 g BT/kg diet + 30 g TA/kg diet. The experimental period was for 35 days (d).

On day 21 of the experiment, all chickens were intravenously immunized with 200 µg of keyhole limpet hemocyanin (KLH; Fujifilm Wako Pure Chemical Corporation). Seven days after immunization (day 28), blood was collected from the wing vein to measure the primary immune response, and on the same day, all chickens received a second immunization with 200 µg of KLH. On day 35, blood samples were collected from each chicken to evaluate the secondary immune response.

Growth performance and sample collection

Chickens were weighed on the first day of the experiment and then weekly for 35 days to calculate body weight gain (BWG). At the end of the experiment (day 35), the chickens were sedated with an intravenous injection of sodium pentobarbital (10 mg/kg, Somnopentyl®, Kyoritsu Seiyaku Corporation, Tokyo, Japan). The spleen, bursa of Fabricius (BF), and cecal tonsil (CT) were removed following the collection of blood samples.

Preparation of cell suspensions

Each lymphoid organ was removed and cut into small pieces. Cell suspensions were prepared by mincing the tissue in ice-cold Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA), and the residual tissue was removed using a cell strainer to obtain single-cell suspensions. The cells were purified by density gradient centrifugation with Ficoll-Paque (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK). The cells were washed with phosphate-buffered saline (PBS), and erythrocytes were lysed with NH_4Cl lysis buffer. The cells were then suspended in RPMI 1640 medium (Fujifilm Wako Pure Chemical Corporation) supplemented with 10% fetal calf serum and antibiotics.

Lymphocyte subsets

For immunofluorescence assays, cells were suspended in PBS supplemented with 0.5% bovine serum albumin and 0.05% sodium azide (BSA-PBS). Viable cells ranging in concentration from 1×10^5 to 1×10^6 were incubated with fluorescence-labeled monoclonal antibodies (mAbs), as described below, at 4°C for 1 hr. Stained cells were washed three times with BSA-PBS and resuspended in BSA-PBS containing propidium iodide (10 µg/mL, Sigma-Aldrich). Relative immunofluorescence intensities were determined by flow cytometry using a FACS Canto™ II system (Becton Dickinson, Franklin Lakes, NJ, USA). Anti-CD4 (400× dilution, CT4), anti-CD8 (400× dilution, CT8), anti-γδ (400× dilution, TCR1), anti-MHC class II (100× dilution, 2G11), and anti-Bu-1b (200× dilution, 5-11G2) mAbs (all from Southern Biotech, Birmingham, AL, USA) were used. For fluorescence labeling of mAbs, Fluorescein Labeling Kit-NH_2, HiLyte™ Fluor

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555 Labeling Kit-NH_2, and HiLyte™ Fluor 647 Labeling (F647) Kit-NH_2 (Dojindo Laboratories, Kumamoto, Japan) were used according to the manufacturer's instructions.

Phagocytosis assay

Spleen cells (3.0×10^6 cells/mL) were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum and an antibiotic and then incubated with 1 µL of a 2.5% FITC-labeled latex bead suspension (1-µm diameter; L1030, Sigma-Aldrich). The cells were then incubated at 37°C for 1 hr in a 5% CO_2 humidified atmosphere. After incubation, cell-free beads were washed with EDTA-PBS, and then the cells were stained with a F647-labeled anti-MHC class II (100× dilution; 2G11, Southern Biotech) mAb and analyzed by flow cytometry using a FACS Canto™ II system. Macrophages were gated according to their relative size (forward scatter) and complexity (side scatter). The phagocytic index of MHC class II^+ macrophages was reported as the ratio of MHC class II^+FITC^+ (phagocytizing latex beads) cells to all MHC class II^+ cells in the macrophage fraction.

Analysis of IL-1β, IL-2, IFN-γ, and IL-4 mRNA expression in spleen cells using real-time polymerase chain reaction (PCR)

Total RNA was extracted from spleen cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Real-time polymerase chain reaction (RT-PCR) was performed using a one-step TB Green Prime Script PLUS RT-PCR Kit (Takara Bio inc, Japan) according to the manufacturer's protocol. Real-time PCR primers pairs were designed using the Oligo 7 software (Molecular Biology Insights, Colorado Springs, CO, USA) and are shown in Table 1. Real-time PCR conditions consisted of reverse transcription at 42°C for 5 min and initial PCR activation at 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec, 57°C for 30 sec and 70°C for 30 sec, and a dissociation curve was added to the protocol. The real-time RT-PCR assay was performed using an Applied Biosystems QuantStudio™ 3 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA expression level of each target gene was normalized to that of GAPDH as the reference gene. Data were analyzed using the Applied Biosystems QuantStudio™ software (Thermo Fisher Scientific).

Enzyme-linked immunosorbent assay (ELISA) for Anti-KLH antibody titer

Each well of a 96-well microplate (Nunc, Roskilde, Denmark) was coated with 60 µL of KLH (10 µg/mL) and incubated at 37°C for 2 hr. After washing with 0.05% Tween 20-PBS, 1% BSA-PBS was added to each well for blocking, and the microplate was then incubated overnight at 4°C. Next, a 100-fold dilution of chicken sera was added to the antigen-coated wells, and the microplate was incubated at 37°C for 1 hr. The wells were then washed as above, and 60 µL of goat anti-chicken IgG (IgY)-Fc fragment (1,000×, Bethyl Laboratories Inc., Montgomery, TX, USA) was added. After incubation at 37°C for 1 hr, the wells were washed, supplemented with 60 µL of peroxidase-labeled rabbit anti-goat IgG-Fc fragment (40,000×, Bethyl Laboratories Inc.), and incubated at 37°C for 1 hr. The wells were washed, supplemented with 60 µL of substrate solution from an ELISA POD substrate A.B.T.S. Kit (Nacalai Tesque Inc., Kyoto, Japan), and then incubated for 30 min at room temperature. Optical density was measured at 405 nm using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistical analysis

The data were subjected to analysis of variance (ANOVA), and were analyzed using the R version 3.5.1 statistical software (R Core Team). Comparisons between normally distributed continuous experimental groups were carried out by two-way ANOVA and Tukey's honestly significant difference (HSD) test. Two-way ANOVA was used to detect some effects of TA, BT, and TA+BT. After detection of the effects, Tukey's HSD test was used for comparison of individual means of each group. A p-value <0.05 was considered significant. Data were expressed as the mean ± SEM.

RESULTS

Effects of dietary supplementation with BT on final body weight, body weight gain, feed intake, and relative weight of lymphoid organs

The results for final body weight (FBW), BWG, feed intake (DFI), and relative weights of lymphoid organs are shown in Table 2. Dietary supplementation with TA, BT, and BT+TA caused significant changes in FBW, BWG, and DFI. The FBW, BWG, and DFI of chickens in the TA group were significantly lower than those in the CON and BT groups ($p<0.01$), respectively. Contrastingly, supplementation with BT+TA considerably improved growth performance in broiler chicken, as evidenced by increased FBW, BWG, and DFI compared with the TA group ($p<0.01$). There were significant differences between the BT+TA, CON, and BT groups ($p<0.01$). FBW was

Table 1. Primer sequences used for real-time PCR

Gene	Primer	Sequences (5′-3′)	Product size	Accession number
GAPDH	F	AAGCGTGTTATCATCTCAGCTC	162	NM204305.1
	R	AATGCCAAAGTTGTATGGAT		
IL-2	F	AATTAAAGAAGAATTGTAACTGC	145	AF000631.1
	R	GGTCATTCATGGAAAATCAG		
IL-1β	F	CTACACCCGCTCACAGTC	126	NM204524.1
	R	TTGAGCCTCACTTTCTGG		
IFN-γ	F	CATGATTTATTATGGACATACTGC	178	NM205149.1
	R	GCTCAGTATGATCCTTTTCTC		
IL-4	F	TGTGCCCACGCTGTGCTT	169	GU119892.1
	R	AACAATTGTGGAGGCTTTGCATA		

PCR: Polymerase chain reaction.

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significantly higher in the BT group than in the CON group ($p<0.05$).

The relative weight of the spleen was significantly affected by dietary supplementation with TA, BT, or BT+TA. Moreover, the relative weights of BF and CT were significantly changed by dietary supplementation with BT or BT+TA. The relative weight of the spleen in the TA group was significantly different from those in the CON, BT, and BT+TA groups ($p<0.01$). The relative weights of the spleen and BF in the BT group were also increased significantly as compared with the CON group ($p<0.01$). The relative weight of the CT in the BT group was significantly higher than those of the CON and BT+TA groups ($p<0.01$). Furthermore, the relative weight of the CT in the TA group was significantly lower than those in the BT and BT+TA groups ($p<0.01$).

Effects of dietary supplementation with BT on lymphocyte subsets in spleen and CT

The results for lymphocyte subsets in the spleen are shown in Fig. 1A. The populations of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ cells in the spleen were affected by dietary supplementation with TA, BT, or BT+TA. The population of $\gamma\delta^+$ cells in the spleen was also significantly changed by dietary supplementation with TA. The populations of $CD4^+$, $CD8^+$, $CD4^+CD8^+$, and $\gamma\delta^+$ cells in the spleen were significantly lower in the TA group than those in the CON and BT groups ($p<0.01$), respectively. Conversely, the populations of $CD4^+$, $CD8^+$, $CD4^+CD8^+$, and $\gamma\delta^+$ cells in the spleen were significantly improved in the BT+TA group compared with those in the TA group ($p<0.01$). The populations of $CD4^+$ and $CD4^+CD8^+$ cells in the spleen were significantly higher in the BT group than those in the CON group ($p<0.05$ and $p<0.01$, respectively). Furthermore, the populations of $CD4^+$ and $CD8^+$ cells in the spleen were significantly higher in the BT group than those in the BT+TA group ($p<0.01$).

The results for lymphocyte subsets in the CT are shown in Fig. 1B. The populations of $CD4^+$ and $CD4^+CD8^+$ cells in the CT were significantly changed by dietary supplementation with TA, BT, or BT+TA. The populations of $CD8^+$ cells were significantly different in the TA and BT groups. In addition, the population of $\gamma\delta^+$ cells in the CT was significantly changed by dietary supplementation with TA.

The populations of $CD4^+$, $CD8^+$, $CD4^+CD8^+$, and $\gamma\delta^+$ cells in the CT were significantly lower in the TA group than those in the CON and BT groups ($p<0.01$), respectively. On the other hand, the BT+TA group showed obvious improvements in the $CD4^+$ and $CD4^+CD8^+$ cell populations compared with those of the TA group ($p<0.01$). The populations of $CD4^+$ and $CD8^+$ cells in the CT were significantly higher in the BT group than those in the CON ($p<0.01$ and $p<0.05$, respectively) and BT+TA groups ($p<0.01$).

Table 2. Effects of BT on final body weight, body weight gain, feed intake, and relative weights of lymphoid organs

Parameters	Groups			
	CON	BT	TA	BT+TA
FBW, g	2,395 ± 3.3	2,680 ± 7.6^{d}	837.6 ± 1.4^{a,b}	1,574.8 ± 3.6^{a,b,c}
BWG, g/day	64.14 ± 2.8	72.28 ± 1.23	19.64 ± 2.1^{a,b}	40.712 ± 1.14^{a,b,c}
ADFI, g	215.8 ± 1.2	220.3 ± 3.5	116.4 ± 1.1^{a,b}	178.62 ± 4.1^{a,b,c}
Organ weights, g				
Bursa	0.14 ± 0.03	0.16 ± 0.04^{a}	0.17 ± 0.01	0.22 ± 0.02^{c}
Spleen	0.11 ± 0.01	0.15 ± 0.02^{a}	0.02 ± 0.01^{a,b}	0.10 ± 0.01^{c}
Cecal tonsil	0.04 ± 0.01	0.08 ± 0.01^{a}	0.03 ± 0.02^{b}	0.06 ± 0.05^{b,c}

CON: control; BT: probiotic preparation Bio-Three; TA: tannic acid; FBW: final body weight; BWG: bodyweight gain; ADFI: average daily feed intake during the last week of the experiment (day 35). The organ weights are shown as the relative weight of lymphoid organs of the four groups and are expressed as g per 100 g of body weight. Data are reported as mean ± SEM. ^{a} ($p<0.01$) vs: CON, ^{b} ($p<0.01$) vs: BT group, ^{c} ($p<0.01$) vs: TA group, and ^{d} ($p<0.05$) vs: CON.

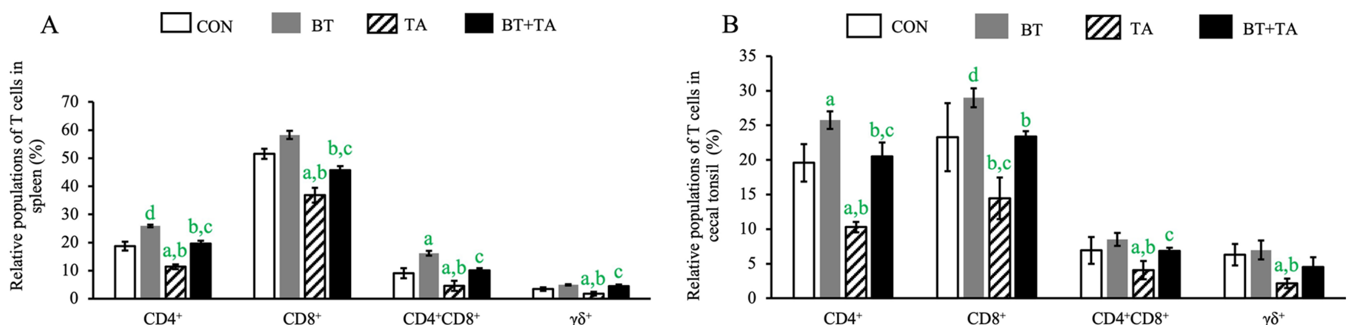

Fig. 1. Effects of dietary supplementation with BT on T lymphocyte subsets in the spleen (A) and CT (B).

CON: control; BT: probiotic preparation Bio-Three; TA: tannic acid. The CON group was fed a normal basal diet, BT group was fed a 3 g probiotic/kg diet, TA group was fed a 30 g TA/kg diet, and BT+TA group was fed a 30 g TA + 3 g probiotic/kg diet. Data represent the mean ± SEM.

$^{a}p<0.01$ vs. CON. $^{b}p<0.01$ vs. BT group. $^{c}p<0.01$ vs. TA group. $^{d}p<0.05$ vs. CON.

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Effects of dietary supplementation with BT on B-lymphocyte subsets in spleen and CT

The results for B-lymphocyte subsets in the spleen and CT are shown in Fig. 2. The populations of MHC class $II^{+}Bu1\text{-}b^{+}$ B cells in the spleen were significantly changed by dietary supplementation with TA and BT+TA. In addition, the populations of B cells in the CT were affected by dietary supplementation with TA. The populations of MHC class $II^{+}Bu1\text{-}b^{+}$ B cells in the spleen and CT were significantly diminished in the TA group compared with those in the CON and BT groups ($p<0.01$). Conversely, the populations of B cells in the spleen and CT were substantially improved in the BT+TA group compared with those in the TA group ($p<0.01$). The populations of MHC class $II^{+}Bu1\text{-}b^{+}$ B cells in the spleen were significantly higher in the BT group than in the BT+TA group ($p<0.01$).

Effect of dietary supplementation with BT on macrophage phagocytosis

The results of phagocytic index are shown in Fig. 3. The phagocytic index was significantly changed by dietary supplementation with BT or TA. In descending order according to their phagocytic indices, the groups were as follows: the BT group, BT+TA and CON groups, and then the TA group. The phagocytic index in the BT group was significantly greater than those in the CON and BT+TA groups ($p<0.01$). In addition, the phagocytic index in BT+TA group was significantly greater than that in the TA group ($p<0.01$).

Effect of dietary supplementation with BT on cytokine mRNA expression

The results for cytokine mRNA expression in the spleen are shown in Fig. 4. The levels of IL-2 and IL-4 mRNA expression were significantly changed by dietary supplementation with TA and BT. IFN-γ mRNA expression was significantly affected by TA, BT, and BT+TA. The levels of IL-2, IFN-γ, and IL-4 mRNA expression in the TA group were significantly lower than in the CON and BT groups ($p<0.01$). Contrastingly, the mRNA expression levels of these cytokines in the BT+TA group were significantly higher than those in the TA group ($p<0.01$). The levels of IL-2 and IFN-γ mRNA expression in the BT group were significantly higher than those in the CON group ($p<0.01$). Furthermore, the levels of IFN-γ and IL-4 mRNA expression in the BT group were significantly higher than those of the BT+TA group ($p<0.01$).

Effect of dietary supplementation with BT on Anti-KLH IgY immune responses

The results for primary and secondary immune responses to KLH are shown in Fig. 5. Primary and secondary anti-KLH IgY immune responses were significantly changed by dietary supplementation with TA and BT+TA. In descending order according to their primary and secondary anti-KLH IgY immune responses, the groups were as follows: the BT group, CON group, BT+TA group, and then TA group. The antibody responses in the TA group were significantly lower than those in the CON and BT groups ($p<0.01$). Conversely, the BT+TA group showed significantly enhanced values compared with the TA group ($p<0.01$). The secondary anti-KLH IgY immune response in the BT group was significantly higher than those in the CON and BT+TA groups ($p<0.05$ and $p<0.01$, respectively).

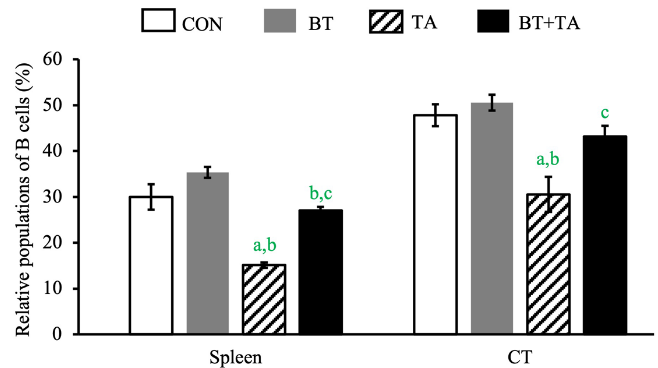

Fig. 2. Effects of dietary supplementation with BT on B-lymphocyte subsets in the spleen and CT.

CON: control; BT: probiotic preparation Bio-Three; TA: tannic acid. The CON group was fed a normal basal diet, BT group was fed a 3 g probiotic/kg diet, TA group was fed a 30 g TA/kg diet, and BT+TA group was fed a 30 g TA + 3 g probiotic/kg diet. Data represent the mean ± SEM.

[a]p<0.01 vs. CON. [b]p<0.01 vs. BT group. [c]p<0.01 vs. TA group.

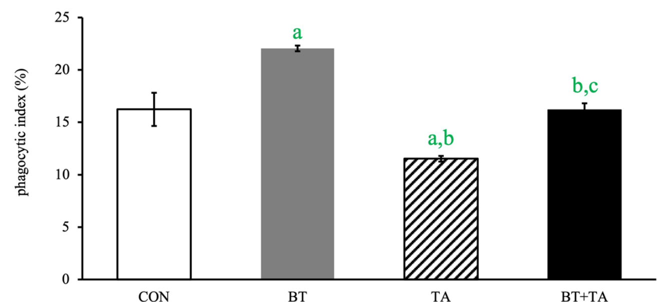

Fig. 3. Effects of dietary supplementation with BT on macrophage phagocytosis.

CON: control; BT: probiotic preparation Bio-Three; TA: tannic acid. The CON group was fed a normal basal diet, BT group was fed a 3 g probiotic /kg diet, TA group was fed a 30 g TA/kg diet, and BT+TA group was fed a 30 g TA + 3 g probiotic/kg diet. Data represent the mean ± SEM.

[a]p<0.01 vs. CON. [b]p<0.01 vs. BT group. [c]p<0.01 vs. TA group.

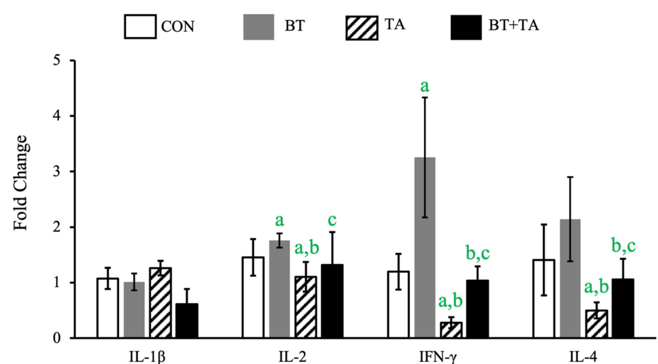

Fig. 4. Effects of dietary supplementation with BT on mRNA expression of cytokines.

CON: control; BT: probiotic preparation Bio-Three; TA: tannic acid. Total RNA was obtained from the splenic tissue of chickens in the CON, BT, TA, and BT+TA groups. Expression levels were measured by real-time PCR. Data represent the mean ± SEM.

[a]p<0.01 vs. CON. [b]p<0.01 vs. BT group. [c]p<0.01 vs. TA group.

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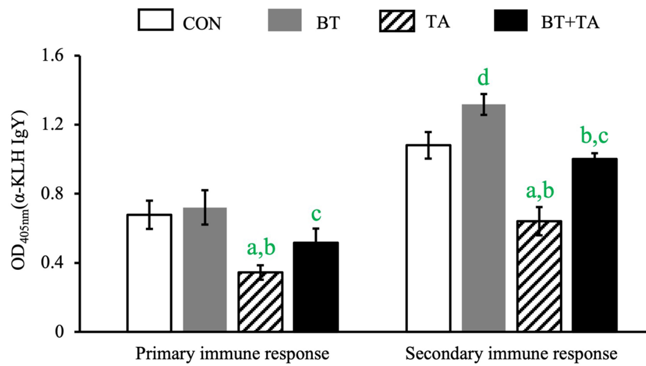

Fig. 5. Effects of dietary supplementation with BT on anti-KLH IgY immune response.

CON: control; BT: probiotic preparation Bio-Three; TA: tannic acid; KLH: keyhole limpet hemocyanin. The figure shows the primary and secondary IgY responses to KLH in the chickens. The CON group was fed a normal basal diet, BT group was fed a 3 g probiotic/kg diet, TA group was fed a 30 g TA/kg diet, and BT+TA group was fed a 30 g TA + 3 g probiotic/kg diet. The chickens were injected with 200 µg of KLH on day 21 of the experiment. After 7 days, sera for primary response evaluation were collected, and the chickens were injected again with 200 µg of KLH. After 7 days, sera for secondary response evaluation were collected. Data represent the mean ± SEM.

[a]p<0.01 vs. CON. [b]p<0.01 vs. BT group. [c]p<0.01 vs. TA group. [d]p<0.05 vs CON.

DISCUSSION

TA in food is associated with toxic anti-nutritional properties and is the main reason for poor poultry productivity [8, 34–38]. Furthermore, it causes deleterious effects, including growth retardation, decreased feed intake, suppressed immune responses, and increased protein catabolism [11–13, 38]. Thus, it is vital to apply nutritional strategies to avoid or reduce the negative effects of TA in poultry. Dietary probiotics are supplements with potential immunostimulatory, anti-inflammatory, and antioxidant activities [19–21, 39]. Our study revealed that dietary BT resulted in improved immune status and growth of broiler chickens exposed to TA, and this was attributed to its ability to reduce the negative effects of TA.

Our findings indicated that FBW, DFI, and organ weights were reduced in chickens treated with TA, which is consistent with previous results [11]. Dietary tannins, such as TA, may have a significant detrimental impact on sugar, amino acids, and mineral absorption. Thus, they not only impair the digestibility of dietary elements by forming complexes with substrates or enzymes involved in their digestion but also disrupt the transport pathways involved in the absorption of simple molecules [38, 40–42]. Similarly, previous reports revealed that dietary intake of TA induced intestinal mucosa necrosis and villi damage in broilers [42] and reduced liver and kidney weights in rats [43–45], sheep, and mice [46]. It has been proven that both hydrolyzable and condensed tannins disrupt intestinal absorption of essential ingredients [47–49].

On the other hand, supplementation with probiotics for diets containing significant amounts of TA may mitigate the adverse effects of the ingested TA. This improvement could be due to the cumulative effects of the probiotic microbes, which include higher digestive enzyme activity and neutralization of the effect of feed toxins in the gut environment in broiler chickens [50–54]. Probiotics consisting of *B. mesenteric*, *C. butyricum*, and *S. faecalis* could prevent the growth of *E. coli*, which is deleterious to the intestines of infant rabbits [55]. Further, these probiotics contain butyric acid, which enhances the regeneration of intestinal mucosal cells [56]. In addition, a previous *in vitro* study reported that microbial fermentation could improve nutrition by decreasing anti-nutritional agents, increasing minerals and carbohydrate bioavailability [57]. Another study discovered that yeast supplementation (*Saccharomyces cerevisiae*) could improve the efficiency of poor-quality fibrous feed in laying pullets [23, 58].

Our results indicated that relative lymphoid organs weights were significantly improved in the BT+TA group compared with the TA group. In previous reports, probiotics, such as *Lactobacillus*-, *Bacillus*-, and *Clostridium*-based probiotics, enhanced the growth performance and nutrient utilization efficiency in chickens [23, 59–61]. These improvements are attributed to the ability of probiotics to create a favorable intestinal environment in animals, allowing efficient digestion and absorption of essential nutrients [62–64]. The BF is a central lymphoid organ of B cells and has important functions in the humoral immunity of poultry [59, 65, 66], while the spleen and CT are secondary lymphoid organs for local immune responses [67–69]. The above reports concluded that probiotics could promote the functions of lymphoid organs, which are responsible for the humoral and cell-mediated immune responses in broiler chickens [69].

In this study, TA supplementation reduced the populations of T and B cells in lymphoid organs and inhibited macrophage phagocytic activity in the spleen. This is attributed to the suppressive effect of high doses of TA, which might elevate corticosterone levels in stressed chickens [11, 70, 71]. Therefore, the probiotic effectively ameliorated the suppression of T and B cell subsets in the BT+TA group compared with the TA group. The improvement of T and B cell subsets in the spleen and CT of the BT+TA group might be because of the effects of probiotic microorganisms on functional activities of the immune response, resulting in increased numbers of lymphocytes [59, 72]. The effects may act directly on hemopoietic organs or indirectly on the intestinal microflora [73]. A previous study showed that the Th1 immune response was stimulated by probiotics containing *B. mesenteric*, *C. butyricum*, and *S. faecalis* [39]. In addition, the number of $CD8^+$ T cells in the intestinal mucosa was increased, which may improve the intestinal immunity of young chicks [74]. Another report observed that supplementation of poor-quality diets with probiotics containing *S. cerevisiae* enhanced the blood hemoglobin concentration [51].

Dietary supplementation of probiotics with TA appears to improve the phagocytic activity of macrophages. Probiotics have a positive effect on the host immune response through increased macrophage activity [75–77]. A previous report emphasized that probiotic supplementation in a chronic stress model enhanced the histological structure of the gut and improved the phagocytic activity of peritoneal and splenic macrophages [78].

In the present study, the splenic expressions of IL-2, INF-γ, and IL-4 following TA exposure were significantly lower than in the CON group, which is consistent with a previous study [11]. Multiple cytokines, such as IL-2 and INF-γ, are secreted

by activated T cells and play essential roles in the maturation, replication, and differentiation of T cells [79–81]. There were marked increases in splenic IFN-γ mRNA expression in the BT and BT+TA groups. Moreover, the BT+TA group showed improvement in IL-1β and IL-4 mRNA expression. We speculated that the improvement of cytokine expression, phagocytosis, and INF-γ indicate increases in the innate immune response and adaptive immune response. Thus, dietary intake of probiotics plays a vital role in regulating the production of cytokines [82].

Our findings also indicated that TA reduced both primary and secondary immune responses in terms of the anti-KLH IgY antibody titers. This could be attributed to the negative immunological impacts of TA, resulting in reduced protein synthesis in lymphoid tissues [12]. Furthermore, the reduction of T cells leads to suppressed cytokine production related to B cell differentiation and IgG class switching [83, 84]. On the other hand, the probiotic improved the anti-KLH IgY antibody immune response in the BT+TA group compared with the TA group. Improvement of the IgY titer was confirmed along with enhanced IL-4 mRNA expression; IL-4 plays an essential role in immunoglobulin class switching [77]. Previous studies reported that probiotics stimulate the immune response, leading to induction of cytokine production, and regulate innate and adaptive immune responses [85–89]. Another study reported that probiotics containing *B. mesenteric*, *C. butyricum*, and *S. faecalis* increased the production of immunoglobulin in the mesenteric lymph node of rats [90]. Furthermore, probiotics improved the structure of the intestinal mucosa and increased the IgA concentration in the small intestine of broiler chickens [91]. Therefore, we suggest that probiotic supplementation in the BT+TA group was protective against the side effects of TA at high doses.

In conclusion, supplementation with probiotics ameliorates the toxic effects of a high dose of TA and shows positive effects on the body performance and immune status of chickens. Therefore, supplementation with probiotics is highly applicable for mitigation of the immunosuppressive action of high doses of TA.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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The authors' responsibilities were as follows: AR and MY designed the research; AR, MY, YO, NI, TK, TA, and MB conducted the research and collected samples; AR and SI analyzed data; AR wrote the initial draft of the manuscript, with strong contributions from MY. AR had primary responsibility for the final content. All authors participated in critical revisions and approved the final manuscript.

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