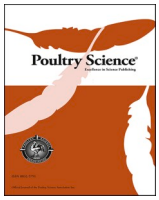
104 (2025) 104605

Contents lists available at ScienceDirect

Poultry Science

journal homepage: www.elsevier.com/locate/psj



Full-Length Article

The mechanism of tea tree oil regulating the damage of hydrogen sulfide to spleen and intestine of chicken☆,☆☆,★

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ARTICLE INFO

Keywords:
Hydrogen sulfide
Intestinal barrier
Spleen
Serum biochemistry
Tea tree oil

ABSTRACT

Intensification of poultry industry has led to a surge in animal product output, but this has also revealed issues with environmental management in poultry houses, particularly the harmful effects of high hydrogen sulfide (H_2S) levels on poultry health. The study aimed to assess the therapeutic impact of tea tree oil (TTO) on H_2S-induced spleen and intestinal injuries in chickens. A total of 240 one-day-old Lohmann Brown chicks were randomly divided into three groups: the control group (CON), the H_2S exposure group (AVG), and the TTO treatment group (TTG), with four replicates, each consisting of 20 chicks. The experiment lasted 42 days. Results showed that TTO treatment alleviated tissue damage in the thymus, kidneys, spleen, and bursa of Fabricius, and improved the organ index ($P < 0.05$) compared with the AVG. Serum analysis revealed that TTO lowered levels of alanine aminotransferase(ALT), aspartate aminotransferase(AST), triglycerides(TG), CD3 positive CD4 positive T cells(CD3+CD4+), CD4 positive to CD8 positive Rratio(CD4+/CD8+), and alkaline phosphatase(AKP), while increasing albumin(ALB), globulin(GLO), immunoglobulin A(IgA), and immunoglobulin G(IgG) levels ($P < 0.05$). Intestinal findings indicated that TTO treatment enhanced villus height, reduced crypt depth, and upregulated the expression of *Claudin 1, Occludin, and ZO-1* mRNA in the jejunum ($P < 0.05$). After TTO treatment, H_2S-induced oxidative stress injury and apoptosis protein expression in spleen were improved ($P < 0.05$). TTO also reduced interferon-γ(INF-γ), tumor necrosis factor-α(TNF-α) and interleukin-1β(IL-1β) proteins ($P < 0.05$), while raising CD3+CD8+ T-cell subsets ($P < 0.05$). Compared with CON, TTO treatment alleviated serum biochemical disorders and intestinal damage caused by H_2S exposure and restored them to normal ($P > 0.05$). In conclusion, TTO can improve spleen and intestinal function and reduce the effects of H_2S on growth performance and health of chickens.

Introduction

The rapid expansion and intensification of poultry industry have increased the supply of animal products, although they have also exposed issues affecting production efficiency. In particular, poor ventilation and delayed manure handling frequently result in elevated levels of H_2S in animal housing facilities (Chen et al., 2019b). High concentrations of H_2S have been shown to reduce animals' stress resistance and impair their immune function, thereby triggering various health issues (Song et al., 2021a). Prolonged exposure to H_2S damages multiple organs and systems, including the digestive and immune systems (Li et al., 2020; Zheng et al., 2019). Previous studies have demonstrated that H_2S caused injury to the jejunum in chickens, inducing intestinal immune inflammation and impairing the gut barrier function (Zheng et al., 2019). The intestinal tract is both a nutrient digestion and absorption organ and an immune organ, which is important to prevent external pathogens and toxins from passing through the intestinal mucosa into other tissues and organs (Chopyk and Grakoui, 2020; Fan et al., 2024; Jia et al., 2023; Leonardi et al., 2022). However, excessive inhalation of H_2S disrupted intestinal structure, leading to barrier dysfunction (Zheng et al., 2019). Consequently, H_2S enters the bloodstream through the damaged intestinal barrier and travels through

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https://doi.org/10.1016/j.psj.2024.104605
Received 11 September 2024; Accepted 26 November 2024
Available online 28 November 2024

body fluids to damage the spleen (Chi et al., 2019).

Tea tree oil (TTO) exhibits multiple benefits, including antioxidant properties, immune enhancement, and the improvement of animal growth performance (Liang et al., 2024). Derived from *Melaleuca alternifolia*, TTO is pale yellow with a distinctive aromatic scent (Nascimento et al., 2023). Previous research has demonstrated that TTO increased the height of intestinal villi in chickens and improved growth performance (Zhang et al., 2021). Additionally, supplementing basal diets with TTO increases the number of gut microbiota in pigs, enhancing nutrient absorption and further promoting growth (Wang et al., 2021). Moreover, studies have shown that adding TTO to drinking water enhances chickens' immune function and decreases the expression of inflammatory factors such as INF-γ and IL-1β. Comparable effects were observed in pigs, where TTO also bolstered immune function and reduced the expression of inflammatory markers (Dong et al., 2024). Recent research further explored the potential of TTO in regulating intestinal barrier function and boosting immune responses (Liu et al., 2022a). Earlier studies also revealed that TTO effectively mitigates tissue damage caused by H_2S, alleviates pathological injuries, increases antioxidant enzyme activities such as SOD (superoxide dismutase) and catalase (CAT), and reduces the expression of inflammatory factors like NF-κB and TNF-γ (Liang et al., 2024). Therefore, TTO showed promising potential in addressing damage caused by external pathogenic factors in poultry.

In summary, previous studies have predominantly focused on the singular toxic effects of H_2S on either the jejunum or spleen, without delving into the interconnected damage effects between these organs. Similarly, research on TTO is mostly limited to its effect on growth performance and antioxidant capacity of animals. Therefore, this study aimed to explore whether TTO could mitigate the effects of H_2S on growth performance and health of chickens by improving the function of the gut-spleen pathway. This study, for the first time, systematically revealed how TTO modulates the interaction between the jejunum and spleen to improve growth performance. To achieve this, we established an animal model of H_2S-exposed chickens treated with TTO. Using enzyme-linked immunosorbent assay (ELISA), real-time quantitative PCR (qRT-PCR), and flow cytometry, we investigated the effects of TTO on the gut and spleen. This study was the first to report the detoxifying effects of TTO on H_2S-exposed chickens, offering new potential therapeutic strategies for addressing immune damage to the spleen and intestine caused by H_2S in poultry farming.

Materials and methods

Test materials

Tea tree oil (TTO) was sourced from Jiangsu Wuxi Chenfang Biotechnology Co., LTD. (Wuxi, China), and its ingredients are shown in Annex Table 1. The reagents including total antioxidant capacity (T-AOC), SOD, TP, CAT, malondialdehyde (MDA), ALB, GLO, glucose, AST, ALT, ALP, TC, TG, GLU were sourced from Nanjing Jiancheng Biological Company. Annexin V-FITC double dye was purchased from ACTGene, USA. The ELISA test kits for chicken Bcl-2 Associated X Protein (Bax), Bak-1, B-Cell lymphoma-2 (Bcl-2), Caspase-3, tumor necrosis factor-α (TNF-α), interleukin4 (IL-4), INF-γ, interleukin1-β (IL-I-β) were purchased from Shanghai Biologic Company. Binding Buffer, hematoxylin and eosin dye solution were procured from Beijing Soleibao Technology Co., LTD. (Beijing, China). Hydrochloric acid, xylene, anhydrous ethanol, 4 % paraformaldehyde and saline were purchased from Chengdu Colon Chemical Co., LTD. (Chengdu, China). Resin glue was acquired from Nanchang Yulu Experimental Equipment Co., LTD. (Nanchang, China). Immunoglobulin A (IgA) and immunoglobulin B (IgB) were sourced from Solebol. The SpectraMax i3x(Meigu, MAX-300, Shanghai, China) spectrometer was purchased from Meigu Molecular Instruments (Shanghai) Co., Ltd.

Table 1
Test design table.

Stage	Group	Amount	H_2S concentration(mg/m^3)
1-21 d	CON	80	$H_2S \leq 0.5$(Safe range)
	AVG	80	$3.5 \leq H_2S \leq 4.5$
	TTG	80	$3.5 \leq H_2S \leq 4.5$ and add 0.02 ml/L tea tree oil to drinking water
22-42 d	CON	80	$H_2S \leq 0.5$
	AVG	80	$19.5 \leq H_2S \leq 20.5$
	TTG	80	$19.5 \leq H_2S \leq 20.5$ and add 0.02 ml/L tea tree oil to drinking water

Experimental design

A total of 240 1-day-old Roman pink laying hens (Sichuan Techlex Food Co., Ltd, Chengdu, China) were selected for the study, which were similar in weight and size. The chickens were randomly divided into three groups, with four replicates per group. The animal test period lasted 42 days. The specific test groups and designed were shown in Table 1. In this study, the three groups of chickens were raised in three environmental control bins and H_2S concentrations were controlled using an H_2S measuring instrument (Shanghai Chromatography Instrument Co., LTD, EXPEC 3700 GC-MS, Shanghai, China). The variation trend of H_2S in the environmental chamber is shown in Fig. 1. The concentrations of H_2S and TTO were set based on previous experimental requirements (Liang et al., 2024; Song et al., 2021b). Specifically, the control group (CON) was treated without any treatment, the H_2S exposure group (AVG) was exposed to a set concentration of H_2S, and the TTO treatment group (TTG) was treated by adding TTO to their drinking water under H_2S exposure. All chickens were fed a basal diet, and the relevant nutrient levels were shown in Annex Table 2 (Zhou et al., 2023). The chickens had access to water freely. In order to ensure the cleanliness of the test environment, the feces in the environmental bin were cleaned every day, and the test temperature (22-24°C) and humidity (40 %-60 %) were kept constant. All procedures used in this experiment have been approved by the Animal Care and Use Committee of Southwest University of Science and Technology, ensuring the legality and ethics of the experiment. In the experiment, 12 chickens were selected from each group for measurement.

Phase growth performance measurement

Before the trial began, all chickens were weighed individually, and their initial weight (IBW) was recorded. At the ages of 21 days and 42 days, the fasting weights are measured and the feed consumption at each stage is analyzed in detail. Based on these data, the average daily gain (ADG), average daily feed intake (ADFI), final body weight (FBW) and feed to gain ratio (F/G) of the chickens at each stage were calculated. The calculation formulas were as follows:

$$\mathrm{ADG} = \mathrm{Stagegain(g)/stagedays(d)}$$

$$\mathrm{ADFI} = \mathrm{stageintake(g)/stagedays(d)}$$

Finally, the F/G ratio was calculated based on ADG and ADFI (Wang et al., 2024).

Sample collection

Prior to the end of the trial, chickens were fasted for 12 hours to ensure blood sample integrity. Blood samples were then collected from the subapex wing veins. (Shan et al., 2023). The collected whole blood samples were treated as anticoagulation, and the flow indexes were detected. Part of the whole blood samples were centrifuged (3000 r/min, 10 min, 4 °C), and the supernatants were removed after centrifugation for biochemical analysis. The rest of of the blood samples were kept at −80 °C for further detailed analysis. After blood was collected,

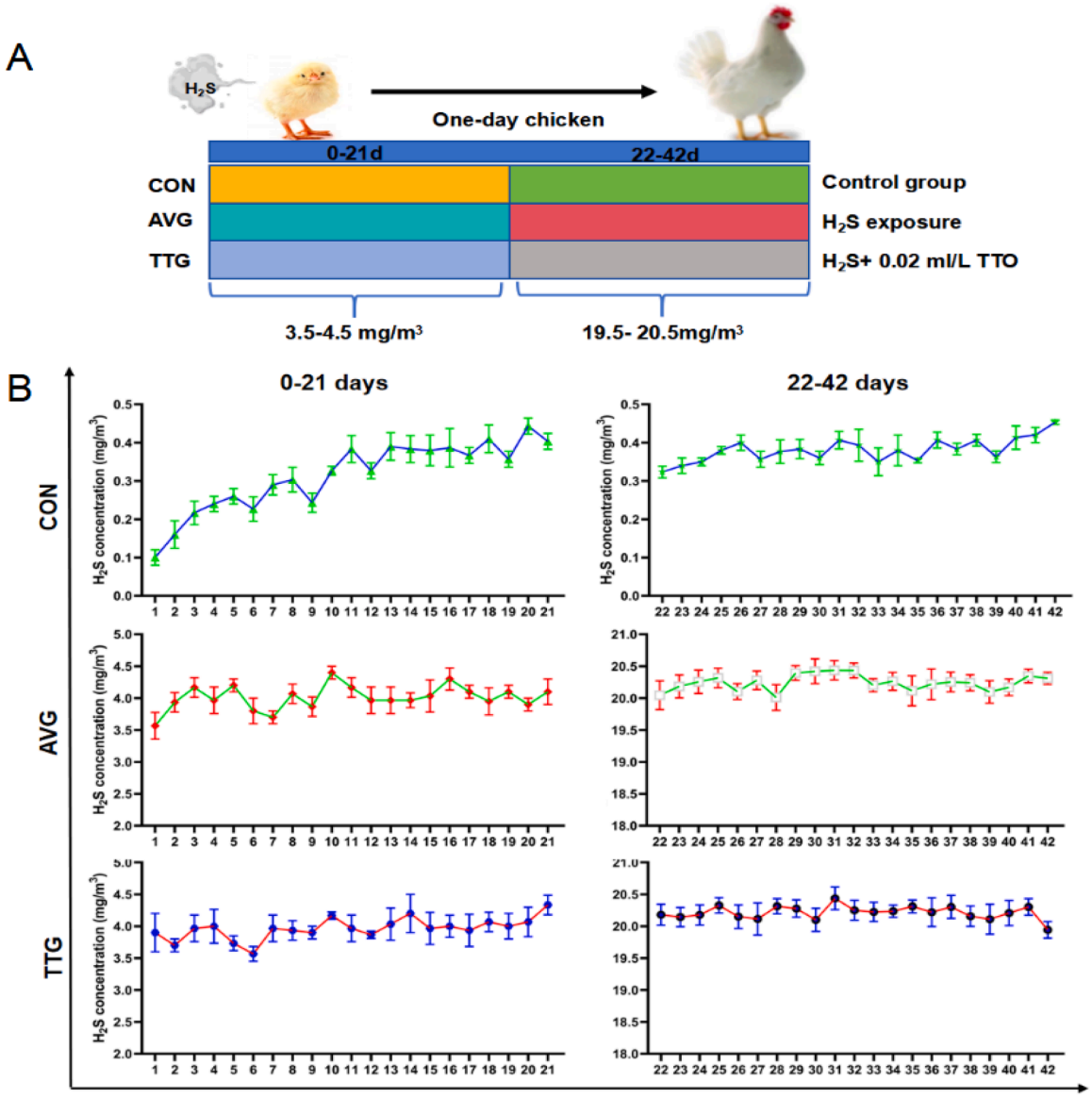

Fig. 1. The overall experimental design and changes in H_2S concentration detection. (A) Model of the first phase of the experiment. (B) Experimental phase II model. (C) H_2S concentration changes. Data are expressed as mean ± standard deviation (M ±SD). CON: control group. AVG: H_2S exposure group. TTO treatment group: TTG.

Table 2
Growth performance changes of chickens at 42 days.

Phase	Group	IBW (g)	FBW (g)	ADFI (g)	ADG (g)	F/G
0-21days	CON	20.16±1.74^{a}	229.00±10.71^{a}	18.05±0.69	9.95±0.46^{a}	1.82±0.06^{b}
	AVG	20.28±2.40^{a}	186.20±7.85b	18.47±0.99	7.90±0.29^{b}	2.34±0.15^{a}
	TTG	19.74±1.51^{a}	207.30±12.14^{b}	17.72±0.86	8.93±0.63^{b}	1.99±0.20^{b}
22-42days	CON	229.00±10.71^{a}	60.30±24.34^{a}	55.50±3.45	20.54±0.99^{a}	2.71±0.20^{b}
	AVG	186.20±7.85^{b}	552.20±15.00^{b}	58.33±3.20	17.43±0.77^{b}	3.48±0.15^{a}
	TTG	207.30±12.14^{b}	585.70±26.66^{b}	53.33±4.08	18.02±1.51^{b}	2.98±0.34^{b}

Three repetitions per group are expressed as mean ± standard deviation. Different lowercase letters of shoulder in the same column indicated significant difference ($P < 0.05$), while no letters or the same letters indicated no significant difference ($P > 0.05$). Average daily feed intake :ADFI. Average daily gain :ADG. Feed/meat ratio : F/G. Initial weight :IBW. Final weight: FBW.CON: control group. AVG: H_2S exposure group. TTO treatment group: TTG.

the chickens were killed by carotid bloodletting under anesthesia. During the dissection, tissue samples were collected from the chicken's thymus, kidneys, spleen, bursa of Fabricius, pancreas, duodenum, jejunum and ileum. Part of the collected samples were immediately put into 4 % paraformaldehyde solution for immobilized treatment for subsequent histological analysis, and spleen tissues were especially put into cold PBS solution for timely determination of relevant flow indicators. The remaining tissue sample was stored at -80°C for subsequent tests.

Organ index

At the end of the 42 days test, the organ indices of the thymus, kidney, spleen, bursa of Fabricius and pancreas were examined after dissection. The formula is as follows: Organ index (g/Kg) = organ wet weight (g)/chicken live weight (Kg) (Choi et al., 2021).

Histomorphological analysis

The morphological and histomorphological characteristics of the intestines were determined in accordance with the research methods outlined by Wang et al. (2019). Briefly, the process involves dehydrating, transparent, and paraffin-embedding fixed samples of spleen, bursa of Fageria, kidney, thymus, pancreas and intestine. Subsequently, the embedded tissue sample was sliced into 6μm sections and stained with hematoxylin-eosin. Finally, the normal tissue and sites with obvious lesions were photographed using an optical microscope (Nikon, IX73-A12FLPH, Tokyo, Japan). Concurrently, the injured organ tissues were scored (Annex Table 3). In addition, the 10 samples were selected from each group for determination when conducting morphological analysis of intestinal tissue. The measurements included villus height (VH, the distance from the opening of the crypt to the end of the villi), crypt depth (CD, the distance from the crypt villi to the base of the crypt) and the VH/CD ratio. These indicators were measured under an electron microscope (Olympus, CX41, Tokyo, Japan) and analyzed with Image-Pro Plus 6.0 software (Miao et al., 2021). In the experiment, the same samples were used to determine the corresponding VH and CD values to ensure the scientific nature of the experiment.

Determination of serum biochemical and immune indices

According to the methods of (Wang et al., 2021), the serum biochemical indices were determined. First, the serum after centrifugation was processed according to the operation method on the instructions. Subsequently, serum biochemical indices were measured, including total protein (TP), albumin (ALB), globulin (GLO), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AKP), total cholesterol (TC), triglyceride(TG) and glucose (GLU). In addition to the above biochemical indicators, serum immune indicators, including IgA and IgB levels, were specifically determined to comprehensively assess the immune status of the serum. This series of measurement steps followed the research method of (Wang et al., 2021) to ensure the accuracy and reliability of the results.

Determination of spleen oxidation indices

According to the previous methods (Liu et al., 2023a), the antioxidant indices of spleen tissue were determined. Firstly, the frozen spleen tissue sample was taken out and an appropriate amount of normal saline was added for homogenization. Upon completion of homogenization, centrifugal treatment was performed (3000 r/min, 10 min, 4°C) (Chen et al., 2024; Liu et al., 2023b), followed by extraction of the supernatant for further use. Subsequently, the T-AOC, SOD, TP, CAT and MDA content in the spleen were determined according to the kit instructions.

Determination of spleen-related indexes using ELISA

Spleen tissue samples collected after the 42 days trial were processed by adding normal saline, followed by grinding and centrifugal separation (3000 r/min, 10 min, 4 °C). After centrifugation, the supernatant was carefully removed for subsequent analysis. Then, the contents of Bcl-2, Bax, Caspase-3, INF-γ, TNF-α, IL-Iβ and IL-4 in the supernatant were determined strictly according to the instructions of the ELISA kit.

Measurement of apoptosis rate by flow cytometry

The spleen tissues were placed on a 200-mesh screen for grinding. After grinding, spleen cells were collected and transferred to a centrifuge tube for centrifugation (1500 r/min, 3 min). After centrifugation, the cells were washed three times with PBS buffer to remove impurities (Liu et al., 2020). After centrifugation again, 500 uL Binding Buffer was added to the cells to prepare cell suspension. At the same time, the cell suspension was added with 10 μL Annexin V-FITC and 5 μL PI, and then reacted at room temperature for 15 min in order to determine the apoptosis rate. As a control reagent, a group of samples without Annexin V-FITC and PI were also set up. When measured by flow cytometry, the excitation wavelength was set to 488 nm. Annexin V-FITC's green fluorescence was detected by FITC channel (FL-1,530 nm), while PI's red fluorescence was detected by PI channel (FL-2,585 nm). Each sample was counted with 10,000 cell counts and the data was analyzed by a standard computer program for flow cytometry (Ramadan et al., 2019).

Determination of mitochondrial membrane potential and reactive oxygen species (ROS)

For the determination of mitochondrial membrane potential, Rhodamine 123, a fluorescent dye with specific binding to mitochondria, was used (Changsha Hanchen Biotechnology Co., LTD., Changsha, China) (Momcilovic et al., 2019). The specific operation steps are as follows: Firstly, the treated cells were suspended with PBS, then Rhodamine 123 dye was added, the final concentration was adjusted to 5 μg/mL, and fully mixed. Then, the cells were incubated at 37°C for 30 min away from light. After incubation, the cell was washed three times with PBS. Finally, flow cytometry was used to measure the mean fluorescence intensity of cells at excitation wavelength 488 nm and emission wavelength 530 nm (Hao et al., 2017), so as to reflect the situation of mitochondrial membrane potential.

To detect the level of ROS in the cells, another specific binding fluorescent dye, DCFH-DA, was used. According to the method of (Hao et al., 2017), the following procedure was performed: the treated cells were suspended with PBS, then DCFH-DA dye was added, the final concentration was adjusted to 100 μM and thoroughly mixed. After incubation at 37 °C for 30 min, the cells were washed three times with PBS. Finally, the average fluorescence intensity of cells was measured by flow cytometry at excitation wavelength of 488nm and emission wavelength of 530 nm, so as to reflect the level of ROS in cells.

Detection of cell subsets by flow cytometry

After the concentration of treated blood cells and spleen tissue cells was adjusted to 1×10^6 /ml, T lymphocyte subsets detection kit (Shanghai Tongwei Industrial Co., LTD., Shanghai, China) was used for detection. Flow cytometry (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., CytoFLEX LX, Shenzhen, China) was used to determine the samples. Prior to the formal test, the instrument voltage was firstly adjusted using an unstained cell suspension. Subsequently, FITC, APC-H7, Percp-Cy5.5, BV510, PE-Cy7, Alexa Fluor 647, PE and Pacific Blue were used to adjust the fluorescence compensation between each fluorescence. Upon completion of the detection, the collected raw data were analyzed by FlowJo software (Becton, Dickin son and Company) (Schultze-Florey et al., 2021).

Quantitative PCR (qRT-PCR) detection

Total RNA was extracted using Trizol kit (Nanjing Jiancheng Biological Company, Nanjing, China) (Sun et al., 2024). The quality and purity of the RNA were evaluated by Agilent Medical Technology (Shanghai) Co., LTD., Shanghai, China. The PrimeScript™ FAST RT reagent Kit with gDNA Eraser Kit (Takara Bio, Tokyo, Japan) was used to reverse transcribed total RNA into cDNA in a cDNA synthesis system

(Invitrogen, CA, USA). The TB Green® Premix Ex Taq™ II kit (Takara Bio, Tokyo, Japan) was used in the fluorescence quantitative PCR apparatus (Murray (Shanghai) Biotechnology Co., LTD, Light Cycler 180, Shanghai, China) for RT-PCR. According to the complete gene sequence of closed junction protein-1 (*ZO-1*), *occludin, claudin-1, β-actin, Bcl-2, BAX, Caspase-3, IL-1β, IL-4, TNF-α* and *INF-γ* of chicken published in GeneBank Specific upper and lower primers were designed using prime 5.0 software and synthesized by Invitrogen Company (Shanghai) after homology search by GenBank Blast. The primer sequence design is shown in Annex Table 4. The specificity of PCR products was evaluated by melting curve analysis. All the reactions were repeated three times. The relative gene expression and blank control were analyzed using the $2^{-\Delta\Delta CT}$ method (Liang et al., 2024).

Statistical analysis

SPSS 22.0 software was used to conduct one-way ANOVA, and Duncan's method was used to compare the mean values of each group. The data are expressed as mean ± standard deviation (M ±SD). The absence of significant differences between groups was indicated by the same letter ($P > 0.05$), while significant differences between groups were denoted by different letters ($P < 0.05$).

Results

Influence of TTO on growth performance during H_2S exposure

The results showed that H_2S exposure led to decrease FBW and ADG ($P < 0.05$) and increase F/G ($P < 0.05$) compared with the CON within 21 days of the experiment (Table 2). During 22-42 days, FBW and ADG were decreased ($P < 0.05$), while F/G was increased in AVG ($P < 0.05$). After TTO treatment, FBW and ADG no difference (Table 2).

TTO ameliorated H_2s-induced organ pathology

Fig. 2 showed the changes of organ index of chickens in each group. Compared with the CON, the indices of kidney, spleen, bursa of fabricius, thymus and other organs were decreased after exposure to H_2S ($P < 0.05$), but the index of the pancreas was not difference ($P > 0.05$).

Fig. 3 showed the histopathological changes of chickens in each group. Compared with the CON, the splenic tissue structure of TTG was normal and no obvious histopathological injury was observed. In contrast, there was significant congestion in the splenic sinus in the AVG ($P < 0.05$). After TTO treatment, the splenic tissue only showed mild hyperemia ($P > 0.05$) (Fig. 3A). The structure of bursa of Fabricius in the CON was normal, with no apparent histopathological damage. However, the number of lymphocytes in the intralymphoid follicles of bursa of Fabricius decreased and the cell arrangement became sparse ($P < 0.05$). The pathological status of bursa of Fabricius was improved after treatment with TTO ($P < 0.05$) (Fig. 3B). The tissue structure of kidney in the CON was also normal, and no obvious pathological injury was observed. Nevertheless, H_2S exposure resulted in necrosis and exfoliation of renal tubular epithelial cells ($P < 0.05$). After treatment with TTO, the necrosis and exfoliation of renal tubular epithelial cells were improved ($P < 0.05$) (Fig. 3C). Compared with the CON, TTG thymus tissue was not injured ($P > 0.05$). Yet, the thymus atrophied and bleeding occurred in the medullary area of the skin in the AVG ($P < 0.05$). After TTO treatment, the thymus and pancreas injury was not obvious (Fig. 3D, E) ($P > 0.05$).

TTO mitigates H_2S-induced intestinal structural damage

Fig. 4A showed the intestinal changes in each group of chickens. It was found that H_2S exposure could cause the destruction of duodenal villi structure and lead to the shedding of villi epithelial cells, and this situation was well improved after treatment with TTO in the TTG (Fig. 4B) ($P < 0.05$). Additionally, TTO treatment also improved the structural blurring and a few cells shedding of jejunum and ileum villous epithelial cells caused by H_2S exposure (Fig. 4C-D) ($P < 0.05$).

Fig. 4E-M showed villus height, crypt depth and ratio of villus height to crypt depth of duodenum, jejunum, and ileum (V/C) in chickens. After 42 days of TTO treatment, TTO increased the villus length in duodenum, jejunum and ileum compared with the AVG ($P < 0.05$). Meanwhile, the crypt depth was also reduced ($P < 0.05$). Further V/C ratio analysis showed that compared with the CON, the V/C ratio was improved after TTO treatment and basically returned to normal level ($P < 0.05$). Compared with the CON, H_2S exposure decreased the mRNA relative expression of *Claudin-1, Occludin* and *ZO-1* genes in jejunum ($P < 0.05$), while TTO improved the damage effect of H_2S on jejunum intestinal barrier ($P < 0.05$) and basically restored the expression of these genes to normal level($P > 0.05$) (Fig. 4N-P).

TTO modulates H_2S-induced changes in blood lymphocyte subsets and serum biochemistry of chickens

Fig. 5 shows the differentiation of T lymphocyte subsets in chicken blood. Compared with the CON, the proportion of CD3+CD4+ and CD4+/CD8+ cells in blood was decreased after H_2S exposure ($P < 0.05$). However, TTO treatment increased the proportion of CD3+CD4+ and CD4+/CD8+ cells ($P < 0.05$).

Fig. 6 showed the changes of serum biochemical indices of chickens in each group. Compared with the CON, H_2S exposure increased the contents of ALT, AST, TG and AKP in serum ($P < 0.05$) (Fig. 5A-D), while decreased the contents of ALB, GLO, IgA and IgB ($P < 0.05$) (Fig. 5E,F,J, K). The contents of ALT, AST, TG and AKP in TTG were lower than those in AVG ($P < 0.05$). Compared with the CON, the difference was not significant and still did not return to the normal level ($P > 0.05$). Meanwhile, the contents of ALB, GLO, IgA and IgB were increased and

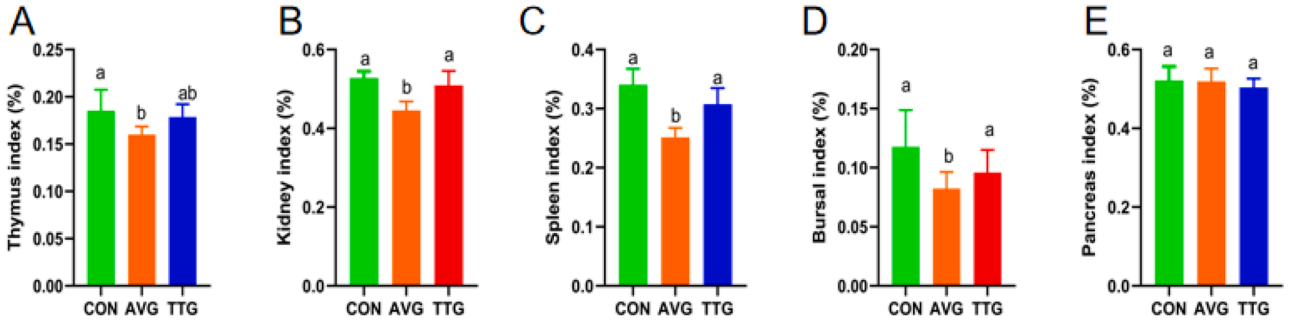

Fig. 2. Changes of organ index of chickens in each group. (A) thymus index. (B) kidney index. (C) spleen index. (D) bursae of Fabricius. (E) pancreas index. Three repetitions per group are expressed as mean ± standard deviation. Different lowercase letters in the shoulder label in the same column indicated significant difference ($P<0.05$), while no letters or the same letters indicated no significant difference ($P>0.05$). AVG: H_2S exposure group. TTO treatment group: TTG.

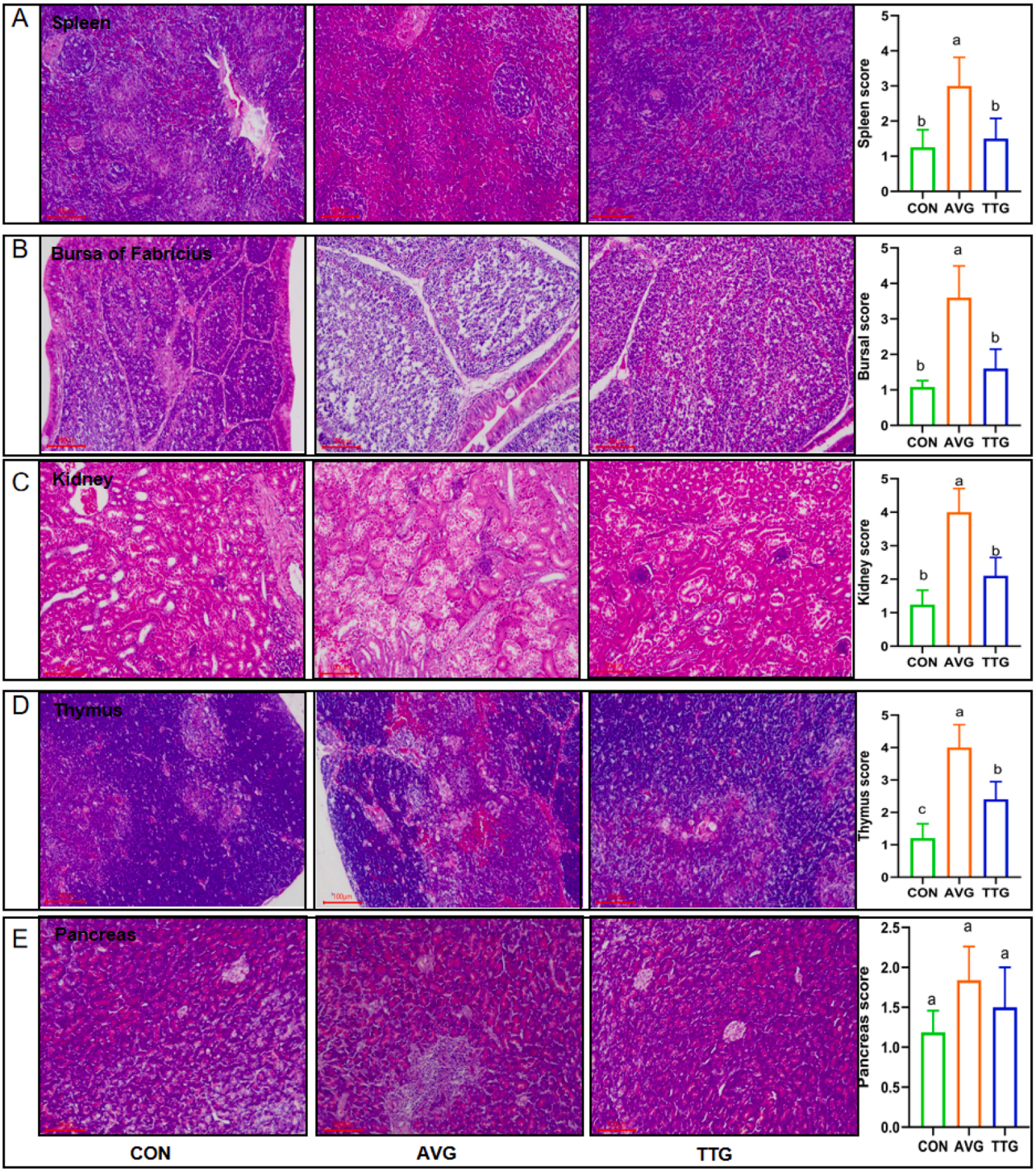

Fig. 3. Histopathological changes of chickens in each group. (A) spleen structure. (B) bursa of Fabricius structure. (C) kidney structure. (D) thymus tissue structure. (E) pancreas and tissue structure. Three repetitions per group are expressed as mean ± standard error. Different lowercase letters in the shoulder label in the same column indicated significant difference ($P < 0.05$), while no letters or the same letters indicated no significant difference ($P > 0.05$). AVG: H_2S exposure group. TTO treatment group: TTG.

basically returned to the normal level ($P < 0.05$). In addition, the content of TC, GLU and TP in blood of chickens no difference among all groups ($P < 0.05$).

TTO inhibits H_2S-induced oxidative stress and apoptosis in spleen

Fig. 7 showed the changes of oxidative stress and apoptosis in spleen of chickens in each group. Compared with the CON, H_2S exposure caused significant increase in MDA content in spleen tissues ($P < 0.05$) (Fig. 7A). At the same time, H_2S also damaged the oxidative defense system of spleen tissue, reduced the contents of CAT, SOD and T-AOC ($P < 0.05$) (Fig. 7B-D). Compared with the AVG, the oxidative stress status of spleen of chickens after TTO treatment was improved ($P < 0.05$) and basically returned to normal level($P > 0.05$). Analysis by flow cytometry showed that ROS content induced by H_2S exposure was reduced after treatment with TTO ($P < 0.05$) (Fig. 7F). Further analysis showed that

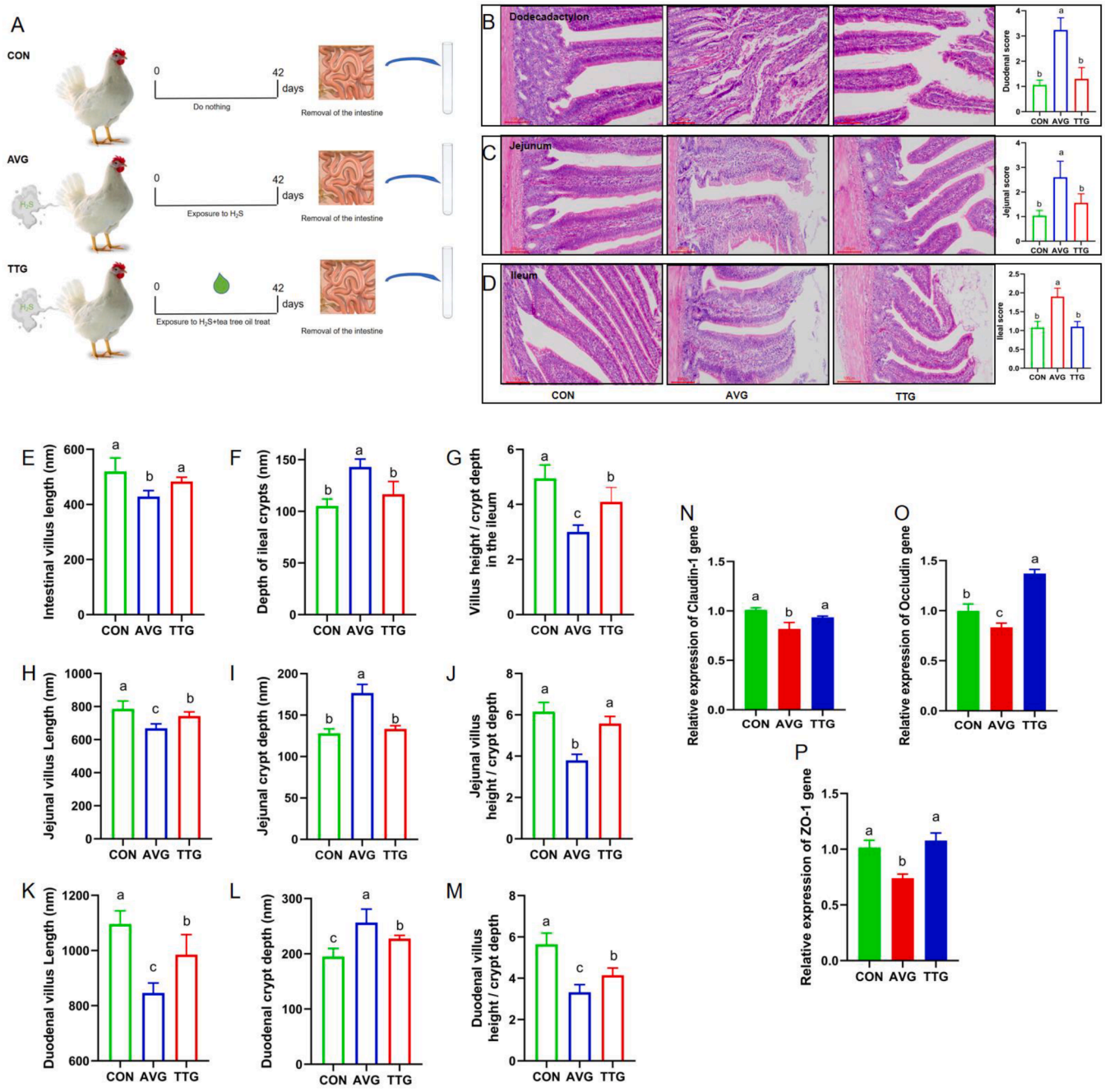

Fig. 4. Intestinal changes in each group of chickens. (A) Intestinal sampling test model. (B) Duodenal sections and scores. (C) jejunal sections and scores. (D) Ileum sections and scores. (E) ileum villi length. (F) Depth of ileal recess. (G) Ratio of ileum villi height to crypt depth (V/C). (H) jejunal villi length. (I) Depth of jejunal recess. (J) Ratio of jejunum villus height to crypt depth (V/C). (K) duodenal villi length. (L) Depth of duodenal recess. (M) Ratio of duodenal villus height to crypt depth (V/C). (N) Relative expression level of Claudin 1mRNA. (O) mRNA relative expression of Occludin. (P) Relative expression of ZO-1 mrna. HE, Bar=100μm, 100 times. Three repetitions per group are expressed as mean ± standard deviation. Different lowercase letters in the shoulder label in the same column indicated significant difference ($P < 0.05$), while no letters or the same letters indicated no significant difference ($P > 0.05$). AVG: H_2S exposure group. TTO treatment group: TTG.

H_2S induced excessive ROS production in mitochondria, which led to mitochondrial depolarization and apoptosis ($P < 0.05$). Compared with CON, cell depolarization returned to normal levels in the TTG ($P < 0.05$) (Fig. 7G). H_2S exposure increased the rate of apoptosis in spleen cells compared with the CON. However, the apoptosis rate of spleen cells in the TTG was reduced and basically returned to normal level ($P < 0.05$) (Fig. 7H). Using RT-PCR and ELISA detection (Fig. 7I-N), it was found that TTO inhibited the expression of Bax and Caspase-3 genes and proteins, while increasing the expression of Bcl-2 proteins and genes ($P < 0.05$).

TTO improves the damage of spleen immune function induced by H_2S

The content of CD3+CD4+ and D4+/CD8+ in the AVG was lower than that in the CON ($P < 0.05$). CD3+CD8+ content was increased ($P < 0.05$). There was no difference in CD3+CD4+ content in TTG compared with AVG($P > 0.05$), but CD4+/CD8+ and CD3+CD8+ contents were increased ($P < 0.05$) (Fig. 8A). The results showed that the mRNA expression levels of INF-γ, TNF-α, IL-1β and IL-4 induced by H_2S exposure were higher than those of CON ($P < 0.05$). The mRNA expression levels of inflammatory cytokines in AVG were compared with TTG ($P <$

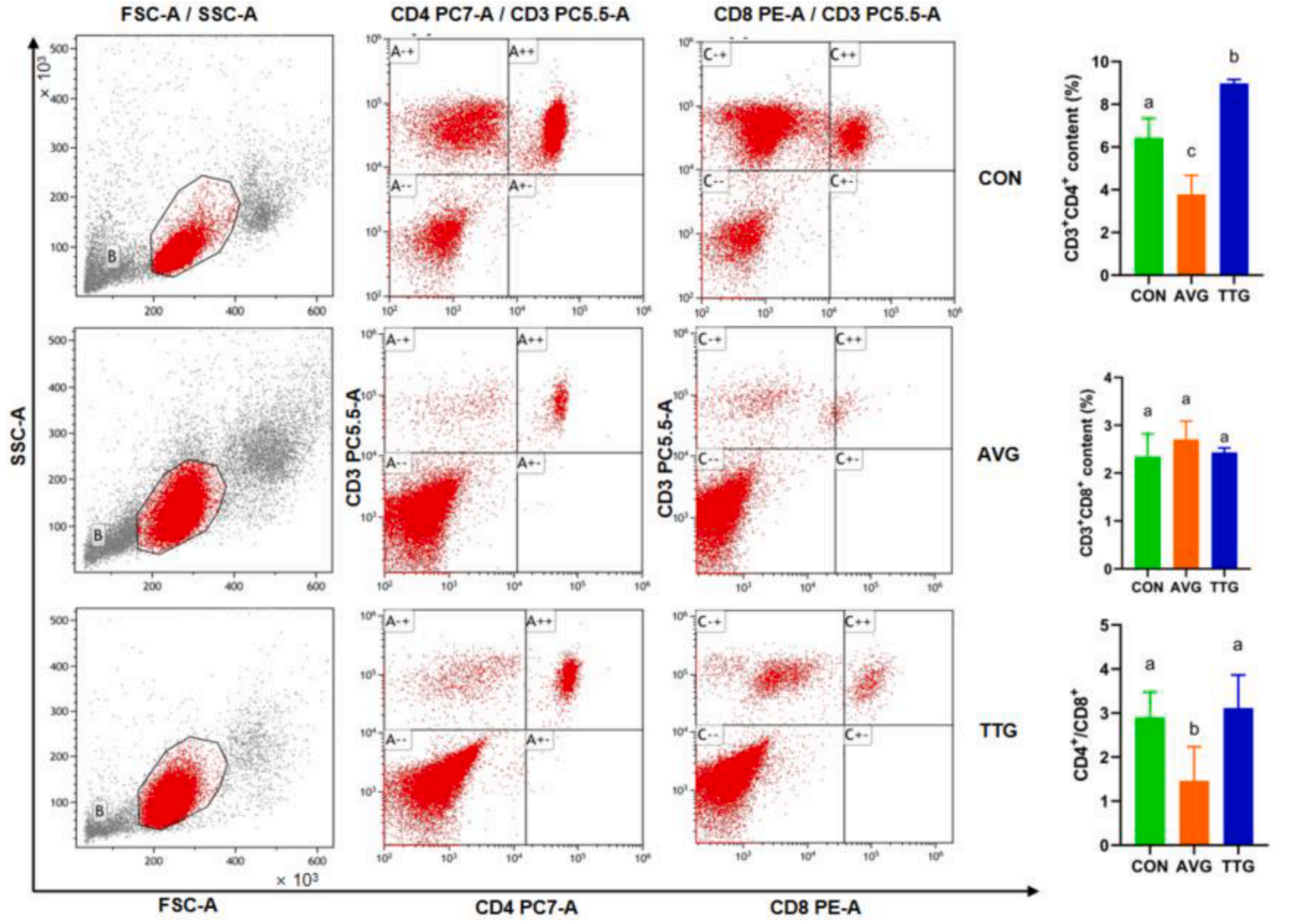

Fig. 5. The differentiation of T lymphocyte subsets in chicken blood. Flow cytometry with double staining of Annexin V and PI. Three repetitions per group are expressed as mean ± standard deviation. Different lowercase letters in the shoulder label in the same column indicated significant difference ($P < 0.05$), while no letters or the same letters indicated no significant difference ($P > 0.05$). AVG: H_2S exposure group. TTO treatment group: TTG.

0.05) (Fig. 8B). ELISA analysis further found that H_2S exposure increased the contents of INF-γ, TNF-α, IL-1β and IL-4 proteins in spleen compared with the CON ($P < 0.05$). TTO treatment reduced the amount of these inflammatory proteins in the spleen (Fig. 8C).

Analysis of the interaction between intestinal, blood biochemical and spleen parameters

The correlation of spleen, blood, and gut-related parameters is shown in Fig. 9A. The results showed that intestinal barrier protein Claudin-1 was negatively correlated with spleen inflammatory protein IL-1β ($P < 0.05$) (Fig. 9A). In addition, intestinal barrier protein ZO-1 was negatively correlated with serum biochemical index TG (Fig. 9A) ($P < 0.05$). Furthermore, Serum biochemical index AKP was positively correlated with spleen inflammatory factor INF-γ.Furthermore, the serum biochemical index AKP exhibited a positive correlation with the spleen inflammatory factor INF-γ. This further reinforces the connection between the intestinal barrier, serum biochemistry, and spleen health. At the same time, this experiment combined intestinal, blood biochemical and spleen function parameters, and based on the effect of TTO, plotted the therapeutic mechanism of TTO on spleen and intestinal damage caused by H_2S via intestinal splenic pathway of chickens (Fig. 9B).

Simultaneously, by integrating parameters from the intestine, blood biochemistry, and spleen function, and based on the effects of TTO, we constructed a therapeutic mechanism model for tea tree oil's action on spleen and intestinal damage induced by H_2S through the chicken's intestinal splenic pathway (depicted in Fig. 9B). The results showed that TTO mitigated oxidative stress, apoptosis, and inflammation of the spleen induced by H2S through the gut ($P < 0.05$).

Discussion

A suitable breeding environment is very important for the growth and development of animals (Liu et al., 2024). When the concentration of H_2S in livestock and poultry houses is high, it will induce the stress response of the body and affect the normal growth and development of chickens. Previous studies have found that H_2S caused damage to intestinal structure and spleen (Chi et al., 2019; Zheng et al., 2019). However, TTO extracts have received attention for their anti-inflammatory, antioxidant, and antibacterial properties (Liang et al., 2024). Studies have shown that TTO can reduce the damage caused by H_2S (Liang et al., 2024). However, no studies have been reported on whether TTO regulates spleen damage caused by H_2S through the gut-spleen pathway. Therefore, this study aimed to investigate whether TTO induces spleen and intestinal damage by regulating H_2S through the gut-spleen pathway.

In this study, it was also found that H_2S inhibited the performance of chickens at different growth stages. Relevant studies have shown that exposureH_2S environment can lead to a significant decrease in daily weight gain, daily feed intake and feed efficiency in broilers (Wang et al., 2011). The results confirm that H_2S has the effect of inhibiting

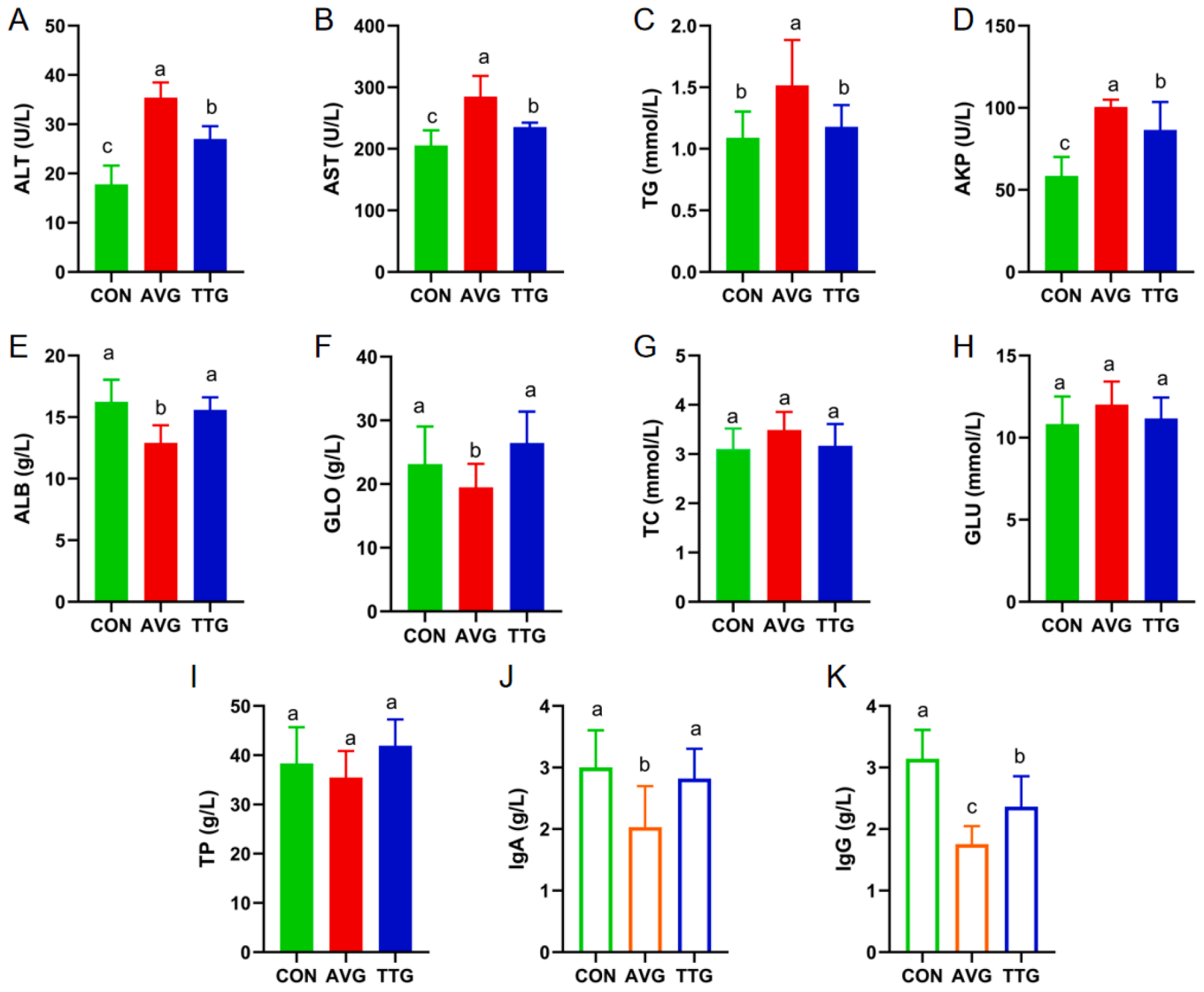

Fig. 6. The changes of serum biochemical indices of chickens in each group. (A) ALT content. (B) AST content. (C) TG content. (D) AKP content. (E) ALB content. (F) GLO content. (G) TC content. (H) GLU content. (I) TP content. (J) IgA content. (K) IgG content. Three repetitions per group are expressed as mean ± standard deviation. Different lowercase letters in the shoulder label in the same column indicated significant difference ($P < 0.05$), while no letters or the same letters indicated no significant difference ($P > 0.05$). AVG: H_2S exposure group. TTO treatment group: TTG.

growth (Liang et al., 2024). However, TTO can promote the gene expression of insulin-like growth factor and growth hormone (Yang et al., 2022), thereby increasing the body weight of chickens and alleviating the inhibitory effect of H_2S on growth performance of chickens. Meanwhile, the H_2S entering the blood through the lungs and intestines can cause damage to immune organs and changes in organ indices under the action of systemic circulation. In this study, it was found that H_2S exposure resulted in a significant decrease in the indices of immune organs (spleen, bursa of Fabricius, and thymus), suggesting that H_2S disrupted immune function in chickens. At the same time, it has also been verified in histopathological injuries. The results indicated that TTO had significant effects on improving immune function and alleviating histopathological damage. Previous studies have found that plant extracts can improve immune organ indexes such as chicken spleen and thymus, because the active ingredients in plant extracts can immunize organs (Liu et al., 2023b). In addition, TTO is rich in active ingredients of terpenoids and alcohols, which can also regulate metabolism and immunity (Nascimento et al., 2023). This may be the important reason why chicken is the increase of immune organ indices and immune enhancement.

As the most important site of digestion and absorption in animal body, the integrity of its mucosal structure and function of the small intestine is crucial to the full absorption of nutrients in the animal body, which directly affects the growth of animals (Miao et al., 2021). Intestinal villi, as an important component of intestinal mucosal morphology and structure, have a direct impact on the body's ability to digest and absorb nutrients (Liu et al., 2023b). Villus height, crypt depth and their ratio (villus height/crypt depth) are important indicators of intestinal mucosal function. Increased villi length, shallower crypts, and higher villus height/crypt depth ratios all reflect enhanced intestinal mucosal absorption (Thaiss et al., 2018). Previous studies have found that adding TTO to drinking water improves intestinal structure in chickens, increasing villus height and the villus height/crypt depth ratio (Liu et al., 2023b). In this study, treatment with TTO alleviated the inhibitory effect of H_2S on villus length in the small intestine, not only increasing the ratio of villus height to crypt depth, but also decreasing crypt depth. These changes help to improve the intestinal absorption capacity of chickens, thereby improving their growth performance. The improvement of intestinal structure also helps to enrich the type and number of bacteria, provide more attachment points for bacteria, and increase the

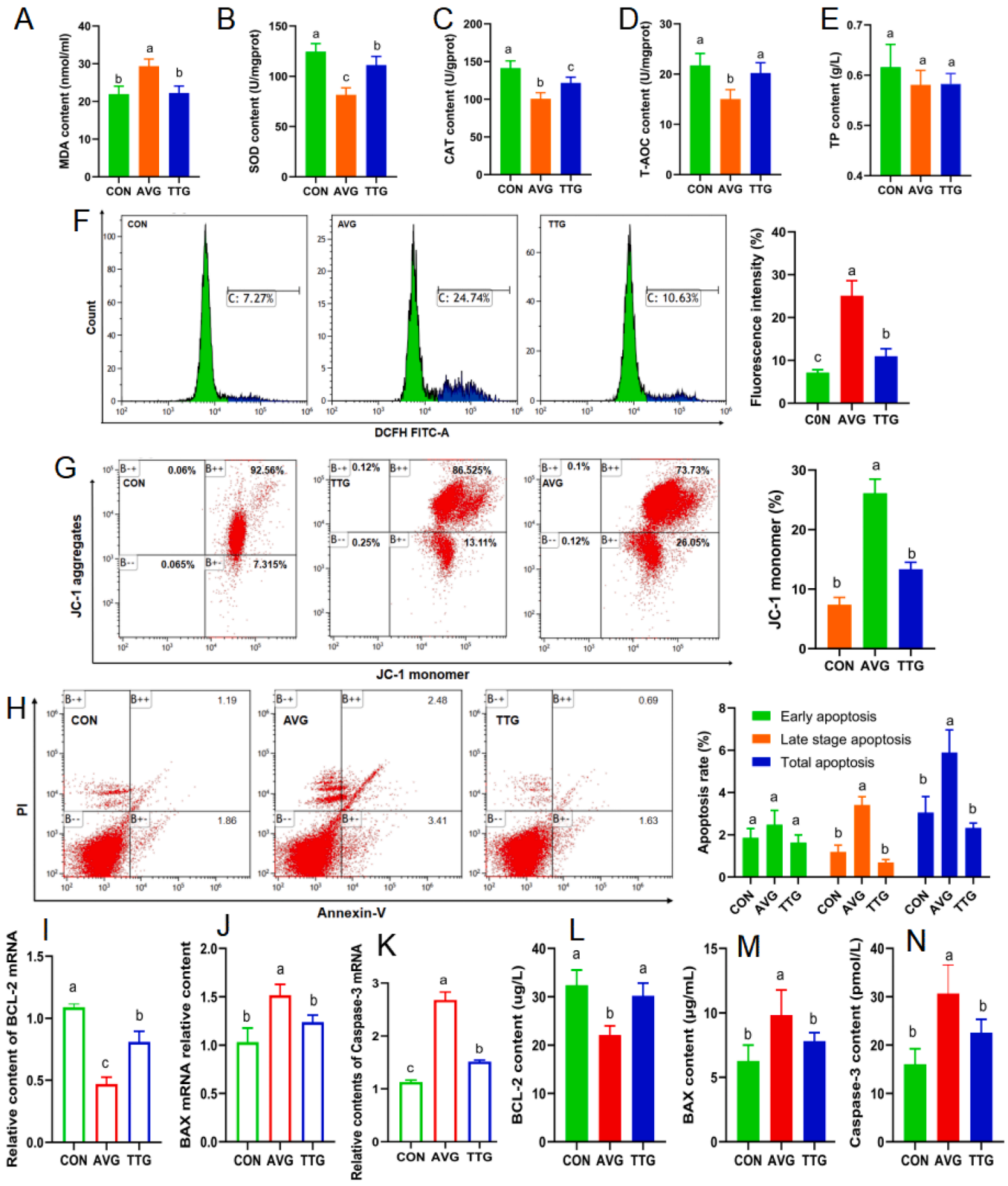

Fig. 7. Changes of oxidative stress and apoptosis in spleen of chickens in each group. (A) MDA content. (B) SOD content. (C) CAT content. (D) T-AOC content. (E) TP content. (F) ROS content in spleen tissue. (G) Changes of mitochondrial membrane potential in spleen cells. (H) Splenic cell apoptosis. (I) Relative mRNA expression of BCL-2 gene. (J) Relative mRNA expression of Bax gene. (K) mRNA relative expression of Caspase-3 gene. (L) Bcl-2 protein content. (M) Protein content of Bax. (N) Caspase-3 protein content. Three repetitions per group are expressed as mean ± standard deviation. Different lowercase letters in the shoulder label in the same column indicated significant difference ($P < 0.05$), while no letters or the same letters indicated no significant difference ($P > 0.05$). AVG: H_2S exposure group. TTO treatment group: TTG.

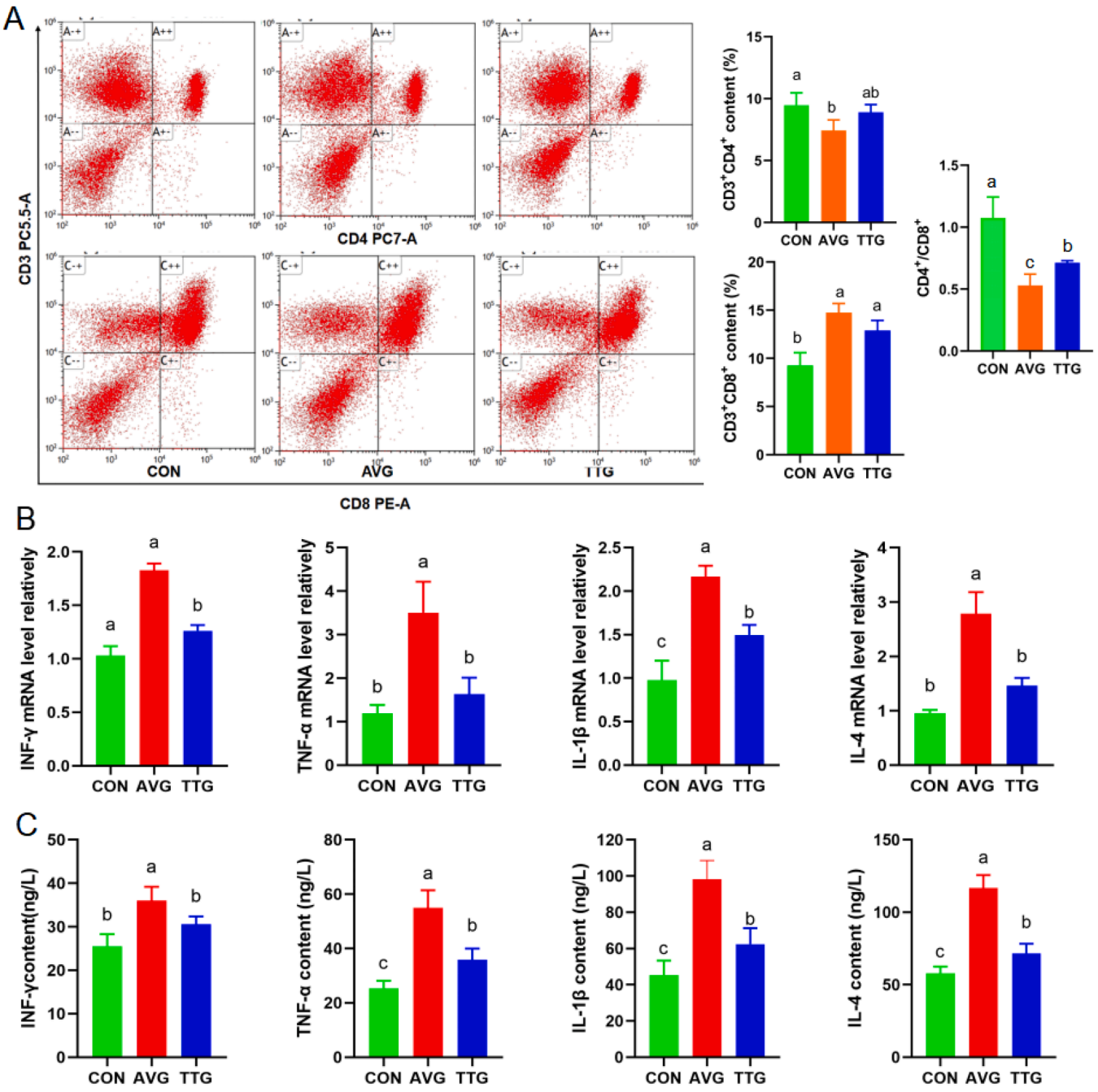

Fig. 8. Changes of spleen immunity and inflammatory factors in each group. Flow cytometry was used to detect immune cells. (B) INF-γ, TNF-α, IL-1β and IL-4 mrna relative expression levels. (C) INF-γ, TNF-α, IL-1β and IL-4 protein content. Three repetitions per group are expressed as mean ± standard deviation. Different lowercase letters in the shoulder label in the same column indicated significant difference ($P < 0.05$), while no letters or the same letters indicated no significant difference ($P > 0.05$). AVG: H_2S exposure group. TTO treatment group:TTG.

residence time of probiotics in the body. In addition, the ratio of villus height to crypt depth means that the gut has better digestion and absorption function. After H_2S enters the intestine, jejunum barrier function is impaired, resulting in decreased expression of *Claudin-1*, Occludin and *ZO-1* proteins (Wu et al., 2023). Decreased expression of *Claudin-1, Occludin* and *ZO-1* genes destroys intestinal barrier function and increases intestinal permeability. The results of this study showed that adding TTO in drinking water can enhance intestinal barrier function by increasing mRNA expression of *Claudin-1, Occludin and ZO-1* in ileum. This suggests that TTO has a role in regulating intestinal barrier function. This effect is mainly attributed to Terpinen-4-ol's ability to up-regulate tight junction proteins, thereby protecting the integrity of the intestinal barrier (Yong et al., 2022).

When H_2S destroys the intestinal structure and barrier function, it will quickly enter the blood and cause oxidative stress, destroy the balance of the internal environment, and lead to the disorder of serum biochemical indexes (Liu et al., 2022b). Serum biochemical indexes can reflect the metabolic state of the animal body and the permeability of tissue cells, and are closely related to the health state of animals (Liu et al., 2022b). In this study, compared with the AVG, TTG increased the contents of ALB and GLO, while decreased the contents of ALT, AST, TG and AKP. Compared with the CON, TTO treatment basically stabilized the serum biochemical indexes of chickens. This indicates that TTO has a significant effect on the improvement of serum biological disorders

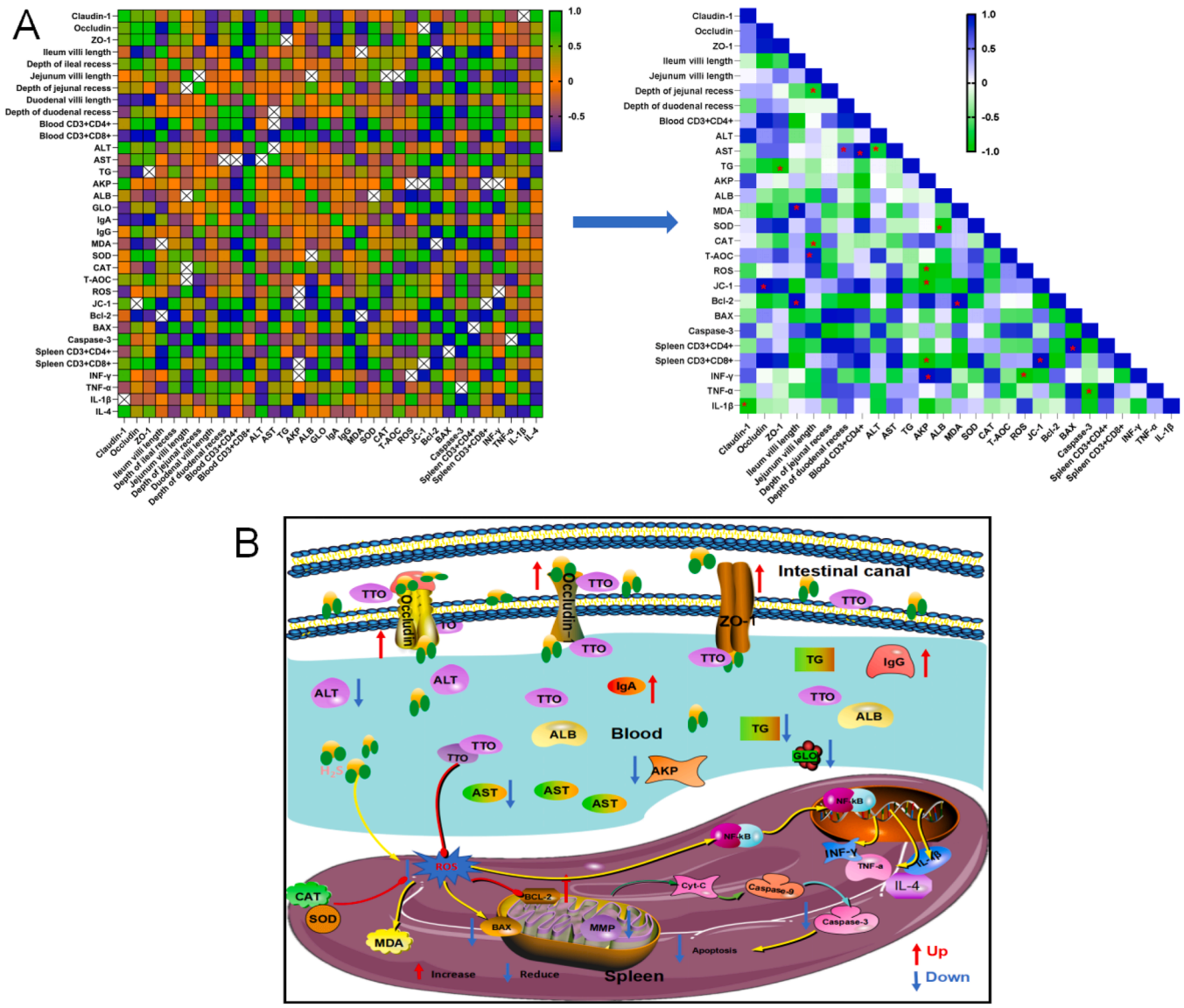

Fig. 9. Correlation analysis of gut-spleen axis parameters and regulation mechanism of gut-spleen axis of tea tree oil. (A) Correlation analysis of gut-spleen axis parameters. (B) Regulation mechanism of gut-spleen axis of tea tree oil. ALB: albumin, GLO: globulin, AST: Aspartate aminotransferase, ALT: alanine aminotransferase, AKP: alkaline phosphatase, TC: cholesterol, IgA: immunoglobulin A, IgB: immunoglobulin B. TTO: Tea tree oil, ZO-1, tight junction protein-1, Occludin-1, Occludin-1, IL-4: Interleukin-4, CAT:Catalase, SOD:Superoxide dismutase, ROS:Reactive oxygen species, H_2S: Sulfuretted hydrogen, MDA: Malondialdehyde, BAX: BCL2 associated X, apoptosis regulator, BCL-2: BCL2 apoptosis regulator, MMP: mitochondrial membrane potential, Cyt-c: Cytochrome C, NF-KB: nuclear factor kappa B subunit, INF-γ: tumor necrosis factor -γ, TNF-α: tumour necrosis factor alpha-like, IL-1β: interleukin-1β. "*" was significantly correlated ($|r| > 0.55$, $P < 0.05$).

caused by H_2S. Relevant studies have shown that TTO can reduce AST and increase AKP content in serum (Yang et al., 2022), which is consistent with the results of this study. In addition, H_2S exposure decreased the serum IgA and IgG levels and the number of CD3+CD4+ T cells in the blood. Meanwhile, CD3+CD4+, IgA and IgG basically returned to normal levels in the CON compared to the TTG. IgA mainly exists in the secretory form (sIgA) and is transported to the airway cavity via the polyimmunoglobulin receptor (pIgR), where it exerts immune rejection against inhaled pathogens (Yuan et al., 2023). IgG is produced by plasma cells of the spleen and lymph nodes and has multiple immune functions (Yuan et al., 2023). CD3+CD4+T cells, as helper T lymphocytes, play a key role in promoting the transformation of T lymphocytes into effector cells and assisting B lymphocytes in producing antibodies, thus assisting humoral and cellular immunity (Kroemer et al., 2020). On the contrary, CD3+CD8+T cells can inhibit the activity of T lymphocytes and the production of antibodies by B lymphocytes, and play an inhibitory role in humoral and cellular immunity (Chen et al., 2022). This finding suggests that TTO may enhance the immune capacity of chickens by increasing the levels of IgA, IgG and the number of CD3+CD4+T cells. Previous studies have found that TTO can increase the body's IgA and IgG levels, thereby enhancing the pigs' and cows' immune function (Shang et al., 2024; Yang et al., 2022; Yuan et al., 2023). This effect is mainly attributed to the component terpinen-4-ol in TTO, which can promote immunity (Liang et al., 2024).

H_2S can also cause spleen damage. This damage often begins in the lungs or intestines, and then H_2S spreads through the blood to various tissues and organs throughout the body, causing widespread oxidative stress disorders and organ damage (Liang et al., 2024; Song et al., 2021b; Zheng et al., 2019). The oxidative stress disorder induced by H_2S in the spleen leads to the overproduction of ROS, which then leads to spleen injury, decline in immune function, and even cell apoptosis (Chi et al., 2019). As a drug with antioxidant properties, TTO can effectively eliminates excess free radicals and improve the antioxidant capacity of animals (Bai et al., 2023). In this study, TTO treatment alleviated SOD,

CAT, T-AOC, and reduced the levels of MDA and ROS caused by H_2S, nearly restored to normal levels. SOD and CAT are the body's first line of defense against oxidative damage, preventing the body from producing excessive ROS (Bai et al., 2023; Liang et al., 2024).

In addition, H_2S-induced ROS causes the opening of mitochondrial permeability transition pores, leading to mitochondrial membrane potential (MMP) depolarization. This study found that adding TTO to drinking water can improve H_2S exposure and lead to mitochondrial depolarization and apoptosis of spleen cells. In the process of apoptosis, mitochondrial damage and changes in membrane permeability can destroy mitochondrial transmembrane potential ($\Delta\Psi m$) and induce apoptosis (Saunders et al., 2024). In order to explore the mechanism of inhibiting apoptosis by TTO, several key apoptosis signaling proteins were analyzed in this study (Newton et al., 2024). Among them, the activation of Caspase-3 and the expression of Bcl-2 family proteins (including the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax) are important indicators of the mitochondrial apoptotic pathway (Poreba et al., 2018). Bcl-2 is antagonistic to Bax (Newton et al., 2024). The activation of Caspase-3 signifies the initiation of apoptos (Jia et al., 2024; Poreba et al., 2018). Therefore, in this study results showed that the levels of pro-apoptotic proteins Bax and Caspase-3 were decreased, while the expression of anti-apoptotic protein Bcl-2 was increased after treatment with TTO compared with the AVG. Meanwhile, compared with the CON, the apoptosis of spleen was improved after treatment with TTO. This result is consistent with the mRNA expression trend of apoptosis genes, indicating that TTO can inhibit apoptosis of spleen cells by regulating mitochondrial pathway. This is mainly due to the fact that α-terpinene, α-terpinolene, γ-terpinene and other components in TTO can remove free radicals and inhibit apoptosis induced by oxidative stress (Liang et al., 2024).

In addition, H_2S caused damage to the immune system of the spleen, as shown by a significant reduction in the number of CD3+CD4+ T lymphocyte subsets in the spleen in the AVG. In contrast, the number of CD3+CD4+ cells in the spleen of the TTG was increased, indicating that TTO has a positive effect on increasing the number of T lymphocyte subsets. Further studies found that contents of inflammation-related proteins such as TNF-α, IFN-γ, IL-1β and IL-4 were higher in the spleen of the AVG than in the TTG, suggesting that TTO treatment can reduce the inflammatory damage caused by H_2S to the spleen. Inflammatory signaling molecules such as TNF-α, IFN-γ and IL-4 synthesized by T cells have been found to play an important role in immune killing (Maggioli et al., 2016). At the same time, IL-1β released by macrophages exacerbates the cellular inflammatory response (Chen et al., 2019a; Shi et al., 2024). Relevant studies have shown that TTO can inhibit the expression of inflammatory factors such as TNF-α and IFN-γ, thereby reducing the inflammatory response caused by H_2S (Liang et al., 2024). This effect is mainly attributed to the active ingredients in TTO, including 1, 8-cineole, terpine-4-ol, and α-terpenol, which inhibit the production of inflammatory factors such as TNF-α and IL-1β, thereby enhancing immunity and reducing inflammatory damage (Finlay-Jones, 2000; Groppo et al., 2002).

Conclusions

This study found that adding TTO to drinking water can enhance intestinal barrier function, thereby inhibiting spleen oxidative stress injury and apoptosis induced by H_2S through the intestine-spleen pathway. These findings reveal potential applications of TTO in maintaining intestinal health and protecting the spleen from H_2S damage.

Disclosures

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Yachao Wang: Conceptualization, Methodology, Data curation, Writing – review & editing. **Yilei Liang:** Formal analysis, Data curation, Investigation. **Yan Huang:** Supervision, Funding acquisition. **Wei wang:** Methodology. **Xiaoyan Long:** Data curation, Investigation. **Li Jiang:** Investigation, Writing – review & editing. **Tingting Cheng:** Methodology, Data curation. **Jinfeng Du:** Formal analysis. **Xuegang Luo:** Writing – review & editing.

Declaration of competing interest

None of the authors of this study have any conflict of interest.

Acknowledgments

Thanks to the support of the National Defense Basic Research Program (16ZG6103). Sichuan Province Science and Technology Department, Key Research and Development Project, 2024YFFK0178, Preparation of Modified Bletilla striata Polysaccharide-Silver Nanocomposite Hydrogel.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.104605.

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