Protective effect of dietary supplementation of *Bupleurum falcatum* L saikosaponins on ammonia exposure–induced ileum injury in broilers

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**ABSTRACT** Ammonia (**NH₃**) at a high concentration has been recognized as a highly poisonous pollutant affecting both air and water quality. **NH₃**, as a stimulus, exerts negative impact on broiler growth and production, but the molecular mechanisms are not clear yet. This study was designed to evaluate the effects of dietary supplementation of *Bupleurum falcatum* L saikosaponins (**SP**) on the growth and ileum health status in broilers exposed to **NH₃**. Day-old Arbor Acers broilers (n = 480) were randomly allocated into 1 of 4 treatments. The main factors were dietary **SP** supplementation (0 or 80 mg/kg of diet) and **NH₃** challenge (with or without 70 ± 5 ppm **NH₃**). The data of growth, intestinal morphology, and mRNA expression related to ileal function were collected from broilers exposed to **NH₃** for 7 d. Results showed that **NH₃** remarkably suppressed growth performance and intestinal development as well as induced biological injuries in the ileum of broilers, resulting from oxidative stress, mucous barrier damage, and immune dysfunction as well as upregulated apoptosis. These negative effects of **NH₃** were alleviated by the **SP** supplement. In conclusion, dietary supplementation of **SP** may be helpful in alleviating the detrimental effects of **NH₃** on the ileum development in broilers.

**Key words:** ammonia, *Bupleurum falcatum* L saikosaponins, ileum, injury, broiler

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**INTRODUCTION**

The intestine is an essential organ for nutrients digestion and absorption as well as an intrinsic barrier in animals including poultry (Ghareeb et al., 2015). A series of intestinal protective mechanisms including physical, chemical, and immunological barriers work combinedly to resist invasion of pathogens and other harmful contaminants to maintain biological homeostasis of animals (Awad et al., 2015). Intestinal health is the basis of optimal healthy condition and plays an important role in improving the growth performance of animals (Tao et al., 2019). Factors such as breeding program, management practices, environmental stressors (temperature, harmful gas, and noise), and nutrient levels can potentially affect intestinal health in broilers (Kers et al., 2018).

Ammonia (**NH₃**), a colorless and highly water-soluble toxicant with pungent odor, is harmful in poultry production (Xing et al., 2016). In animal houses, ammonia mainly emits from animal manure by hydrolysis, mineralization, and volatilization (Zhang et al., 2015). The concentration of atmospheric **NH₃** inside poultry houses is relevant to its harmful degree. According to the National Institute of Safety and Health Administration and the Occupational Safety and Health Administration, the highest acceptable level of **NH₃** in poultry houses is within a range from 25 ppm to 50 ppm (Tao et al., 2019). However, to meet the demand for poultry meat products and reduce production costs, farmers increase the stocking size and density of broilers; hence, the levels of **NH₃** inside poultry houses often exceed 80 ppm, especially during winter. Ammonia is regarded as an important factor with great impacts on broiler’s health and welfare (David et al., 2015; Naseem...
and King, 2018). Reports showed that excessive exposure of \( \text{NH}_3 \) may directly decrease growth and productive performance of poultry (Soliman et al., 2017), endanger the respiratory tract (Wei et al., 2015), cause neurotoxicity and immunotoxicity (Xiong et al., 2016), and severely interfere with broiler welfare (Wei et al., 2012), resulting in a variety of diseases such as necrotic enteritis or even death (Olanrewaju et al., 2008). Moreover, studies have also revealed that the gastrointestinal tract is one of the target organs damaged by \( \text{NH}_3 \) inhalation; high concentrations of this toxin can significantly induce upregulation of some genes and proteins expression in the intestine as well as trigger intestinal flora disturbance, inflammatory response, immune dysfunction, oxidative stress, or apoptosis (Zhang et al., 2015). Compared with other animals, poultry produces the highest ammonia emission (Aardenne et al., 2001), whereas poultry is one of the most sensitive animals to \( \text{NH}_3 \) (Watthes et al., 1984). However, the effects of \( \text{NH}_3 \) on the small intestine of poultry are often neglected and inconclusive. Therefore, it is necessary to find out the molecular mechanisms that underlie the toxicity of \( \text{NH}_3 \) on the ileum of broilers. Meanwhile, effective methods for prevention and treatment of the \( \text{NH}_3 \) exposure-induced intestine injury are in great demand. Because \( \text{NH}_3 \) is reported as one of the most harmful stressors in animal husbandry, the use of antistress agents to enhance the host defense is considered the most promising solution to this toxin (Quarles and Kling, 1974; Guo, 2017).

Recently, traditional Chinese herb medicinal products have been showed to possess potential antistress properties and strengthen the host resistance to \( \text{NH}_3 \) stress (Ma et al., 2012). Saikosaponins (SP) represent a group of oleanane derivatives, usually as glucosides, which are ubiquitously found in plants (Cheng et al., 2010). Saikosaponins (a, c, and d) extracted from the roots of \textit{Bupleurum falcatum L} are biologically active components (Tsai et al., 2010). Saikosaponins have been used as a treatment supplement for various medicinal efficacy including immunomodulation (Chiang et al., 2010), hepatoprotection (Zhang et al., 2018), antitumor (Li et al., 2017), antiviral (Chang et al., 2007), and anti-inflammation (Benito et al., 1998). Furthermore, SP are widely used as antistress agents in traditional Chinese medicine. It has been reported that \textit{Radix Bupleuri} extracts containing SP mitigated negative effects of heat stress (Pan et al., 2014). However, its protective effect against \( \text{NH}_3 \) stress in chickens is poorly understood. Therefore, with the aim to develop a novel anti-\( \text{NH}_3 \) stress agent, we conducted experiments to investigate the potential for the use of SP to protect broilers from \( \text{NH}_3 \) exposure-induced intestinal injuries. In addition, the possible damage mechanism of \( \text{NH}_3 \) exposure and protective mechanism of SP in broilers were revealed.

**MATERIALS AND METHODS**

**Reagents**

\textit{B. falcatum L} saikosaponins (purity\( >99\% \), mainly contained 32.1\% saikosaponin a, 11.8\% saikosaponin c, 43.9\% saikosaponin d, 4.7\% saikosaponin e, 7.5\% saikosaponin f) were provided by Zhejiang Shengshi Biotechnology Co. Ltd. (Zhejiang, P. R. China). Total reactive oxygen species (ROS) assay kit, TRIzol reagent (Invitrogen, China), mitochondrial membrane potential (MMP) detection JC-1 kit (BD Bioscience, Franklin Lakes, NJ), and reverse transcription reagents (TaKaRa, Shiga, Japan) as well as the chicken-specific ELISA assay kits for total antioxidant capacity (T-AOC), malondialdehyde (MDA), superoxidase dismutase (SOD), glutathione (GSH), and secretory immunoglobulin A (sIgA) (Nanjing Jiancheng Bioengineering Institute, Jiangsu, P. R. China) were purchased from companies.

**Experimental Animals**

The animal experiment was performed at the Poultry Farm of Sichuan Agricultural University. All procedures were conducted in accordance with the national standard Laboratory Animal-Requirements of Environment and Housing Facilities (GB 14925-2001). Animal handling and care were approved by the Sichuan Agricultural University’s Institutional Animal Care and Use Committee under permit number DYY-2018203007.

A total of four hundred eighty 1-day-old male Arbor Acers (AA) broilers with initial body weight of 38.83 ± 3.45 g were used in this study (Poultry Breeding Farm of Sichuan Agricultural University). Broilers were randomly assigned to environmentally controlled chambers (4,500 \( \times \) 3,000 \( \times \) 2,500 mm; length \( \times \) width \( \times \) height) of 30 broilers per chamber (Wei et al., 2012).

The airflow, photoperiod, temperature, and relative humidity were basically maintained at the same level according to poultry management guidelines (Zheng et al., 2018). Gas concentration in the chicken chambers was continuously monitored using LumaSense Photoacoustic Field Gas-Monitor Innova-1412 (Santa Clara, CA). Water and regular diets were provided based on the standard brooding practices throughout the whole period. The formulation and nutrient levels of the regular diets were based on the National Research Council requirements for chicken (Supplementary Table 1) (Pesti, 1995).

**Experimental Design**

The 16 chambers were randomly assigned into 4 groups, and 120 broilers per group with 4 replicates (a chamber was a replicate, and 30 broilers per replicate): 1) negative control group (CON; birds fed regular diets, the concentration of \( \text{NH}_3 < 5 \text{ ppm} \)); 2) SP control group (CSP; birds fed the regular diets mixed with 80 mg/kg SP, the concentration of \( \text{NH}_3 < 5 \text{ ppm} \)); 3) \text{NH}_3\)-exposed group (NH; birds fed the regular diets and exposed to 70 ± 5 ppm \( \text{NH}_3 \)); and 4) \text{NH}_3\)-exposed group + SP (NSP; birds fed the regular diets mixed with 80 mg/kg SP and exposed to 70 ± 5 ppm \( \text{NH}_3 \)). After acclimation for day 1, the treatment was applied from day 2 to 8. The concentration of \( \text{NH}_3 \) was selected based on previous...
studies, which caused severe intestinal damage (Wei et al., 2012; Zhang et al., 2015; Tao et al., 2019). The dose of SP was chosen based on our previous experiment (Bai et al., 2020). Daily weight gain (DWG), daily feed intake (DFI), and final body weight were measured and recorded from all the experimental birds and were ultimately used to calculate the feed conversion ratio (FCR) as DWG/DFI. These growth performance parameters were adjusted by mortality.

**Sample Collection and Ileal Cell Separation**

On day 8, 12 chickens per group close to the average body weight were euthanized by injecting pentobarbital sodium intravenously at a dose of 100 mg/kg within 2 min from the time removed from its chamber. Thereafter, each segment of the small intestine was immediately weighed after removing the content, and the relative weight of the intestine was expressed as the ratio of the intestinal weight to body weight (mg/g). Then, the tissue samples at the midpoint of each segment of the small intestine (2 cm of the duodenum, jejunum, and ileum) were dissected and fixed in 4% (wt/vol) buffered paraformaldehyde for histological observation, and the remaining ileum tissue samples were collected for further analyses. The mucosa layer of the ileum tenue was scraped as directed by Luo et al. (2013), whereas the ilea cells were harvested following the method described by Lin et al. (Lin et al., 2019). In brief, the ileum mucosa was cut, centrifuged, and suspended to 1 × 10^8 cell/mL in 4°C PBS solution which was used for flow cytometry analysis (Lin et al., 2019).

**Relative Organ Index, Antioxidant Indices, and Immunoglobulin**

In brief, 1 g of ileum mucosa was homogenized with 0.9% saline at 4°C and centrifuged; then, the supernatant was collected for subsequent test. Specific ELISA kits were used to measure the contents of T-AOC, SOD, MDA, GSH, and sIgA in the ileum tissues according to the manufacturer’s instructions.

**Histopathological Observation and Morphometric Parameters of the Intestine**

Histological analysis was performed adhering to the guidelines described in the previous study (Uni et al., 2001). Trimmed samples were dehydrated, cleared, and then embedded in paraffin. The samples were sectioned into 5 µm slices using a RM2235 microtome (Leica, Wetzlar, Germany), flattened onto glass slides, and then dried. After dewaxing with xylene, the sections were stained with hematoxylin and eosin (Thermo, Waltham, MA) and then sealed with neutral resin. Five sections of each of the chicken’s intestinal specimen (duodenum, jejunum, and ileum) were taken, and pictures (400 × ) taken on them (5 each) were examined under a CX22 microscope (Olympus, Tokyo, Japan) with DM1000 micro-imaging system (Leica) randomly. The villus height as well as crypt depth were determined by Image Pro Plus 6.0. The villus/crypt ratio was calculated according to villus height/crypt depth.

**Alcian Blue/Periodic Acid-Schiff Stain for Goblet Cells**

Dewaxed sections were stained with 1% Alcian blue and Schiff’s reagent. After the samples were dehydrated and cleared, then they were sealed with neutral resin, and photographed under light microscope. The mucus secretion was calculated by mean optical density value, and the number of goblet cells was expressed as the total number per µm^2 (Wang et al., 2018).

**Transmission Electron Microscope Observation**

Transmission electron microscopy analysis was performed according to the procedure described in the literature (Wang et al., 2018). The ileum pieces were carved into small pieces and immediately fixed in 2.5% glutaraldehyde (pH = 7.4, 4°C), and OsO4 was used for postfixation. After dehydrating, the samples were soaked in epoxy resin acetone solution and finally embedded in epoxy resin. Microtome was used to slice the trimmed tissue blocks into 80 nm slices. The sections were double stained with lead citrate and uranyl acetate solution. Ultrastructural architectures of the ileum were observed by HT7700 transmission electron microscopy (Hitachi, Tokyo, Japan) and photographed with GANTAN830.10 W CCD camera (Hitachi).

**Reactive Oxygen Species of Ileum by Flow Cytometry**

The prepared ileal cells were incubated for 15 min at 37°C with DCFH-DA (total ROS assay kit) in the dark. The ROS (%) generation in ileum was determined using a flow cytometer.

**Mitochondrial Membrane Potential, Apoptosis, and T lymphocyte Cell Subsets of Ileum by Flow Cytometry**

The prepared ileal cells were incubated for 15 min at 37°C with JC-1 in the dark, and the MMP was measured using flow cytometry analysis. The result of MMP was described as mitochondrial depolarization ratio. In addition, the cells were incubated for 15 min at 37°C with 5 µL Annexin V-FITC and 5 µL proliferation index (PI). Another sample was subsequently incubated for 20 min at 22°C with Mouse Anti-Chicken CD3-SPRD, Mouse Anti-Chicken CD4-PE/CY7, and Mouse Anti-Chicken CD8α-PE in dark. Furthermore, the percentage of apoptosis and T lymphocyte cell subsets were measured using flow cytometry (Bio-Rad), and all the data collected were analyzed by Kaluza 2.1 software.
Cell Life Cycle of Ileum by Flow Cytometry

The separated ileal cells were exposed for 30 min at 4°C with PI (5 mL/L propidium iodide, 0.5% Triton-X100, RNase) in the dark, and the life cycle was immediately measured by flow cytometry. The percentage of cells at the rest phase (G0/G1 phase), DNA synthesis phase (S phase), and division phase (G2 + M phase) were analyzed by Kaluza 2.1 software. The PI was calculated as (S + G2M)/(G0/1 + S + G2M).

Fluorescence Real-Time Quantification PCR

The samples of ileum mucosa were washed with prefrozen diethyl pyrocarbonate water and immediately frozen in liquid nitrogen and stored at −80°C. Approximately 60 mg of the preserved sample was ground thoroughly with liquid nitrogen in a precooled mortar. Total RNA was extracted with TRIzol (Invitrogen). RNA concentration was determined by a nucleic acid protein analyzer, of which the D260/D280 range eligible for reverse transcription was 1.8–2.0. The cDNA was stored at −80°C.

According to the specific steps of SYBR Green Remix Ex Taq kit specification of TaKaRa and with GAPDH as the internal reference, QRT-PCR was used to detect the expression levels of the following genes: Claudin1, Zo1, Muc2, Nrf2, HO-1, Caspase 3, Caspase 9, Bax, Bcl-2, VDAC, TNF-α, and IFN-γ. The 2^−ΔΔCT method was used to calculate the relative expression levels of these genes. All primers (Supplementary Table 2) were designed using Premier 5 (PREMIER Biosoft International, Palo Alto, CA) and synthesized by Chengke BioTech Co., Ltd.

Statistical Analysis

One-way analysis of variance was performed using statistical software SPSS 19.0 (SPSS Inc., Chicago, IL). Duncan’s multiple range test was used to determine the differences among treatment groups. All parameters determined in this study were presented as mean ± SE. Significance was determined at a level of P < 0.05.

RESULTS

Effect of SP on Growth Performance of NH3-Exposed Broilers

In general, the negative effect of NH3 on growth performance indices was reversed by SP during the experimental period (Figure 1). The results showed that the NH groups had the lowest DWG (P < 0.05). Compared with the CON, the NH group recorded lower DFI and final body weight (P < 0.05) as well as higher FCR (P < 0.05).

Effect of SP on Intestinal Development of NH3-Exposed Broilers

The treatment-related morphometric variables are presented in Figure 2. The dietary supplementation of SP had an advanced effect on both the relative organ index and morphometric parameters of intestine (the duodenum, jejunum, and ileum) in broilers. Compared with the CON group, exposure to high concentration of NH3 (the NH group) significantly reduced the villus height in the jejunum and ileum as well as the ratio of villus/
crypt in the duodenum and ileum \( (P < 0.05) \), whereas these parameters in the NSP group suggested SP normalized the alterations of the intestinal morphology. Moreover, the relative organ index of the duodenum and jejunum in CSP group was higher than that in CON and in NH group \( (P < 0.05) \).

Histopathological examinations (Figure 3A) exhibited a normal structure in the ileum of the CON group with complete villi, densely arrayed columnar cells as well as obvious crypt. The CSP (Figure 3B) and NSP group (Figure 3D) also showed a normal structure, similar to the CON group. Nevertheless, in NH group

**Figure 2.** The data of relative organ index (\%), villus height (\( \mu \)m), crypt depth (\( \mu \)m), and villus:crypt ratio of duodenum (A–D), jejunum (E–H), and ileum (I–L) in different groups. \( P < 0.05 \) was considered statistically significant. **\( \times \times \)P < 0.05 vs. control group; **\( \# \# \)P < 0.05 vs. NH\(_3\)-treated group.

Abbreviations: CON, negative control group; CSP, SP control group; NH, NH\(_3\)-exposed group; NSP, NH\(_3\)-exposed group + SP; PI, proliferation index.

**Figure 3.** *Bupleurum falcatum* L saikosaponins protect the epithelial cells of ileum in NH\(_3\)-exposed broilers. Histopathological observation of ileum in different experimental groups. (A) CON group, (B) CSP group, (C) NH group, (D) SP group. H.E. stain, scale bar = 100 \( \mu \)m. Abbreviations: CON, negative control group; CSP, SP control group; NH, NH\(_3\)-exposed group; NSP, NH\(_3\)-exposed group + SP.
(Figure 3C), the epithelia of villi in the ileum were slightly shedding, and the lamina propria became loose because of edema, resulting in the widened gap between the intestinal epithelium and the lamina propria.

The ileum ultrastructure is presented in Figure 4. The ultrastructure of the ileum in the CON group (Figure 4A) appeared normal. They displayed closely packed microvilli of the mucosal epithelial cells and abundant organelles with normal ultrastructure located in the cytoplasm, which seems to be an enlarged endoplasmic reticulum. The CSP group (Figure 4B) also showed a normal structure, similar to that of the CON group. In the ileum of NH group, the round vesicles could easily be observed in the cytoplasm of the mucous epithelia (Figures 4C and 4D). The mitochondria in the cytoplasm became swollen and vacuolated with degenerated cristae and membranes, and some of high electron density inclusions can be found within the mitochondria (Figure 4E), leading to greater amount of electron density (Figures 4D and 4E). At the same time, the vesicles of Golgi expanded, thereby resulting in increased vacuoles with very low electron density (Figure 4F). This ultrastructural damage was alleviated in the NSP group compared with those in the NH group. However, some broken and shed microvilli, swollen mitochondria as well as secondary lysosomes were still observed in the NSP group (Figure 4B).

Figure 5 summarized the changes of the ileal cell life cycle of the experimental birds. The amount of ileal cells in the S phase of NH group was the lowest ($P < 0.05$), concomitant with the reduction of the PI index ($P < 0.05$). However, SP promoted ileal cells into both the S and G2M phase in the NH3-exposed chickens, which gave a clear indication of an effective ileum protective characteristic of SP (Supplementary Figure 1).

Figure 4. Bupleurum falcatum L saikosaponins protected mitochondria in NH3-exposed broilers. The ultrastructural changes of the ileum in different groups: (A) CON group, (B) NSP group, (C) NH group, scale bar = 10 μm; (D) the enlargement of the red frame area in the image of C, (E–F) NH group, scale bar = 2 μm. The arrow in red pointed to the mitochondrial degeneration, the triangles in red marked the vesicle, and the circle in red pointed to the expansion of Golgi vesicles. Abbreviations: CON, negative control group; NH, NH3-exposed group; NSP, NH3-exposed group + SP.
**SP Protects the Epithelial Cells of Ileum of NH₃-Exposed Broilers**

Regarding the total number of globule cell (GC) (Figure 6A), results revealed that the NH group recorded the lowest count of GC in the ileum compared with other groups ($P < 0.05$). The reduction in the GC density indicated a decreased mucus secretion. The mucus secretion record (Figure 6B) in the NH group was significantly lower than that in the CON and CSP group ($P < 0.05$).

**SP Modulates the Ileal Immune of NH₃-Exposed Broilers**

After NH₃ exposure, the generation of sIgA and the content of T lymphocyte cell subsets in the broiler ileum are presented in Figure 6C, respectively. The NH₃ significantly decreased the sIgA values in birds ($P < 0.05$). The sIgA value in the NSP group was higher than that of the NH group ($P < 0.05$); however, it was still lower than that of the CON group ($P < 0.05$).

As shown in Figure 6, the NH group recorded the lowest CD₃⁺, CD₃⁺CD₄⁺, and CD₃⁺CD₈⁺ counts in ileum compared with other groups ($P < 0.05$). Compared with the NH group, SP exhibited greater ileum immunomodulation ability via elevated levels of T lymphocyte cell subsets (NSP group), whereas the CD₃⁺ and CD₃⁺CD₄⁺ content in the NSP group were still lower than those in the CON groups ($P < 0.05$). The flow cytometry quadrant diagrams were showed in Supplementary Figure 2.

**SP Regulates Activities of Antioxidant Enzymes/Indices in the Ileum of NH₃-Exposed Broilers**

Figure 7 showed how SP reverted the negative effects of NH₃ on the ileal antioxidant-related biochemistry. Levels of SOD, T-AOC, and GSH were reduced in the NH group compared with other groups ($P < 0.05$). Moreover, these levels in the NSP group had a remission in comparison to the NH group ($P < 0.05$). Furthermore, all the experimental groups recorded no significant differences for MDA levels, but the NH group recorded the highest level ($P > 0.05$).

Furthermore, ROS level in the ileum of the chickens was quantified to evaluate oxidative stress. As shown in Figure 7E, the ROS level in the NH group was significantly higher than that in all other groups ($P < 0.05$). Conversely, SP had a positive effect to resist oxidative stress in NH₃-exposed chickens, because NSP group recorded the lower ROS level compared with the NH group ($P < 0.05$). The flow cytometry quadrant diagrams were showed in Supplementary Figure 3.

**SP Reduces Ileal Apoptosis and Protect Mitochondria in NH₃-Exposed Broilers**

Figures 7F and 7G showed SP supplementation reduced ileum profound apoptosis which was stimulated by NH₃ in chickens. The highest percentage of apoptosis was found in the NH group compared with other groups ($P < 0.05$). Moreover, NH₃ caused an
excessively higher ratio of mitochondrial depolarization in the ileum than all other groups \((P < 0.05)\), whereas compared with the NH group, SP normalized mitochondrial membrane potential and resulted in a reduction of mitochondrial depolarization ratio in the NH3-exposed chickens (the NSP group). The flow cytometry quadrant diagrams were showed in Supplementary Figure 4.

Figure 6. *Bupleurum falcatum* L saikosaponins protected the epithelial cells of ileum as well as modulated the ileal immune in NH3-exposed broilers. The data of global cell (A) and Mucus secretion content (B) of ileum in different groups. Note: mm² was the unit area of one field under 400 × magnification, OD represented the mean optical density value. The secretory immunoglobulin A content (sIgA, g/g) of ileum in different groups (C). The T lymphocyte cell subsets (D) CD³⁺ T lymphocytes, (E) CD³⁺ CD⁴⁺ peripheral T lymphocytes, (F) CD³⁺ CD⁸⁺ peripheral T lymphocytes; (%) of ileum in different groups. \(P < 0.05\) was considered statistically significant. **\(P < 0.05\) vs. control group; ##\(P < 0.05\) vs. NH3-treated group. Abbreviations: CON, negative control group; CSP, SP control group; NH, NH3-exposed group; NSP, NH3-exposed group + SP.

Figure 7. *Bupleurum falcatum* L saikosaponins balanced ileal antioxidant system against oxidative stress, reduced the ileal apoptosis and protected mitochondria in NH3-exposed broilers. (A) MDA (malondialdehyde, mmol/L), (B) SOD (superoxide dismutase, U/mL), (C) T-AOC (total antioxidant capacity, U/mL), (D) GSH (glutathione, μmol/g), (E) Reactive oxygen species (ROS, %) generations, (F) Apoptosis, (G) Mitochondrial depolarization ratio levels of ileum in different groups. \(P < 0.05\) was considered statistically significant. **\(P < 0.05\) vs. control group; ##\(P < 0.05\) vs. NH3-treated group. Abbreviations: CON, negative control group; CSP, SP control group; NH, NH3-exposed group; NSP, NH3-exposed group + SP.
**SP Inhibits NH₃-Induced Ileal Damage by Regulating mRNA Expressions Associated With the Functions of Mucosal Barrier, Immunity, Oxidative Stress, and Apoptosis in Broilers**

Figure 8 illustrated that NH₃ impaired mucosal barrier of ileum via downregulation of tight junction proteins (Claudin1, Zo1) and the primary secreting mucoprotein (Muc2) genes in comparison to the CON and CSP groups (P < 0.05). The SP only significantly affected Zo1 upregulation in birds after NH₃ exposure in comparison to the NH group (P < 0.05). Moreover, Figure 8 summarized the changes in the expression of ileal immune-related genes. The mRNA level of TNF-α in NH group was the highest among all the experimental groups (P < 0.05), while it was mildly upregulated in the NSP group compared with CON group (P < 0.05). The NH group recorded lower expression of IFN-γ than CSP group (P < 0.05), comparatively. In addition, the mRNA levels of the oxidative stress-related gene Nrf2 and HO-1 were significantly reduced in the ileal of chickens in the NH group compared with those in all other groups (P < 0.05), but SP effectively increased the expressions of both Nrf2 and HO-1 to the same level as the CON group. Figure 8 showed the influence of NH₃ inhalation on apoptotic-related genes. In the NH group, the mRNA levels of caspase 3, caspase 9, and Bax were higher (P < 0.05), whereas the Bcl-2 expression in the same group (NH group) was lower (P < 0.05) in the ileum than all other treatments. Meanwhile, the expression of VDAC in NH group was the highest compared with CON and CSP groups (P < 0.05).

**DISCUSSION**

Ammonia is a highly toxic pollutant which can influence different organs and physiological functions in animals including chickens. The poultry management guidelines have set the limit of 20 ppm ammonia concentration in chicken house to protect their health and welfare (Zhang et al., 2015). Numerous evidence has demonstrated that the concentrations of NH₃ beyond the prescribed limit may impair the gastrointestinal tract by pathways of apoptosis and mitochondrial damage, causing health problems to animals (Tsujii et al., 1992; Igarashi et al., 2001). The molecular mechanisms underlying the damages caused by exogenous NH₃ on broilers’ ileum remain unclear. In the current study, we examined the growth parameters, intestinal morphology and gene expression, and ileum injury in broilers exposed to excess concentration of NH₃ with or without SP supplementation. Results in this study showed that exposure to high concentrations of NH₃ can induce growth inhibition with multiple ileum injuries in broilers. In addition, our study, for the first time, confirmed that SP can protect the ileum against NH₃-induced lesion in the ileum of broilers through several pathways: (1) improving intestinal development; (2) protecting ileal mucosal barrier via immunity promotion; (3) antagonizing oxidative stress; (4) suppressing apoptosis. The
SP have therapeutic potential against NH3-associated damage.

Studies have reported that high concentrations of NH3 directly resulted in poor growth performance, low DWG and high mortality rate in poultry (Miles et al., 2004; Soliman et al., 2017). Similarly, NH3 showed inhibitory effect on growth with lower DWG and higher FCR in our study. Moreover, supplementation of SP regained the growth performance of chickens exposed to NH3 through enhancing DWG and reducing FCR. With regard to the pharmacological effects of SP, we hypothesized that this phenomenon may be attributed to its improvement in immune functions and inhibition of emotional disorder caused by NH3-stress (Li et al., 2018); however, further studies are recommended to prove this hypothesis.

In addition to the reduction of growth performance, exposure to excess concentrations of NH3 also interfered with intestinal health of broilers. The intestinal layers, including the lining epithelium, lamina propria with gland, and lamina muscularis, maximize nutrients digestion and absorption by providing a large mucosal surface which has been studded with the intestinal villi (Hernández et al., 2006). An increase in the intestinal villus resulted in a great mucosa surface area, and the migration of proliferating crypt cells to the villi ensures the rapid renewal of the villus (Awad et al., 2009). Therefore, the height of the intestinal villus and the ratio of villus/crypt are positively related to the absorptive efficiency of the small intestine in chickens, while the crypt depth is negatively related (Geyra et al., 2001). Our study indicated that exposure to high concentrations of NH3 exerted negative impacts on intestinal mucosal structure and development in broilers by decreasing the indices, villus height, and villus/crypt ratio of different intestinal segments, which may inhibit nutrients absorption. Interestingly, this observation is in line with previous studies (Uni et al., 2001; Zhang et al., 2015). Moreover, histopathology analysis demonstrated that NH3 could cause shedding of the epithelia of villi in the ileum of broilers. Improvement in those morphometric variables in the NSP group suggested that SP maintained the integrity of intestinal morphology and structure to improve the intestinal development. In addition, our study also showed that NH3 blocked the entry of ileal cells into the S and G2/M phases, resulting in the remarkable reduction of the PI index. The loss of cell-cycle control in the NH3 treatment represented a hallmark of severe lesion in the ileum. Comparably, the results in the NSP group indicated that SP promoted the proliferation activity of ileal cells to improve the development of ileum in the NH3-exposed broilers.

To further identify the mechanism by which NH3 impaired the ileum, we examined the number of goblet cells and mucus secretion. The density of GC and relative functions in mucus synthesis and secretion are direct indicators reflecting the health status of intestinal epithelial cells of the animal, which can exert the protective activities of the mucous barrier (Taupin and Podolsky, 2003). NH3 reduced the total GC count as well as the mucus secretion in the ileum, by which it leads to mucous barrier dysfunction. Moreover, supplementation of SP enhanced both total GC count and mucus production as observed in our study. As yet, the underlying effect of NH3 on the differentiation of the GC has not been clearly illustrated in broilers. The GC differentiation is associated with the mucin gene expression (Baurhoo et al., 2007) and the intestinal immunity (McDole et al., 2012). Therefore, we speculated that falling of the ileal GC count in NH group might relate to the repressed immunity in ileum. Furthermore, relative mRNA expression of ileal mucosal barrier-related genes supported this speculation, since NH3 exposure downregulated the expression of genes which encoded tight junction proteins (Claudin1 and Zo1) and the primary secreting muco-protein (Muc2) in ileum. Previous studies suggested that reduced gene expression of tight junction proteins and Muc2 was usually associated with pathological conditions of the mucous immunity (Lee et al., 2017; Palamidi and Mountzouris, 2018). The noted increased expression of those (ileal mucosal barrier-related) genes in the NSP group could be regarded as beneficial recovery of the mucous barrier.

Generally, the intestine is the largest immune organ that continuously defends against pathogenic invasion when exposed to an array of challenges (Buchan, 1999). Hence, a damage on the potential of intestinal immunity prompts the pathological status. Zhang et al. had reported that high concentration of NH3 impaired the adaptive immunity of the small intestine in chickens by downregulating 8 differential protein species related to immune response in chickens (Zhang et al., 2015). Similarly, our results demonstrated that NH3 decreased the content of slgA, T cell subsets (CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺), and affected the expression of cytokine profile (TNF-α and IFN-γ) in the ileum of broilers. Importantly, the status of intestinal immunity is associated with the levels of slgA, the main immunoglobulin prohibits the entry of pathogen into subepithelial areas (Dasgupta et al., 2014), the capability of mucous T cell proliferation in response to exogenous antigens (Grewal and Flavell, 1998) as well as cytokine profile (Lee et al., 2013). In this study, the changes observed in regard to these parameters in the NSP group suggested that SP attenuated NH3-induced ileal immune dysfunction to protect ileal mucous barrier in NH3-exposed broilers, which probably inhibits or prevents inflammation and infection, leading to a greater growth rate.

Emerging evidence demonstrated that the oxidative stress is one of the extreme important factors by which the immune dysfunction is easily induced. In broilers jejunum, H2S, a harmful gas, induces inflammatory reaction via redox homeostasis disorder (Zheng et al., 2018). In mice, oxidative stress induces immune dysfunction by upregulating the expression of proinflammatory cytokines (TNF-α, IFN-γ and the like) (Tewari et al., 2015). Based on the above studies, we revealed the immune dysfunction in the ileum caused by NH3 might
link to the oxidative stress. Oxidation as well as reduction is a procedure for energy production, which is common to many fundamental responses of organisms. Oxidative stress disrupts the redox balance (reduction-oxidation) with high levels of ROS generation (Noctor et al., 2016). In our study, increased levels of ileal ROS and MDA along with decreased activity of ileal antioxidiant compounds (SOD, T-AOC, and GSH) were observed in the NH group, which revealed that oxidative stress has been induced in NH3-exposed broilers (Zhang et al., 2015). NH3-induced oxidative stress was also revealed by the downregulation of the Nrf2/HO-1 pathway. Nrf2/HO-1 pathway plays a crucial function in stress-protection, which exerts beneficial effects through inhibiting oxidative injury, regulating apoptosis as well as modulating inflammation (Loboda et al., 2016). Specifically, the results obtained in this study showed a marked decrease in the expression of Nrf2 and HO-1 genes following NH3 treatment, suggesting that NH3 could break down the redox homeostasis and cause oxidative stress in the broiler ileum. Moreover, SP in broilers can restore ileal antioxidative status by directly scavenging ROS and attenuating NH3-induced disturbances in cellular redox state.

Apoptosis is another adverse effect induced by oxidative stress in organisms, whereas excessive ROS promotes cell degradation and death (Zhu et al., 2013). Igarashi et al. reported that excess concentration of NH3 initiates apoptosis by oxidative stress pathway (Igarashi et al., 2001). Our study corroborated with the previous study, suggesting that NH3 exposure significantly elevated the incidence of apoptosis via redox homeostasis disorder in the ileum of broilers. Mitochondrion not only functioned in producing ATP but also involved in controlling cell death processes, including apoptosis (Borutaite, 2010). Once the free radicals attack mitochondrion, the mitochondrial transmembrane potential collapses, which elevates the ratio of mitochondrial depolarization, a sensitive index used in evaluating mitochondrial function. Loss of transmembrane potential in cells is considered as a major determinant of apoptosis (Gao et al., 2012; Sinha et al., 2013). Therefore, the microstructural and functional integrity of the mitochondria is important in resisting apoptosis and maintaining the normal function of cells. This study revealed that NH3-induced cytotoxicity damaged the integrity of the mitochondria and further increased mitochondrial depolarization ratio, which resulted in an increased apoptosis. In addition, the expression of mitochondrial porin VDAC, which is in positive correlation with the mitochondria-mediated apoptosis, was upregulated in the NH3 group. The outcome further indicated that NH3 aroused apoptosis in the ileum by damaging mitochondrion. Again, this study revealed that SP exerted protective effect against NH3-induced ileal injury and reduced incidence of apoptosis by the inhibition of oxidative stress and protection of mitochondrion.

The mitochondrial dysfunction leading to disorders of energy generation might be an explanation of the poor growth performance observed in NH3-exposed broilers (Zhang et al., 2015). The mRNA expressions of the 4 apoptosis-related biomarkers (caspase 3, caspase 9, Bax and Bcl-2) were examined. Caspase family is essentially involved in cell apoptosis, such that caspase 3 and caspase 9 are the 2 downstream effectors involved in death-receptor pathway of apoptosis, and their activation commits the cells to apoptosis (Zhu et al., 2012). Bcl-2 is an antiapoptotic protein, whereas Bax protein plays a proapoptotic effect; they together participate in the control of apoptosis (Gobé et al., 2002). Upregulation of caspase 3, caspase 9, and Bax along with downregulation of Bcl-2 in the NH3 treatment group was correlated with the high incidence of apoptosis in the ileum. Integrating with our previous results, it showed that NH3 could trigger ileum apoptosis from variety of pathways. Therefore, antiapoptotic therapy may be beneficial for the NH3-associated damage. Our results also demonstrated that SP blocked NH3-induced apoptosis by regulating caspase 3, caspase 9, Bax, and Bcl-2 to normal physiological levels. However, further studies are required to elucidate specific signal pathways, especially the inflammation pathway.

CONCLUSION

In this study, reduced growth rate was observed in NH3-exposed broilers, and it may be relevant with the intestinal health status. The SP showed a positive effect on anti-NH3 damage via alleviating growth inhibition and intestinal lesions. Collectively, the current study indicated that exposure to high concentrations of ambient NH3 causes poor development of the small intestine and ileum injury in broilers. These impacts might be resulted from the NH3-induced oxidative stress along with the immune dysfunction and apoptosis because the mRNA levels of genes related to mucosal barrier, immune, oxidative stress, and apoptosis were upregulated in NH3 exposed broilers. In addition, this study identified the potential mechanisms underlying the SP protective functions in the NH3-exposed broilers. The SP effectively promote intestinal development, modulate the immunity of the ileum, and potentially block the apoptosis by exerting antioxidant and mitochondrial protective effects. This study provides new information about pathways of NH3 impacting broiler health and reveals that SP could be a potential feed additive to defense against NH3 damage in chickens.

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DISCLOSURES

There is no competing interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.10.057.

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