Immunopotentiators improve the antioxidant defense, apoptosis, and immune response in Shaoxing ducklings

Tiantian Gu [ORCID],* Lizhi Lu,* Wenwu Xu,* Tao Zeng,* Yong Tian,* Bindan Chen,[†] Li Chen,* Junda Shen,* and Guoqin Li[1],*

*Institute of Animal Husbandry and Veterinary Medicine, Zhejiang Academy of Agricultural Sciences, Hangzhou, Zhejiang 310021, China; and [†]Zhejiang Guowei Technology Co., Ltd, Zhuji, Zhejiang 311800, China

ABSTRACT The abuse of antibiotics for agricultural purposes has been under scrutiny. Therefore, there is an urgent need to find antibiotic substitutes in animal production. The effects of chlorogenic acid, β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG on spleen antioxidant capacity, apoptosis, and the immune response in Shaoxing ducklings were investigated in this study. The ducklings treated with β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG showed significant reduction in catalase and superoxide dismutase activities. The five immunopotentiators facilitated caspase 3 expression and reduced Bcl2 expression in the spleen. Compared to the control group, the protein level of COX2 was significantly upregulated in the chlorogenic acid, CpG-DNA, and chicken IgG groups. The protein level of iNOS expression was significantly improved in all immunopotentiator groups, except for the astragalus flavone group. The five immunopotentiators induced *IL-1β, IFN-α, IFN-β, TNF-α, RIG-I, TLR3*, and *TLR7* gene expression. In summary, chlorogenic acid, β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG, as immunopotentiators, improved the innate immune response in the ducklings, which not only provides a new avenue for the development of efficient approaches to prevent pathogen infections, but also offers an alternative to antibiotics in animal production.

Key words: immunopotentiators, antioxidant activity, apoptosis, immune-related gene expression, Shaoxing ducklings

INTRODUCTION

Duck, as one of the main poultry foods for human consumption, has become increasingly popular in China (Zeng et al., 2016; Bai et al., 2020). However, outbreaks of endemic diseases have always been a major limiting factor in duck breeding. Some research have demonstrated that antibiotics can enhance the resistance to microbial invasion with an effective treatment mode, while its abuse in animal food causes the direct selection of antibiotic-resistant microbes and turns the animal food systems into reservoirs of antibiotic resistance genes (Barton, 2000; Davies and Davies, 2010), which not only leads to massive economic losses in the animal industry but also causes dangerous issues for human public health. Reducing antibiotic intake and eliminating antibiotic residues in animal food has become a priority in food safety and public health. Thus, it is important to find alternative antibiotics in animal production.

To minimize these problems and secure animal health, modulating immune function is discussed as a potential means to decrease disease susceptibility in farm animals (Berends et al., 2001). Immunopotentiators, as potential immune modulators, may increase resistance to infectious diseases by enhancing specific immune responses and enhancing nonspecific defense mechanisms (Sakai, 1999; Masihi, 2000).

Adding immunopotentiators is an effective way to increase immunocompetency and disease resistance in animal production. Immunopotentiators from various natural sources can modulate the immune system (Abou-Elkhair et al., 2020). Immunopotentiators have been widely studied in mice (Jie et al., 2020), fish (Gantner et al., 2003; Herre et al., 2004), and pigs (Hiss and Sauerwein, 2003). Cook et al. (2001) has reported that β-glucan could significantly improve the efficiency of macrophages against pathogens by recognizing the cell wall of Gram-negative

fish pathogenic bacteria. Hiss and Sauerwein (2003) showed that pig treated with β-glucan could enhance the pattern recognition receptor (**PRR**) titers to enhance the immunity of weaned piglets. Phytoremedies seem to be a significant source of immunomodulatory agents. Ayurveda documents the role of "Rasayana" as a "system of rejuvenation" (Ali, 1998).

Hence, the availability of commercial immunopotentiators is necessary for field application in animals. Moreover, there are very few studies on the effects of immunopotentiators in ducks. Our previous studies demonstrated that chlorogenic acid, β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG could enhance the immune effects of duck embryonic fibroblast cells (**DEFs**) in vitro (Gu et al., 2020). In the present study, the effects of these 5 immunopotentiators were evaluated in vivo by determining spleen tissue antioxidant activity, apoptosis, proinflammatory cytokines, and immune-related gene expression. The purpose of this study was to validate the immunoenhancement of chlorogenic acid, β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG, which could emphasize the tremendous potential of immunopotentiators as antibiotic substitutes in ducks as well as in other animals.

MATERIALS AND METHODS

Ethics Approval

The animals used in this study were raised and slaughtered in accordance with the National Standard of Laboratory Animal Guidelines for Ethical Review of Animal Welfare (GB/T 35892-2018), issued by the General Administration of Quality Supervision, Inspection, and Quarantine of the People's Republic of China and Standardization Administration of the People's Republic of China. All experimental procedures were approved by the Institute of Animal Husbandry and Veterinary Science, Zhejiang Academy of Agricultural Sciences (Hangzhou, China). We also confirm that all efforts were made to minimize suffering.

Reagents and Ducklings

One hundred and fifty 1-day-old healthy Shaoxing ducklings were obtained from Guiliu Breeding Company (Zhoukou, Henan, China). Saline was purchased from Kelun Pharmaceutical Co., Ltd. (Henan, China), chlorogenic acid, β-D-Glucan, and astragalus flavone were purchased from Yuanye Bio-Technology Co., Ltd (Shanghai, China), CpG-DNA (5′-GATATGCGACC-GATT-3′) was obtained from TSINGKE Biological Technology (Beijing, China), and chicken IgG was purchased from Solarbio (Beijing, China). All reagents were dissolved in sterile water, the stock solutions were kept at −20°C and protected from light until use. The selected ducklings were immediately anesthetized with sodium pentobarbital (intraperitoneal injection, 100 mg/kg) and killed by exsanguination.

Treatment Protocol

Based on our previous in vitro screening study on 22 kinds of immunopotentiators to determine the positive immune regulatory effects of immunopotentiators (Gu, et al., 2020), chlorogenic acid, β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG were chosen for further in vivo study. Shaoxing ducklings in 1-day-old were divided into 6 groups and subcutaneously injected with 0.5 mL saline, 0.4 mL chlorogenic acid (1 mg/mL), 0.7 mL β-D-Glucan (1 mg/mL), 0.4 mL astragalus flavone (5 mg/mL), 0.5 mL CpG-DNA (5 μg/mL), and 0.5 mL chicken IgG (1 mg/mL), respectively, once daily for 3 d. Fifteen days after the last immunopotentiator injection, the ducklings (5 ducklings per group) were injected with pentobarbital sodium (100 mg/kg). Spleen tissues were quickly separated on an ice platform, and the large spleen leaves were placed in 4% formalin fixative for in situ hybridization analysis. The remaining spleen tissues were frozen at −80°C for antioxidant indicators, protein, and gene expression analysis.

Assay for Spleen Superoxide Dismutase, Catalase, and MDA Levels

Spleen samples of 5 Shaoxing ducklings per group were obtained for the determination of antioxidant enzyme activities. Spleen superoxide dismutase (**SOD**) (U/mL), catalase (**CAT**) (U/mL), and MDA (nmol/ mL) levels were assayed using a superoxide dismutase assay kit (HY-60001), catalase assay kit (HY-60015), and malondialdehyde assay kit (HY-60003), respectively, in accordance with the manufacturer's protocols.

In Situ Hybridization

Sense and antisense digoxigenin-labeled *IL-1β, IFN-α*, and *IFN-β* gene-specific oligonucleotides were prepared through 3′ end-labeling reactions with kits (Exiqon, Woburn, MA). In situ hybridization tissue slides were incubated at 37°C for 2 min, fixed in 4% paraformaldehyde on ice for 15 min, and then washed twice for 5 min in 1 × phosphate-buffered saline (**PBS**). The slides were treated for 10 min with 3% H_2O_2 to quench the endogenous peroxidase. The slides were pre-hybridized for 2 h at room temperature, and IL-1β, IFN-α, and IFN-β hybridization was carried out at 4°C overnight. Subsequently, the slides were washed with 5 × standard saline citrate (**SSC**) for 10 min and 0.2 × SSC for 1 h, respectively, and the slides were incubated in a blocking solution for 1 h at room temperature and then incubated with alkaline phosphatase (**AP**)-conjugated antibody to digoxigenin (Roche Diagnostics, Indianapolis, IN) at 4°C overnight. The next day, the slides were brought to room temperature, washed twice in PBS, washed twice with AP buffer, and the peroxidase conjugates were subsequently localized using the substrate diaminobenzidine tetrahydrochloride and counterstained with Mayer's hematoxylin.

Table 1. Primers used in the study.

Primer name	Primer sequence(5'→3')	Annealing temperature (°C)	Application
qTNF-α-F	CACAGGACAGCCTATGCCAACAA	60	qRT-PCR
qTNF-α-R	CTGAACTGGGCGGTCATAAAATAC		
qRIG-I-F	AGCAGCAGGCATAACTAAACTCA	60	qRT-PCR
qRIG-I-R	TCACTGTCAACATCTTTGGCATTA		
qTLR3-F	GCAACACTCCGCCTAAGTATCA	60	qRT-PCR
qTLR3-R	CAGTAGAAAGCTATCCTCCACCCT		
qTLR7-F	GACAACCTTTCCCAGAGCATTC	60	qRT-PCR
qTLR7-R	ACAGCCTTTTCCTCAGCCTAAC		
β-actin-F	ATGTCGCCCTGGATTTCG	60	qRT-PCR
β-actin-R	CACAGGACTCCATACCCAAGAA		

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA). The cycling procedure was as follows: 95°C for 3 min, 95°C for 10 s and 60°C for 30 s. Steps 2−3 were repeated for 40 cycles. Relative expression levels of target genes were normalized to β-actin, analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and presented as a ratio compared with the control. The primer sequences are shown in Table 1.

Western Blot

Proteins in the spleen tissues were obtained as described in the lysis buffer (Beyotime, Shanghai, China), following the manufacturer's instructions. The protein concentrations were measured using the BCA Protein Assay Kit (Beyotime), and all samples in the experiment were normalized to equal protein concentrations. Protein samples were separated by SDS-PAGE, transferred onto a PVDF membrane (Millipore, Billerica, MA), blocked with 5% non-fat milk in TBS-Tween-20 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20), and incubated with specific primary antibodies against Bcl-2 (1:500; Abcam, Cambridge, UK), caspase 3 (1:1,000; Abcam), iNOS (1:1,000; Abcam), COX2 (1:500; Bioybyt, Shanghai, China), and GAPDH (1:10,000; Abcam) overnight at 4°C. The membranes were incubated for 1 h with appropriate HRP-conjugated secondary anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG (1:5,000; Thermo Fisher Scientific), followed by enhanced chemiluminescence (**ECL**) detection. The results were normalized to GAPDH as an internal control. Protein levels were quantified using the ImageJ software.

Statistical Analysis

Data are represented as mean ± standard deviation (**SD**) and analyzed using t test analysis with SPSS 13.0 and GraphPad Prism 8 software. Statistical significance was set at $P < 0.05$; $P < 0.01$ was considered extremely significant.

RESULTS

Effects of Immunopotentiators on Spleen Antioxidant Activity

To verify the effect of the 5 screened immunopotentiators on the antioxidant capacity of Shaoxing ducklings, the MDA level and SOD and CAT activities of spleen tissue were measured. The levels of MDA in the 5 treated groups showed no significant difference (Figure 1A). The activities of CAT in the β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG groups were significantly lower than those in the control group (Figure 1B). SOD activity was significantly reduced in
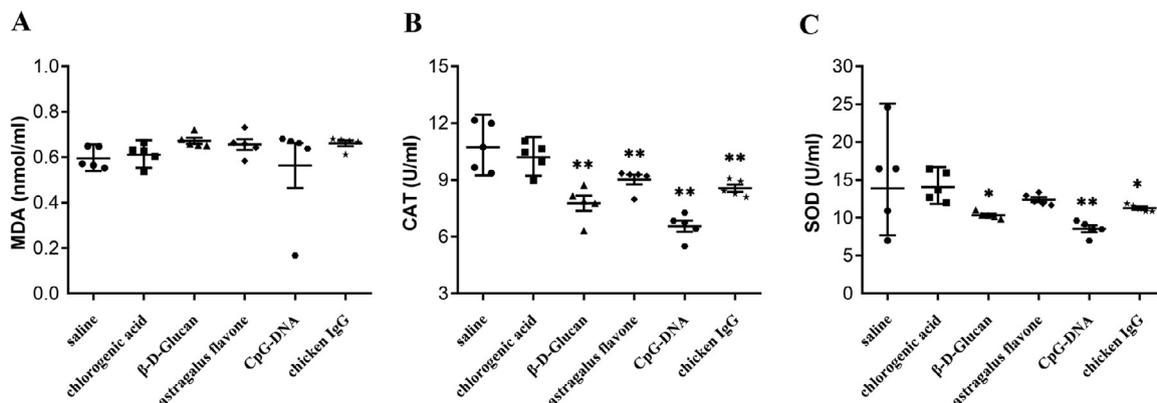

Figure 1. Effects of immunopotentiators on spleen MDA level and CAT and SOD activities in ducklings. Vertical bars represent the mean ± SD (n = 5). Abbreviations: CAT, catalase; SOD, superoxide dismutase.
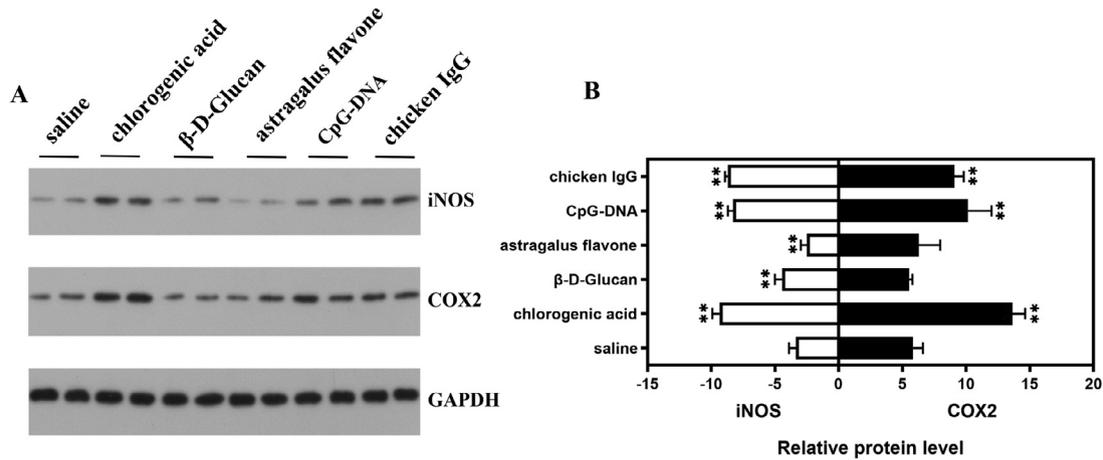
Figure 2. The protein levels of iNOS and COX2 after administration of five immunopotentiators in ducklings. Vertical bars represent the mean ± SD (n = 3).

β-D-Glucan, CpG-DNA, and chicken IgG groups comparing with control group (Figure 1C).

Effects of Immunopotentiators on Spleen iNOS and COX2 Protein

To determine whether immunopotentiators could activate the expression of iNOS and COX2 in response to innate immunity in ducklings, the expression of spleen iNOS and COX2 was detected using western blot. The expression of COX2 was upregulated in the spleen of ducklings injected with chlorogenic acid, β-D-Glucan, CpG-DNA, and chicken IgG, compared to that in the control group, while the ducklings treated with astragalus flavone showed significantly reduce in COX2 protein level (Figures 2A and 2B). Similarly, the expression of iNOS was increased significantly in the chlorogenic acid, CpG-DNA, and chicken IgG groups, compared to that in the control group (Figures 2A and 2B).

Effects of Immunopotentiators on Spleen Bcl2 and Caspase 3 Protein

Western blot analysis of Bcl2 and Caspase 3 protein levels was performed to estimate the expression of

apoptosis-related genes (Figure 3). Compared to the control, the expression level of caspase 3 protein was significantly increased in the chlorogenic acid, β-D-Glucan, astragalus flavone, and chicken IgG groups, while the ducklings treated CpG-DNA showed significantly reduce in caspase 3 protein level (Figures 3A and 3B). The expression levels of Bcl2 were decreased significantly in the chlorogenic acid, β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG groups, compared to those in the control group (Figures 3A and 3B).

Effects of Immunopotentiators on Spleen Inflammatory Cytokine Production

To further examine the effect of immunopotentiator treatments on immune responses, the expression of the cytokines (IL-1β, IFN-α, and IFN-β) was measured using in situ hybridization. The IL-1β and IFN-α levels in the spleen were significantly upregulated with the chlorogenic acid, β-D-Glucan, and astragalus flavone treatments, while the IL-1β and IFN-α levels during CpG-DNA and chicken IgG treatment showed weak expression comparing with that of the control (Figures 4A and 4B). In addition, IFN-β levels were enhanced significantly after all immunopotentiator
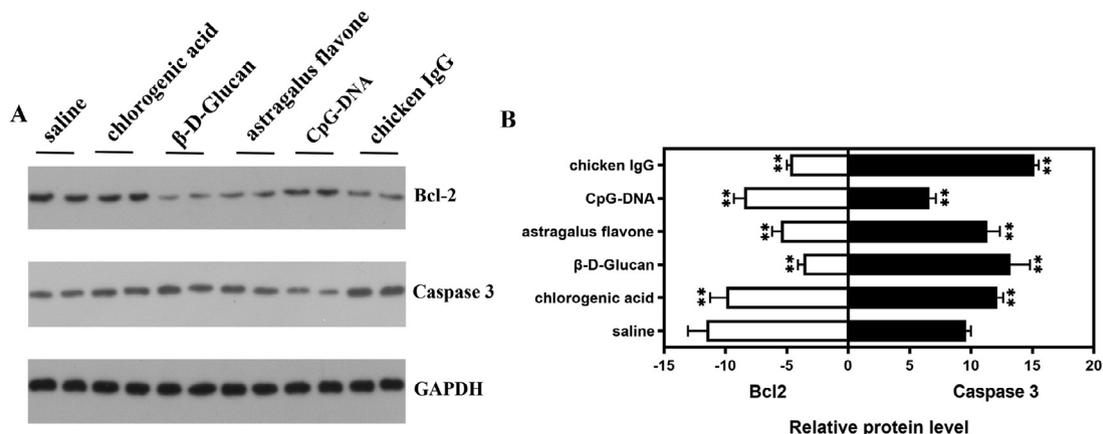

Figure 3. The protein levels of Bcl2 and Caspase 3 in the spleen of ducklings after administration of five immunopotentiators. Vertical bars represent the mean ± SD (n = 3).
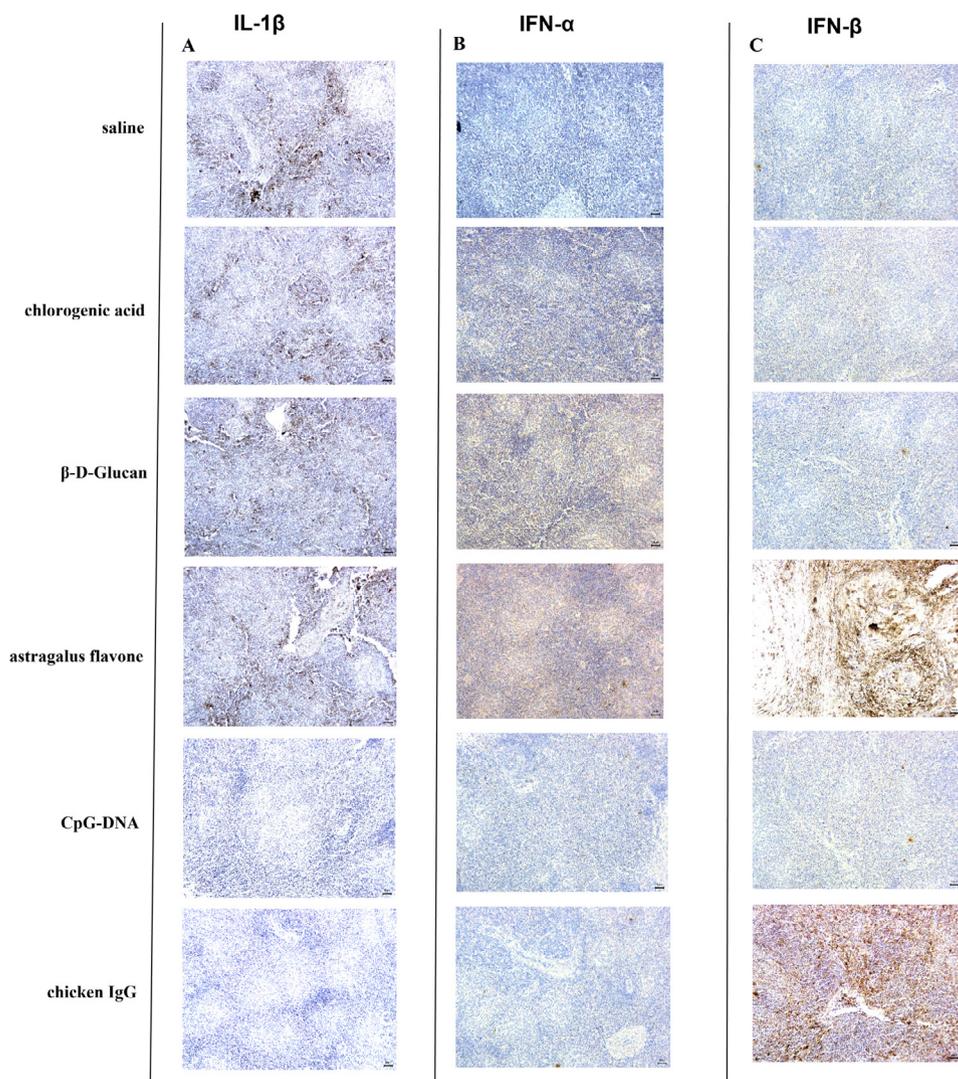
Figure 4. Effects of immunopotentiators on inflammatory cytokine production in the spleen of ducklings administered five different immunopotentiators. Spatial detection of *IL-1β* (A), *IFN-α* (B), and *IFN-β* (C) transcripts in the spleen of ducklings was determined by in situ hybridization (ISH, 200 ×).

treatments, especially with astragalus flavone and chicken IgG (Figure 4C).

Effects of Immunopotentiators on Spleen PRRs mRNA Expression

To further determine the role of different immunopotentiators in the regulation of the innate immune response by PRRs, the expression levels of the *TNF-α*, *RIG-I*, and *TLR3/7* genes in the spleen tissues were analyzed (Figure 5). The mRNA expression of *TNF-α* and *TLR7* were significantly upregulated in all immunopotentiator treatments (Figures 5A and 5D). The RIG-I levels in the spleen were significantly increased with the astragalus flavone, CpG-DNA, and chicken IgG treatments (Figure 5B). Besides, the TLR3 mRNA level was detected, which showed more significant higher in *β*-D-Glucan, astragalus flavone, CpG-DNA and chicken IgG group than that in the control group (Figure 5C).

DISCUSSION

Outbreaks of endemic diseases have become a limiting factor in duck cultivation, and the abuse of antibiotics in duck has been under scrutiny. Thus, finding alternative antibiotics in animal production became more and more imminent. Many studies have been conducted to protect ducks from epidemic diseases (Yang et al., 2020; Liu et al., 2021). Liu et al. (2021) has reported that *Hericium erinaceus polysaccharide* (**HEP**) alleviated immune organs damage and regulated the level of serum IgA, IgM, and IgG in MDRV-infected ducklings. However, efficient disease control strategies are not currently available. Immunopotentiators can activate nonspecific defense mechanisms to increase animal resistance to infectious disease (Sakai, 1999). In the present study, chlorogenic acid, *β*-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG were chosen to evaluate the efficiency of immune regulation in Shaoxing ducklings with regard to antioxidant capacity, apoptosis, and inflammatory cytokines.
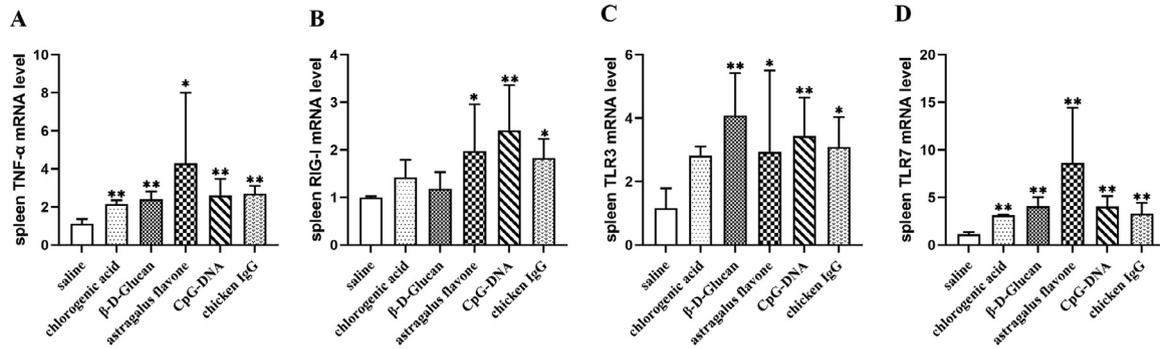
Figure 5. Effects of immunopotentiators on PRR mRNA expression in the spleen of ducklings administered five different immunopotentiators. RT-qPCR was used to detect the mRNA expression of *TNF-α* (A), *RIG-I* (B), *TLR3* (C), and *TLR7* (D). *β-actin* was used as the RT-qPCR control. The experiments were done in triplicate. Vertical bars represent the mean ± SD (n = 5). Abbreviation: PRR, pattern recognition receptor.

Immune parameters are always related to antioxidant abilities. To avoid or repair the damage, the compounds should possess adequate protection systems, such as key enzymatic antioxidant defenses (i.e., SOD and MDA) in tissues (Bruce et al., 2004). As the main end product of lipid peroxidation, MDA can be used as an index of lipid peroxidation (Sakin et al., 2012; Ural et al., 2015; Yonar et al., 2015). As the first-line of enzymatic antioxidant defense, SOD and CAT protect cells from oxidative stress as a result of the excessive generation of ROS (Suliman et al., 2021). Thus, they are important biochemical parameters for antioxidant defense (Zhang et al., 2013). Moreover, the balance between oxidants and antioxidants is fundamental for immune cell function because it preserves cell membrane integrity and functionality, cellular proteins, and nucleic acids (Yilmaz, 2019). Mujahid et al. (2007) showed that excessive accumulation of MDA further inhibited the activities of antioxidant enzymes and accelerated oxidative damage to proteins and DNA. In this study, the MDA levels in the 5 immunopotentiator-treated groups showed no significant change compared to those in the control group, while the enzymatic activities of SOD and CAT were lower than those obtained from control ducklings. This is an important mechanism for the prevention of damage induced by reactive oxygen species (Ghanima et al., 2021). There is evidence that the 5 immunopotentiators may play a protective role against oxidative stress.

The proinflammatory cytokines and NO produced by activated macrophages play critical roles in inflammatory diseases (Martel-Pelletier et al., 2003). The main finding of this study was that all 5 immunopotentiators could induce COX2 and iNOS expression, leading to cell apoptosis. Western blot analysis of the protein levels of Bcl2 showed a significant decrease, and those of caspase 3 were increased significantly by the 5 immunopotentiator treatments. Numerous studies have shown that the caspase 3 pathway has antiapoptotic effects, which has been implicated in a variety of biological processes (Yang et al., 2009). An early study suggested that β-glucan treatment in human SNU-C4 cells could decrease the mRNA level of Bcl-2 and increase that of caspase 3 (Kim et al., 2009). The main finding of the current study is that chlorogenic

acid, β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG could lead to spleen tissue cell apoptosis via upregulation of COX2 and iNOS expression.

Numerous studies have shown that iNOS-derived NO can regulate the immune response and serves as a vital molecule of protective innate immunity against pathogen invasion (Wink et al., 2011; Bogdan, 2015). The current results showed that these 5 immunopotentiators promoted the expression of iNOS protein in the spleen. In addition, the expression profiles of immune-related genes and PRRs (*IL-1β, IFN-α, IFN-β*, and *TNF-α*) were observed using in situ hybridization and RT-qPCR. IL-1β, a major coordinator of the immune response, can stimulate immune responses by inducing the release of cytokines. TNF-α as a cell signaling protein can induce inflammation and apoptosis (Liu et al., 2018). Similar to IL-1β, TNF-α was detected in all the examined groups, and the expression level was upregulated in immunopotentiator treatments. PRRs are central players in the early host immune response to acute viral infection and have been shown to play a crucial role in defense against microorganisms, mediating the innate antiviral immune responses to induce the production of IFN (Yoneyama and Fujita, 2009). In the current study, RIG-I, TLR3, and TLR7 were detected in spleen tissues, and higher expression levels were found in all immunopotentiator treatment groups than in the control group. This induced the production of *IFN-α* and *IFN-β*, suggesting that the five immunopotentiators could modulate the immune-related genes. Chlorogenic acid, β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG might activate immune responses to protect against pathogen invasion and maintain body homeostasis by balancing proinflammatory and anti-inflammatory responses.

Taken together, the results of this study demonstrate that chlorogenic acid, β-D-Glucan, astragalus flavone, CpG-DNA, and chicken IgG could improve the antioxidant capacity, apoptosis, pro-inflammatory factors, and PRRs in vivo to regulate the immune response. These results imply that the key immunopotentiators could be used to improve the duck innate immune response and can be considered as a reliable alternative to antibiotics in animal production.

ACKNOWLEDGMENTS

DISCLOSURES

The authors declare that they have no conflict of interest

REFERENCES

Abou-Elkhair, R., H. Abdo Basha, W. Slouma Hamouda Abd El Naby, J. S. Ajarem, S. N. Maodaa, A. A. Allam, and M. A. E. Naiel. 2020. Effect of a diet supplemented with the Moringa oleifera seed powder on the performance, egg quality, and gene expression in Japanese laying quail under heat-stress. Animals 10:809–821.

Ali, M. 1998. Rasayana therapy in classical literature of Ayurveda: a review. Bull. Indian Inst. Hist. Med. Hyderabad 28:95–110.

Bai, H., Q. Bao, Y. Zhang, Q. Q. Song, B. Liu, L. Zhong, X. Zhang, Z. X. Wang, Y. Jiang, Q. Xu, G. B. Chang, and G. H. Chen. 2020. Research note: effects of the rearing method and stocking density on carcass traits and proximate composition of meat in small-sized meat ducks. Poult. Sci 99:2011–2016.

Barton, M. D. 2000. Antibiotic use in animal feed and its impact on human healt. Nutr. Res. Rev 13:279–299.

Berends, B. R., A. E. Bogaard, F. V. Knapen, and J. M. Snijders. 2001. Human health hazards associated with the administration of antimicrobials to slaughter animals. Part II. An assessment of the risks of resistant bacteria in pigs and pork. Vet. Q 23:10–21.

Bogdan, C. 2015. Nitric oxide synthase in innate and adaptive immunity: an update. Trends Immunol 36:161–178.

Bruce, M., L. Daly, and N. Towers. 2004. Lean or agile: a solution for supply chain management in the textiles and clothing industry? Int. J. Opera. Produc. Manage 24:151–170.

Cook, M. T., P. J. Hayball, W. Hutchinson, B. Nowak, and J. D. Hayball. 2001. The efficacy of a commercial beta-glucan preparation, EcoActiva, on stimulating respiratory burst activity of head-kidney macrophages from pink snapper (Pagrus auratus), Sparidae. Fish Shellfish Immunol. 11:661–672.

Davies, J., and D. Davies. 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev 74:417–433.

Gantner, B. N., R. M. Simmons, S. J. Canavera, S. Akira, and D. M. Underhill. 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J. Exp. Med 197:1107–1117.

Ghanima, M. M. A, M. E. A. El-Hack, M. S. Abougabal, A. E. Taha, V. Tufarelli, V. Laudadio, and M. A. E. Naiel. 2021. Growth, carcass traits, immunity and oxidative status of broilers exposed to continuous or intermittent lighting programs. Anim. Biosci 34:1243–1252.

Gu, T. T., G. Q. Li, X. S. Wu, T. Zeng, Q. Xu, L. M. Li, S. Vladyslav, G. H. Chen, and L. Z. Lu. 2020. Effects of immunopotentiators on biochemical parameters, proinflammatory cytokine, and nonspecific immune responses in Shaoxing ducklings. Poult. Sci 99:5461–5471.

Herre, J., S. Gordon, and G. D. Brown. 2004. Dectin-1 and its role in the recognition of beta-glucans by macrophages. Mol. Immunol 40:869–876.

Hiss, S., and H. Sauerwein. 2003. Influence of dietary ss-glucan on growth performance, lymphocyte proliferation, specific immune response and haptoglobin plasma concentrations in pigs. J. Anim. Physiol. Anim. Nutr 87:2–11.

Jie, D., T. T. Gao, Z. S. Shan, J. Y. Song, M. Zhang, O. Kurskaya, K. Sharshov, L. X. Wei, and H. T. Bi. 2020. Immunostimulating effect of polysaccharides isolated from Ma-Nuo-Xi decoction in cyclophosphamide-immunosuppressed mice. Int. J. Biol. Macromo 146:45–52.

Kim, M. J., S. Y. Hong, S. K. Kim, C. Cheong, H. J. Park, H. K. Chun, K. H. Jang, B. D. Yoon, C. H. Kim, and S. A. Kang. 2009. beta-Glucan enhanced apoptosis in human colon cancer cells SNU-C4. Nutr. Res. Pract 3:180–184.

Liu, X., X. X. Ying, Y. L. Li, H. Yang, W. S. Hao, and M. L. Yu. 2018. Identification differential behavior of Gd@C$_{82}$(OH)$_{22}$ upon interaction with serum albumin using spectroscopic analysis. Spectrochim. Acta A Mol. Biomol. Spectrosc 203:383–396.

Liu, Z. N., L. Y. Liao, Q. Chen, S. Q. Lin, Y. Luo, T. Qin, J. Li, Q. X. Wang, B. C. Wu, Y. F. Huang, and Y. J. Wu. 2021. Effects of Hericium erinaceus polysaccharide on immunity and apoptosis of the main immune organs in Muscovy duck reovirus-infected ducklings. Int. J. Biol. Macromol 171:448–456.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408.

Martel-Pelletier, J., J. P. Pelletier, and H. Fahmi. 2003. Cyclooxygenase-2 and prostaglandins in articular tissues. Semin. Arthritis Rheum 33:155–167.

Masihi, K. N. 2000. Immunomodulatory agents for prophylaxis and therapy of infections. Int J Antimicrob Agents 14:181–191.

Mujahid, A., Y. Akiba, C. H. Warden, and M. Toyomizu. 2007. Sequential changes in superoxide production, anion carriers and substrate oxidation in skeletal muscle mitochondria of heat-stressed chickens. FEBS Lett 581:3461–3467.

Sakai, M. 1999. Current research status of fish Immunostimulants. Aquaculture 172:63–92.

Sakin, F., S. M. Yonar, M. E. Yonar, and N. Saglam. 2012. Changes in selected immunological parameters and oxidative stress responses in different organs of oncorhynchus mykiss exposed to ivermectin. Rev. Chimie -Bucharest- Orig. Ed. 63:989–995.

Suliman, G. M., A. N. Alowaimer, S. I. Al-Mufarrej, E. O. S Hussein, E. H. Fazea, M. A. E Naiel, R. A. Alhotan, and A. A. Swelum. 2021. The effects of clove seed (Syzygium aromaticum) dietary administration on carcass characteristics, meat quality, and sensory attributes of broiler chickens. Poult. Sci 100:100904–100912.

Ural, M., M. E. Yonar, and S. Mişe Yonar. 2015. Protective effect of ellagic acid on oxidative stress and antioxidant status in Cyprinus carpio during malathion exposure. Cell Mol. Biol. (Noisy-le-grand) 61:58–63.

Wink, D. A., H. B. Hines, R. Y. Cheng, C. H. Switzer, W. Flores-Santana, M. P. Vitek, L. A. Ridnour, and C. A. Colton. 2011. Nitric oxide and redox mechanisms in the immune response. J. Leukoc. Biol 89:873–891.

Yang, S. L., R. C. Yang, X. Zhou, S. H. Yang, L. L. Luo, Y. C. Zhu, and S. Boonanuntan. 2020. Effects of feeding diets with processed Moringa oleifera stem meal on growth and laying performance, and immunological and antioxidant activities in laying ducks. Poult. Sci 99:3445–3451.

Yang, Z. G., A. Q. Chen, and B. Liu. 2009. Antiproliferation and apoptosis induced by evodiamine in human colorectal carcinoma cells (COLO-205). Chem. Biodivers 6:924–933.

Yilmaz, S. 2019. Effects of dietary blackberry syrup supplement on growth performance, antioxidant, and immunological responses, and resistance of Nile tilapia, Oreochromis niloticus to Plesiomonas shigelloides. Fish Shellfish Immunol 84:1125–1133.

Yonar, M. E., S. M. Yonar, A. Pala, S. Silici, and N. Sağlam. 2015. Trichlorfon-induced haematological and biochemical changes in Cyprinus carpio: ameliorative effect of propolis. Dis. Aquat. Organ 114:209–216.

Yoneyama, M., and T. Fujita. 2009. RNA recognition and signal transduction by RIG-I-like receptors. Immunol. Rev 227:54–65.

Zeng, T., L. Chen, X. Du, S. J. Lai, S. P. Huang, Y. L. Liu, and L. Z. Lu. 2016. Association analysis between feed efficiency studies and expression of hypothalamic neuropeptide genes in laying ducks. Anim. Genet 47:606–609.

Zhang, C. N., X. F. Li, W. N. Xu, G. Z. Jiang, K. L. Lu, L. N. Wang, and W. B. Liu. 2013. Combined effects of dietary fructooligosaccharide and Bacillus licheniformis on innate immunity, antioxidant capability and disease resistance of triangular bream (Megalobrama terminalis). Fish Shellfish Immunol 35:1380–6138.