frontiers | Research Topics

Treatment of animal diseases with veterinary phytotherapy

Edited by
Fazul Nabi and Dayou Shi

Published in
Frontiers in Veterinary Science

ISSN 1664-8714
ISBN 978-2-8325-2228-8
DOI 10.3389/978-2-8325-2228-8

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Treatment of animal diseases with veterinary phytotherapy

Topic editors

Fazul Nabi — Lasbela University of Agriculture, Water and Marine Sciences, Pakistan

Dayou Shi — South China Agricultural University, China

Topic coordinator

Qingxia Wu — Tibet Agricultural and Animal Husbandry University, China

Citation

Nabi, F., Shi, D., eds. (2023). *Treatment of animal diseases with veterinary phytotherapy*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2228-8

Table of contents

frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 06 April 2023
DOI 10.3389/fvets.2023.1171987

OPEN ACCESS

EDITED AND REVIEWED BY
Arturo Anadón,
Complutense University of Madrid, Spain

*CORRESPONDENCE
Fazul Nabi
✉ fazulnabishar@yahoo.com

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 22 February 2023
ACCEPTED 28 March 2023
PUBLISHED 06 April 2023

CITATION
Nabi F, Shi D, Wu Q and Baloch DM (2023) Editorial: Treatment of animal diseases with veterinary phytotherapy.
Front. Vet. Sci. 10:1171987.
doi: 10.3389/fvets.2023.1171987

Editorial: Treatment of animal diseases with veterinary phytotherapy

Fazul Nabi[1,2]*, Dayou Shi[3], Qingxia Wu[4] and Dost Muhammad Baloch[1]

[1]Faculty of Veterinary and Animal Science, Lasbela University of Agriculture, Water and Marine Sciences, Uthal, Balochistan, Pakistan, [2]Department of Traditional Chinese Veterinary Medicine, College of Veterinary Medicine, Southwest University, Chongqing, China, [3]College of Veterinary Medicine, South China Agricultural University, Guanzghou, China, [4]Animal Science College, Tibet Agriculture and Animal Husbandry University, Linzhi, China

KEYWORDS

traditional Chinese medicine, animals disease, animal health, plant, phytotherapy, animal nutrition

Editorial on the Research Topic
Treatment of animal diseases with veterinary phytotherapy

The authors have been invited to serve as Guest Editors for this Research Topic. As a Guest Editors we are responsible for identifying the emerging trends, highlights the important topics, or presenting a particular perspective on a subject. Additionally, we ensured the quality and relevance of the publication, as well as bringing fresh ideas and diverse viewpoints to the readers. Phytotherapy, also known as herbal medicine or botanical medicine, is a type of complementary and alternative medicine that uses plant extracts and other natural substances to treat various health conditions (1). These herbs are believed to have various medicinal properties that can help to alleviate symptoms of different health conditions, such as anxiety, depression, insomnia, pain, and inflammation (2, 3). With the advent of antibiotic resistance phytobiotic gain much attention among researchers as an alternative treatment option for livestock, poultry, and aquaculture. Some commonly used herbs in phytotherapy include herbs and their derivatives, plant derived bioactive compounds including lycopene, carotenoids, L-theanine, fucoidan, and humic acid (4, 5). Herbal medicine used in veterinary practice has been gaining popularity as an alternative or complementary treatment option for a wide range of conditions in animals, and it's great to see that there is ongoing research in this field (6, 7).

Numerous natural phytochemical compounds found in plants have medicinal properties that have been used for centuries to promote healing and prevent disease. In veterinary and medical clinical practice, herbal medicine is used in a variety of ways. For example, some veterinarians may use herbal remedies to treat animals with conditions such as bacterial infection, oxidative stress and digestive problems. Similarly, some veterinarian recommended in-feed herbal supplements as an alternative source of antibiotic growth promoter to promote or maintain the productivity of domestic animals. The herbal medicine used for therapeutic tool should be more natural and less harmful than conventional pharmaceutical drugs, it is important to note that not all herbal remedies are safe or effective (8–10). Therefore, the main objective of this Research Topic is to provide the new insight regarding the beneficial application of medicinal plant as source of phytobiotic for the treatment of pathophysiological disorder in veterinary practices. Studies considered for publication in this topic have been classified into following sections.

Anti-inflammatory potential of phytobiotics

Inflammation is a natural response of the body to injury or infection. However, chronic inflammation can lead to various diseases such as arthritis, diabetes, and cardiovascular disease. Phytobiotics are the natural compounds derived from plants that are known to have various beneficial effects on human and animal health. One of the benefits of phytobiotics is their anti-inflammatory properties. Phytobiotics can help to reduce inflammation by inhibiting the production of inflammatory cytokines and enzymes and by scavenging free radicals that contribute to oxidative stress (11). Examples of phytotherapeutic compounds with anti-inflammatory properties include curcumin, found in turmeric; resveratrol, found in grapes and red wine; quercetin, found in fruits and vegetables; and omega-3 fatty acids, found in fish and some plants.

Several studies have been reported that phytotherapeutic compounds, which are derived from plants, have been found to have anti-inflammatory properties that can help to regulate the body's proinflammatory mediators. These compounds can act on various pathways involved in inflammation, including the inhibition of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-alpha), interleukin-1 beta (IL-1beta), and interleukin-6 (IL-6) (12). It has been reported that chronic inflammation has been linked to an increased the risk of cancer development and progression. Inflammation is a complex biological process that involves the activation of immune cells, the release of inflammatory mediators, such as cytokines and chemokines, and the production of reactive oxygen and nitrogen species (ROS/RNS) by immune and non-immune cells, they play a role in the transformation of normal cells to the development of cancer cells. Natural compounds such as resveratrol, curcumin, quercetin, and gingerol have been found to have antioxidant properties, which can help to reduce oxidative stress, prevent inflammation, and modulate these signaling pathways, thereby reducing inflammation (13, 14). In recent study it has been reported that the dietary supplementation of traditional Chinese medicine (TCM) formula significantly increased the levels of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, and reduced the levels of reactive oxygen species in the liver of piglets compared to the control group. The TCM formula also decreased the expression of inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, in the liver (15). Similarly, another study found that treatment with *Jasonia glutinosa* extract resulted in significant reduction in inflammation and oxidative stress markers in the colon tissue of mice with induced colitis. Additionally, *J. glutinosa* extract improved the integrity of the intestinal barrier by reducing the leakage of gut bacteria and endotoxins into the bloodstream (16).

Antibacterial potential of phytotherapeutic compounds

The beneficial application of herbal medicine has increased over the past decade, and there has been a growing emphasis on natural plant-based therapeutic agents for the treatment and control of microbial infections. Many plants contain bioactive compounds with antimicrobial properties that can help to inhibit the growth and spread of microorganisms. These compounds include alkaloids, flavonoids, terpenoids, and phenolic acids, among others. Research has shown that many herbal medicines have potent antimicrobial properties and can be used as an alternative or complementary therapy to conventional antibiotics. However, it is important to note that the efficacy and safety of herbal medicines may vary depending on factors such as the plant species, plant part used, method of preparation, and dosage.

Several studies have shown that phytobiotics can inhibit the growth of various bacterial species, including those that are pathogenic to humans and animals. For example, the essential oil of oregano has been shown to be effective against several types of bacteria, including *Escherichia coli, Salmonella enterica,* and *Staphylococcus aureus*. Other commonly studied phytobiotics with antibacterial properties include garlic, cinnamon, thyme, and tea tree oil. These substances have been shown to be effective against a range of bacteria, including those that are antibiotic-resistant.

The antibacterial effects of phytobiotics are believed to be due to their ability to disrupt bacterial cell membranes, interfere with bacterial metabolic processes, and/or inhibit bacterial enzyme activity. Additionally, some phytobiotics may stimulate the immune system to help fight bacterial infections. However, more research is needed to fully understand their mechanisms of action and potential side effects.

Antiviral potential of phytotherapeutic compounds

Mutations in viruses are a natural occurrence and can lead to the emergence of new strains that may have different characteristics than the original virus. In some cases, mutations can lead to the development of resistance to against antiviral agents (17). The development of resistance to antiviral agents is a concern in the field of virology, as it can make it more difficult to control viral infections. To address this, researchers are continuously working to develop new antiviral agents that are effective against a wide range of viral strains, including those that have developed resistance to existing treatments. There has been growing interest among researchers in exploring the potential of phytotherapeutic compounds as effective treatments for viral infections due to their antiviral properties.

Several studies have suggested that certain phytotherapeutic compounds may have antiviral effects, and could potentially be used as alternative or complementary treatments for viral infections in animals. For example, compounds such as flavonoids, terpenes, and alkaloids found in various plants have been shown to possess antiviral activity against number of viruses including influenza, herpes simplex virus, HIV, and coronavirus (18). On the other hand, some studies have shown that compounds found in plants such as echinacea, elderberry, and garlic have antiviral effects and can help to boost the immunological response. However, it's important to note that the efficacy and safety of these phytotherapeutic compounds in treating viral infections in animals still needs to be thoroughly evaluated through clinical trials and

further research. Additionally, it's essential to ensure that the use of such treatments is in compliance with applicable laws and regulations.

Phytobiotics modulate gut microbiota in animals

These microbiotas, also known as gut microbiota or gut flora, is a complex and diversified collection of microorganisms that reside in the gastrointestinal tract of animals, and poultry birds. The microbiota consists of thousands of different microbial species, including bacteria, archaea, viruses, and fungi, which together form a complex ecological system that plays a crucial role in maintaining the health and wellbeing of the host organism. The study of the gut microbiota is a rapidly growing field of research, as scientists continue to uncover the many ways in which these microorganisms interact with the host and influence various aspects of health and disease.

Phytobiotics, have been shown to have a significant impact on the composition and function of the gut microbiota. These compounds can act as prebiotics, which are substances that promote the growth of beneficial bacteria in the gut, or as antimicrobials, which can help to control the growth of harmful bacteria (19). Some examples of phytobiotics include polyphenols, flavonoids, and terpenes, which are found in a wide range of plant-based foods such as fruits, vegetables, herbs, and spices. These compounds can help to maintain a healthy balance of bacteria in the gut, which is important for overall health and wellbeing of host.

There are numerous bacterial species in the intestine that contribute to the maintenance of the body's homeostatic balance. Some of the most well-known bacterial species that play a crucial role in gut health include *Bifidobacterium, Lactobacillus*, and *Akkermansia muciniphila*. These bacterial species have been found to interact with plant medicines or herbs to improve the health of the bowel and balance of the animal's body. In addition, some herbs such as ginger, peppermint, and fennel have been traditionally used to treat digestive issues and have been found to have antimicrobial properties against harmful bacteria in the gut. These herbs may also help to promote the growth of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus*, and improve gut health.

Immunomodulatory role of phytobiotics

The immune system is responsible for protecting the body against infections and diseases. Immunosuppressive diseases are a significant concern in livestock and poultry species because they can negatively impact animal health and productivity, leading to economic losses for producers. Immunosuppressive diseases initiated through several factors, including viral, bacterial, and parasitic infections, as well as environmental and managemental stressors such as poor nutrition or overcrowding. These diseases can weaken the animal's immune system, making it more vulnerable to secondary infections and reducing its ability to fight off pathogens (20).

Previous studies have been suggested that phytobiotics significantly regulated the immune system by activating or suppressing certain immune responses. For example, some phytobiotics can stimulate the production of cytokines, which are signaling molecules that helps to boost immune response (21, 22). Other phytobiotics can inhibit the activity of inflammatory cells, such as macrophages and neutrophils, which can reduce inflammation and tissue damage. Phytobiotics can also have antioxidant properties, which can help to protect the immune system from damages caused by free radicals of reactive oxygen species. Additionally, some phytobiotics can stimulate the growth of beneficial gut bacteria, leading to enhance immunological response. Overall, the immunomodulatory role of phytobiotics is an area of active research, and many plant-derived compounds are being studied for their potential therapeutic effects on immune-related disorders.

Conclusion

In recent years, there has been an increasing interest in the application of TCM in animal husbandry, particularly as a feed additive for poultry and livestock species. It's great to observed that research reports on the application of TCM as a feed additive have contributed to the improvement of the health and economy of poultry and livestock species. The use of phytotherapeutic compounds or extracts from TCM can indeed have positive effects on the health and performance of animals. It's important to continue research and develop new ways to improve animal health and productivity in a sustainable and ethical manner. The contributions of all the participants involved in this Research Topic are greatly appreciated, as their work will help to advance the field and benefit the industry as a whole.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

This study was supported and funded by the Special Funding for Chongqing Post-Doctoral Research Project 2020 (number 7820100603).

Acknowledgments

The suggestion and idea for this Research Topic was supported by Professor Juan Liu, College of Veterinary Medicine, Southwest University, Chongqing China.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Alagawany M, Abd El-Hack ME, Saeed M, Naveed M, Arain MA, Arif M, et al. Nutritional applications and beneficial health applications of green tea and l-theanine in some animal species: a review. *J Anim Physiol Anim Nutr.* (2020) 104:245–56. doi: 10.1111/jpn.13219

2. Arain MA, Mei Z, Hassan F, Saeed M, Alagawany M, Shar A, et al. Lycopene: a natural antioxidant for prevention of heat-induced oxidative stress in poultry. *Worlds Poult Sci J.* (2018) 74:89–100. doi: 10.1017/S0043933917001040

3. Arif M, Alagawany M, Abd El-Hack M, Saeed M, Arain M, Elnesr S, et al. Humic acid as a feed additive in poultry diets: a review. *Iran J Vet Res.* (2019) 20:167.

4. Changxing L, Chenling M, Alagawany M, Jianhua L, Dongfang D, Gaichao W, et al. Health benefits and potential applications of anthocyanins in poultry feed industry. *Worlds Poul Sci J.* (2018) 74:251–64. doi: 10.1017/S0043933918000053

5. Nabi F, Arain MA, Rajput N, Alagawany M, Soomro J, Umer M, et al. Health benefits of carotenoids and potential application in poultry industry: a review. *J Anim Physiol Anim Nutr.* (2020) 104:1809–18. doi: 10.1111/jpn.13375

6. Tamminen L-M, Emanuelson U, Blanco-Penedo I. Systematic review of phytotherapeutic treatments for different farm animals under European conditions. *Front Vet Sci.* (2018) 5:140. doi: 10.3389/fvets.2018.00140

7. Saeed M, Babazadeh D, Naveed M, Arain MA, Hassan FU, Chao S, et al. Reconsidering betaine as a natural anti-heat stress agent in poultry industry: a review. *Trop Anim Health Prod.* (2017) 49:1329–38. doi: 10.1007/s11250-017-1355-z

8. Bisi-Johnson M, Obi C, Kambizi L, Nkomo M. A survey of indigenous herbal diarrhoeal remedies of OR Tambo district, Eastern Cape Province, South Africa. *Afr J Biotechnol.* (2010) 9. doi: 10.5897/AJB09.1475

9. Stark TD, Mtui DJ, Balemba OB. Ethnopharmacological survey of plants used in the traditional treatment of gastrointestinal pain, inflammation and diarrhea in Africa: future perspectives for integration into modern medicine. *Animals.* (2013) 3:158–227. doi: 10.3390/ani3010158

10. Arain MA, Nabi F, Shah QA, Alagawany M, Fazlani SA, Khalid M, et al. The role of early feeding in improving performance and health of poultry: herbs and their derivatives. *Worlds Poul Sci J.* (2022) 78:499–513. doi: 10.1080/00439339.2022.2043133

11. Saeed M, Babazadeh D, Arif M, Arain M, Bhutto Z, Shar A, et al. Silymarin: a potent hepatoprotective agent in poultry industry. *Worlds Poul Sci J.* (2017) 73:483–92. doi: 10.1017/S0043933917000538

12. Saeed M, Babazadeh D, Naveed M, Alagawany M, Abd El-Hack ME, Arain MA, et al. *In ovo* delivery of various biological supplements, vaccines and drugs in poultry: current knowledge. *J Sci Food Agric.* (2019) 99:3727–39. doi: 10.1002/jsfa.9593

13. Wang Q, Li W, Hu H, Lu X, Qin S. Monomeric compounds from traditional Chinese medicine: new hopes for drug discovery in pulmonary fibrosis. *Biomed Pharmacother.* (2023) 159:114226. doi: 10.1016/j.biopha.2023.114226

14. Saeed M, Arain MA, Ali Fazlani S, Marghazani IB, Umar M, Soomro J, et al. A comprehensive review on the health benefits and nutritional significance of fucoidan polysaccharide derived from brown seaweeds in human, animals and aquatic organisms. *Aquac Nutr.* (2021) 27:633–54. doi: 10.1111/anu.13233

15. Wang X, Wang Y, Mao Y, Hu A, Xu T, Yang Y, et al. Corrigendum: The beneficial effects of traditional Chinese medicine on antioxidative status and inflammatory cytokines expression in the liver of piglets. *Front Vet Sci.* (2022) 9:1063573. doi: 10.3389/fvets.2022.1063573

16. Valero MS, González M, Ramón-Gimenez M, Andrade PB, Moreo E, Les F, et al. Jasonia glutinosa (L) DC, a traditional herbal medicine, reduces inflammation, oxidative stress and protects the intestinal barrier in a murine model of colitis. *Inflammopharmacology.* (2020) 28:1717–34. doi: 10.1007/s10787-019-00626-0

17. Syed SF, Zhao Q, Umer M, Alagawany M, Ujjan IA, Soomro F, et al. Past, present and future of hepatitis E virus infection: zoonotic perspectives. *Microb Pathog.* (2018) 119:103–8. doi: 10.1016/j.micpath.2018.03.051

18. Setayesh M, Karimi M, Zargaran A, Abousaidi H, Shahesmaeili A, Amiri F, et al. Efficacy of a Persian herbal medicine compound on coronavirus disease 2019 (COVID-19): a randomized controlled trial. *Integr Med Res.* (2022) 11:5–5. doi: 10.1016/j.imr.2022.100869

19. Nabi F, Arain MA. Rising stars in comparative and clinical medicine: 2021. *Front Vet Sci.* (2022) 9:1030960. doi: 10.3389/fvets.2022.1030960

20. Nabi F, Ahmed J, Tao W, Lu Q, Bhutto ZA, Qadir A, et al. An updated review on efficiency of penthorum chinense pursh in traditional uses, toxicology, and clinical trials. *BioMed Res Int.* (2023) 2023:4254051. doi: 10.1155/2023/4254051

21. Nabi F, Arain MA, Fazlani SA, Khalid M, Bugti F, Ali S, et al. Effect of *in ovo* trace element supplementation on immune-related cells of the small intestine of post-hatched broiler chicken. *Biol Trace Elem Res.* (2022) 19:1–10. doi: 10.1007/s12011-022-03492-0

22. Wu S, Wang Y, Iqbal M, Mehmood K, Li Y, Tang Z, et al. Challenges of fluoride pollution in environment: mechanisms and pathological significance of toxicity–a review. *Environ Pollut.* (2022) 304:119241. doi: 10.1016/j.envpol.2022.119241

frontiers | Frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 21 June 2022
doi: 10.3389/fvets.2022.905382

Effect of Traditional Chinese Medicine on the Gut Microbiota in Heat-Stressed Laying Hens

Chunxin Ye[1,2†], *Qian Qu*[2†], *Lin Bai*[3], *Jiaxin Chen*[2], *Zhuoke Cai*[2], *Jiaqi Sun*[2], *Cui Liu*[2*] and *Dayou Shi*[2*]

[1] Guangdong Polytechnic of Science and Trade, Guangzhou, China, [2] College of Veterinary Medicine, South China Agricultural University, Guangzhou, China, [3] Wens Foodstuff Group Co., Ltd, Yunfu, China

OPEN ACCESS

Edited by:
Xiaofei Shang,
Lanzhou Institute of Husbandry and Pharmaceutical Sciences (CAAS), China

Reviewed by:
Asghar Kamboh,
Sindh Agriculture University, Pakistan
Aoyun Li,
Huazhong Agricultural University, China

***Correspondence:**
Dayou Shi
shidayou@scau.edu.cn
Cui Liu
liuc@scau.edu.cn

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

Received: 27 March 2022
Accepted: 16 May 2022
Published: 21 June 2022

Citation:
Ye C, Qu Q, Bai L, Chen J, Cai Z, Sun J, Liu C and Shi D (2022) Effect of Traditional Chinese Medicine on the Gut Microbiota in Heat-Stressed Laying Hens. Front. Vet. Sci. 9:905382. doi: 10.3389/fvets.2022.905382

Gut microbiota plays an important role in health and disease. To determine whether the traditional Chinese formula Zi Huang Huo Xiang San (ZHHXS) modulates gut microbiota under heat stress, a heat stress model was prepared in Roman layer hens by housing them at temperatures of 32–36°C and administering ZHHXS for 4 weeks. The Roman egg layers were randomly divided into three groups with 10 hens in each: a ZHHXS treatment group (ZHHXS-HS), a heat-stressed group (HS), and a blank control group (BC). The ZHHXS-HS and HS groups were housed in a 34 ± 2°C environment, while the BC group was housed at 25 ± 1°C. The ZHHXS-HS hens were fed a diet supplemented with 1% ZHHXS from 1 to 28 days, while the other groups were not. Gut microbiota in the hens' feces was assessed through 16S rRNA high-throughput sequencing on days 1, 3, 7, 14, and 28. A plot of the PCA scores showed that the gut microbiota composition in the BC group was a similar trend in the ZHHXS-HS group on days 1 and 3. The principal coordinate analysis (PCoA) unweighted distribution showed that the gut microbiota composition had no significant differences between the BC and ZHHXS-HS groups on days 1 and 7. The PCoA weighted distribution showed that the gut microbiota composition had no significant differences between the BC and ZHHXS-HS groups on days 1 and 3. This study showed that the composition of gut microbiota in layer hens with heat stress was modulated by ZHHXS treatment. ZHHXS treatment caused key phylotypes of gut microbiota to match the BC group, particularly *Actinobacteria*, *Bacteroidetes*, *Bacteroides*, and *Enterococcus*. The effect of ZHHXS in alleviating heat stress could be achieved by altering the composition of gut microbiota and regulating some key phylotypes.

Keywords: traditional Chinese medicine, gut microbiota, heat stress, laying hen, 16S rRNA

INTRODUCTION

Stress is a disorder associated with environmental factors, including temperature, diet, radiation, and so on. Heat stress is the most serious stress that causes adverse effects on growth performance, immunity, mortality, and breast meat quality (1–4). Emerging evidence demonstrates that heat stress can aggravate metabolic disorders and intestinal diseases (5–10). Based on our previous

TABLE 1 | The composition of the Zi Huang Huo Xiang San.

Items	*Chinese names*	Actions
Echinacea root	*Zi Zhui Ju Gen*	Strengthens immune system
Scutellaria	*Huang Qin*	Clearing heat, drying dampness, purging fire, and detoxification
Patchouli	*Guang Huo Xiang*	eliminating dampness with aromatics, stopping vomiting and relieving summer-heat
Elsholtzia	*Xiang Ru*	Diaphoresis, detumescence and removing dampness for regulating stomach
Gypsum	*Shi Gao*	clearing away heat and purging fire, except vexed, slake the thirst
Dried tangerine peel	*Chen Pi*	Regulating Qi and strengthening the spleen, drying dampness, and eliminating phlegm
White atractylodes rhizome	*Bai Zhu*	Strengthen the Spleen, dry up Dampness, tonify Qi, and prom
Licorice	*Gan Cao*	Strengthen the Spleen and tonify Qi, Harmonize the effects of other herbs

TABLE 2 | Composition and nutrient levels of the basal diet.

Items	Content (%)	Nutritional level	Content (%)
Corn	59.50	Crude protein	17.19
Wheat bran	4.00	Calcium	3.5
Soybean meal	14.60	Phosphorus	0.42
Cottonseed meal	6.00	Lysine	0.71
Rapeseed meal	4.00	Methionine	0.36
Dried meat floss	2.25	Cystine	0.19
Stone powder	8.4	Metabolizable energy (MJ/Kg)	12.82

study, we found that heat stress has a critical impact on the composition of gut microbiota, growth performance, circulating levels of HSP70, and cortisol (11). Therefore, the gut microbiota may be the key factor in the treatment of heat stress.

The research has demonstrated that gut microbiota has responded and reacted to the treatment mechanisms of Traditional Chinese Medicine (TCM) (12–15). Gut microbiota is an important, complex, and substantial bacterial microecological system. Microorganisms in the intestine form a mutually compatible and beneficial harmonious relationship under long-term interactions with the organism. Under the mutual effect of the external environment, the host's health, and the bacterial microecological system, the balance with gut microbiota and the promotion of an organism's healthy development (16–18) can be maintained well. Gut microbiota, an ecological environment with a dynamic balance of organism and TCM, can regulate the overall balance of the body. Therefore, the regulation of TCM on the health of gut microbiota may be beneficial to balancing the body (19, 20). After Jia Wei Er Shu San was administered to weanling piglets, the diversity and structure of their gut microbiota significantly increased. The gut damage in a rat model with spleen deficiency was relieved through the regulation of gut microbiota *via* the decoction of four mild drugs. Gut microbiota in patients with type II diabetes can be regulated using a decoction of Ge Gen Huang Lian to treat diabetes (21). The turbulence of

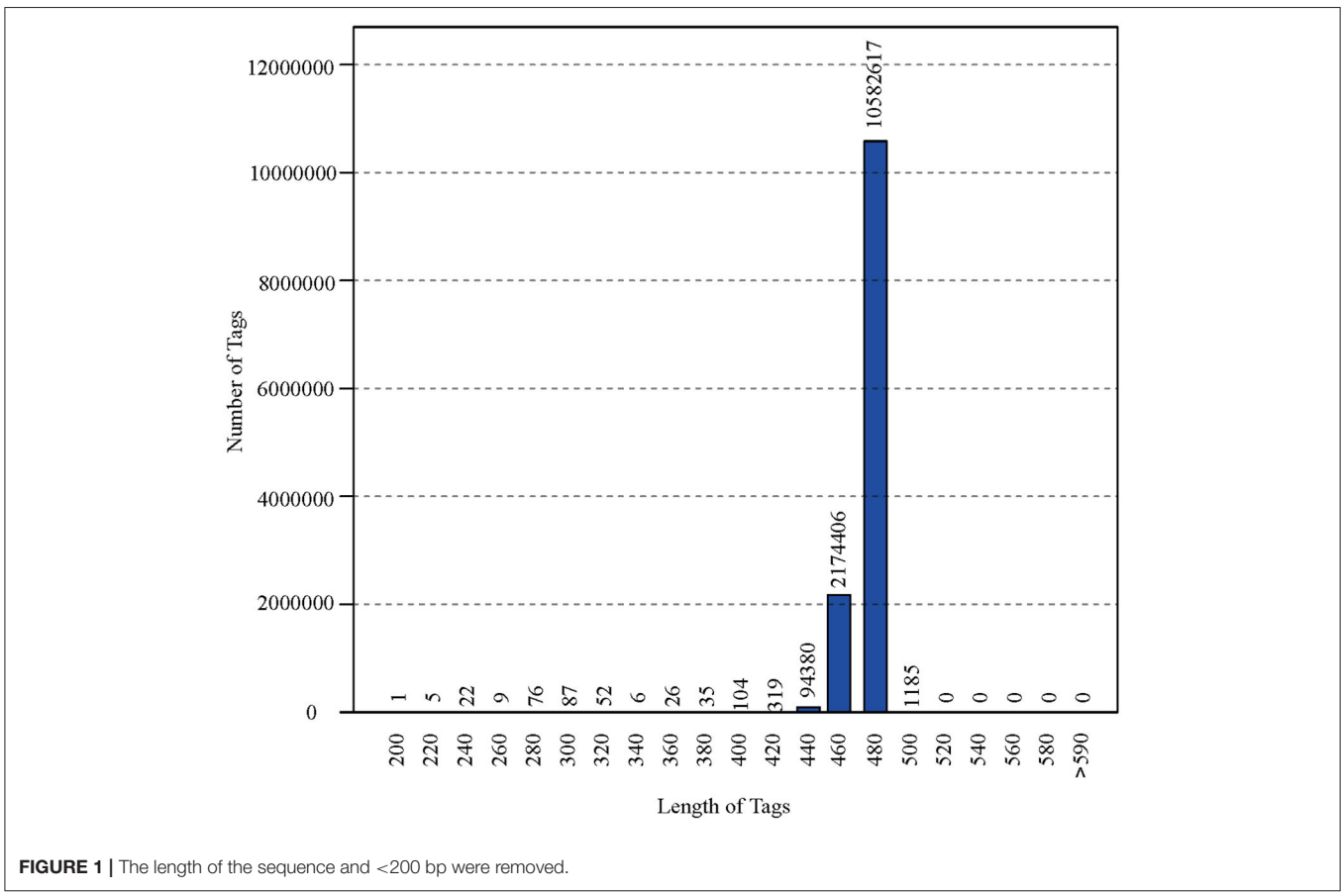


FIGURE 1 | The length of the sequence and <200 bp were removed.

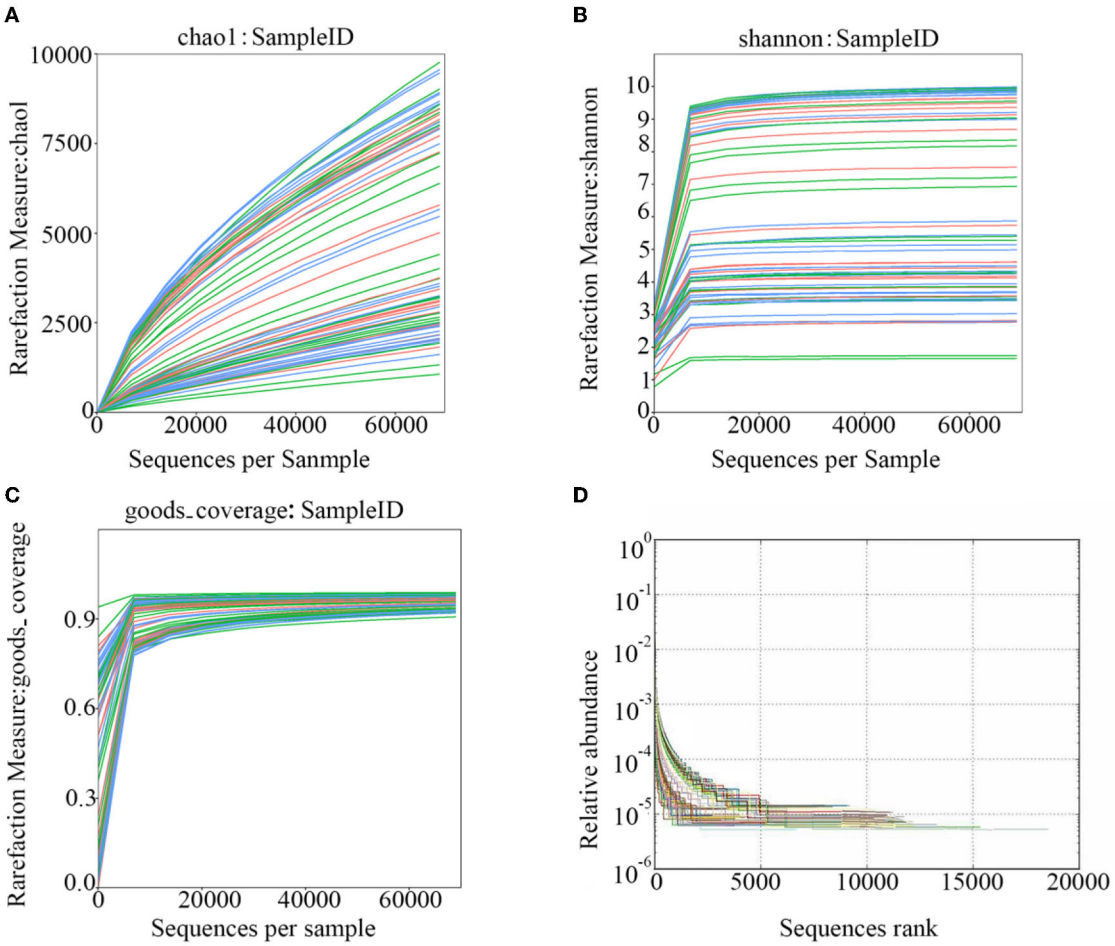


FIGURE 2 | The rarefaction curves that were generated from the OTUs. **(A)** shows the chao1; **(B)** shows the Shannon; **(C)** shows the goods-coverage; and **(D)** shows the Rank-Abundance.

gut microbiota metabolites, such as short-chain fatty acids and hosts, was extensively modulated by Atractylodes macrocephala to achieve the therapeutic effects (22–24). Scutellaria baicalensis Georgi polysaccharide can improve intestinal barrier function and modulate gut microbiota on dextran sulfate sodium-induced ulcerative colitis (25). However, the study is required to further investigate the complexity of the interactions among TCM, heat stress, and gut microbiota.

In this study, the traditional Chinese formula of ZHHXS changed the diversity and structure of gut microbiota in layer hens under heat stress, and the change of some key phylotypes in gut microbiota may be responsible for the anti-heat stress effects of TCM.

MATERIALS AND METHODS

Animals

Thirty 35-week-old Roman egg-laying hens were purchased from Yuan Shi Laying Hens Breeding Co., Ltd. (Guangzhou, China) and housed in standard environmental conditions. The ethical approval of the Animal Experiment Administration Committee of South China Agricultural University was obtained before the experiments began and all efforts were made to minimize the hens' suffering during the experiments. All the procedures involving the hens including their selection, management, and preparation throughout the experiments were conducted in strict accordance with Chinese legislation on the use and care of laboratory animals. The animals' housing, care, and handling were conducted at the Laboratory Animal Center of South China Agricultural University, Guangzhou, China.

Preparation of Zi Huang Huo Xiang San

The traditional Chinese medicine formula used in this study was Zi Huang Huo Xiang San (ZHHXS) (**Table 1**). It was composed of eight dried Chinese herbs, namely Echinacea root, Scutellaria, patchouli, Elsholtzia, Gypsum, dried tangerine peel, white atractylodes rhizome, and licorice, which were mixed in the dry weight ratio of 4:4:3:3:2:2:1:1. The herbs were purchased from qualified suppliers based on standards specified in the *Chinese Pharmacopoeia* (Guangzhou, China). The herbs were crushed

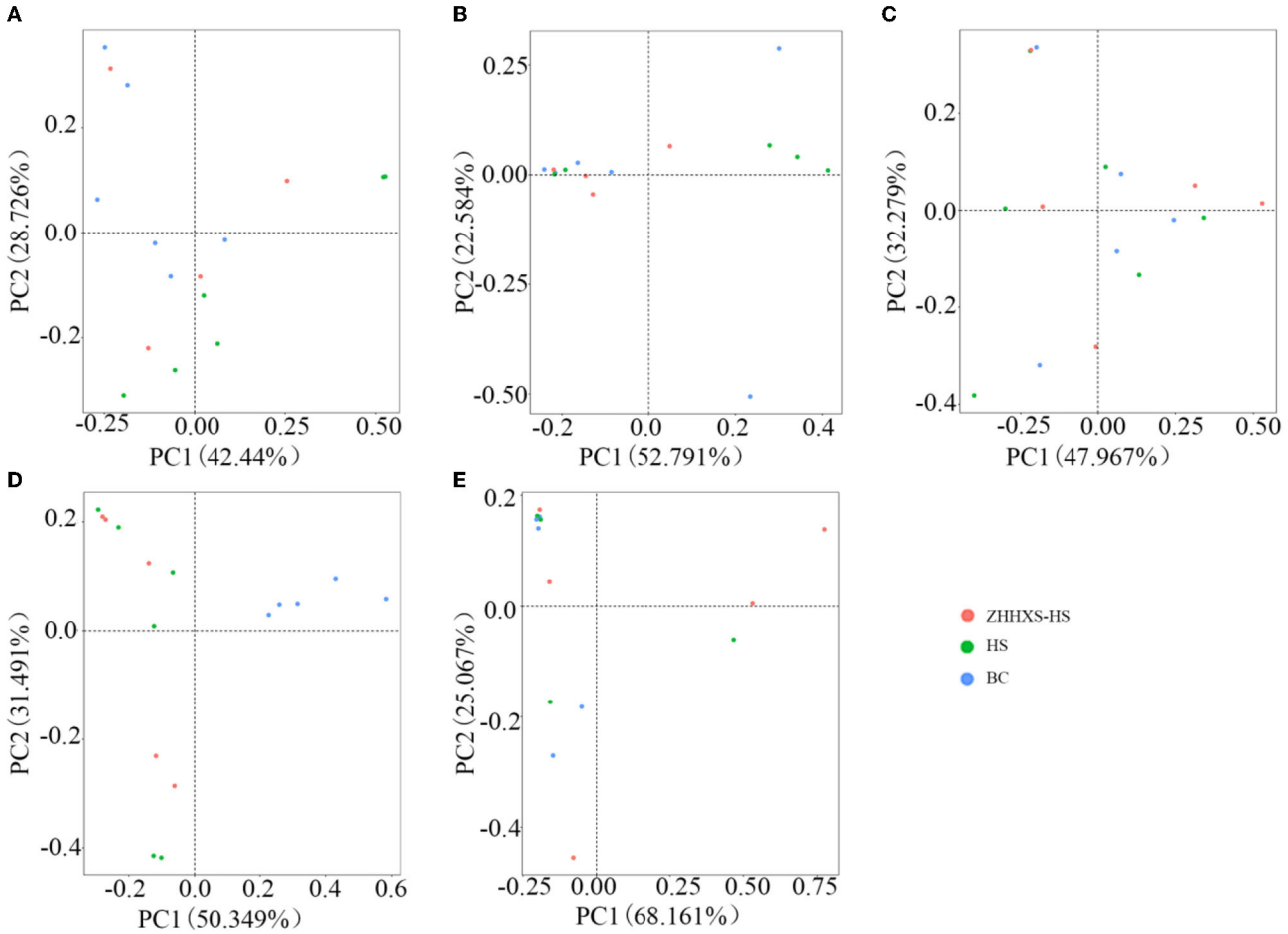


FIGURE 3 | The structural changes of gut microbiota based on Principal component analysis (PCA). **(A)** The PCA on day 1 in different groups; **(B)** PCA on day 3 in different groups; **(C)** PCA on day 7 in different groups; **(D)** PCA on day 14 in different groups; **(E)** PCA on day 28 in different groups.

with a pulverizer and sifted using 80 mesh sieves. The materials were mixed to feed the hens for a basal diet supplemented with 1% of the mixture.

Reagents

A TIANamp Stool DNA Kit (DP328) manufactured by Tiangen Biotech Co., Ltd. (Beijing, China) was used in this study. D1K ScreenTape and D1K Reagent manufactured by Agilent Technologies were also utilized. A Qubit dsDNA HS Assay Kit was purchased from Life Technologies. A TruSeq Custom Amplicon Sample Prep Kit and a MiSeq Reagent Kit v3 (600 Cycles PE) were purchased from Illumina.

Experimental Design

Thirty Roman egg layers were randomly divided into 3 groups with 10 hens in each group: a heat-stressed group (HS), for which the heater temperature is maintained at 34 ± 2°C for 28 days, a ZHHXS treatment group (ZHHXS-HS), maintained at a temperature 34 ± 2°C with a basal diet supplemented with 1% ZHHXS from 1 to 28 days, and a blank control group (BC) maintained at a temperature 25 ± 1°C for 28 days. Hens in the HS and BC groups were provided a basal diet and free access to drinking water. The basal diet was shown in **Table 2**. The 200-mg feces were collected on days 1, 3, 7, 14, and 28. All feces samples were stored in a −80°C freezer until analysis.

Bioinformatics Statistical Analysis

The sequence length, OTU (Operational Taxonomic Unit) numbers, and rarefaction curve (Chao1, Shannon, Good's coverage, and rank abundance) were performed using mothur on a single-summary command. Beta diversity analysis consisted of principal component analysis (PCA) and PCoA based on the Unifrac distance metric. The statistical analyses of the relative abundance of the phylum and genus levels, a taxonomy-based analysis, were carried out by analysis of variance (ANOVA) with SPSS 19.0. Values of $P < 0.05$ were considered statistically significant. The bar graph of the phylum and genus was produced with GraphPad Prism 5 software.

RESULTS

The Sequence of Gut Microbiota

After polymerase chain reaction (PCR), all of the feces samples were sequenced by an Illumina MiSeq sequencer, which was

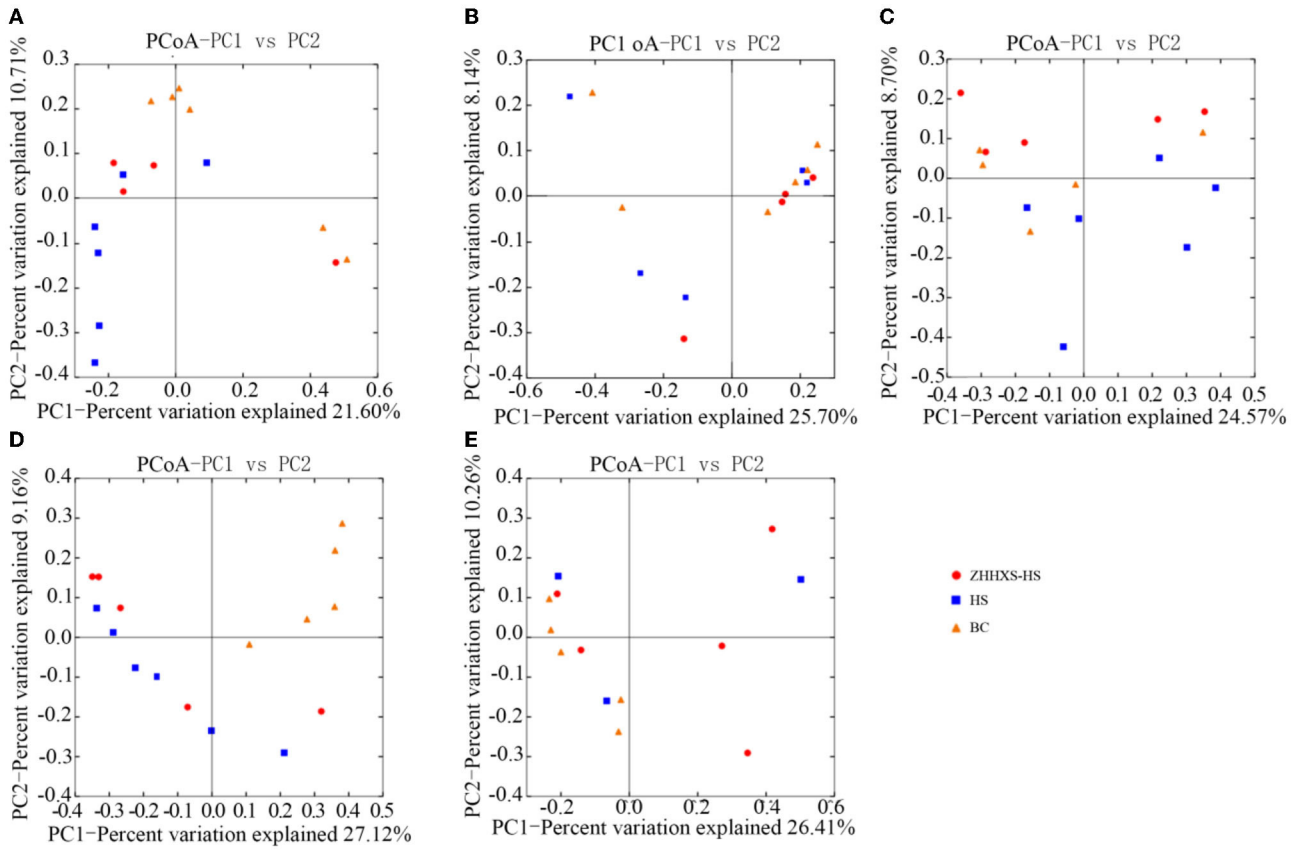


FIGURE 4 | Changes of intestinal microflora composition based on unweighted principal coordinate analysis. The PCoA distribution with unweighting shows on day 1 **(A)**, on day 3 **(B)**, on day 7 **(C)**, on day 14 **(D)**, and on day 28 **(E)** in different groups.

used to monitor the structural changes in the three groups' gut microbiota. Sequence lengths of <200 bp were removed, 12,853,330 sequences were gained, and the average length was 480 bp (**Figure 1**). A total of 737,136 OTUs were generated through clustering analysis for high-quality sequences at a 97% similarity cut-off. The Chao1, Shannon, Good's coverage, and rank abundance curves that were generated from the OTUs suggested that high-sample coverage was captured with the sequencing depth (**Figure 2**), and further increases in the sequencing depth were unlikely to achieve greater gut microbiota diversity.

The Gut Microbiota Composition Overtime After ZHHXS

Principal component analysis, an unsupervised multivariate statistical method, was used to analyze the composition changes in the hens' gut microbiota (**Figure 3**). A plot of the PCA scores showed that the gut microbiota composition in the BC group was significantly different from the HS group along PC2 on day 1 and PC1 on days 3 and 14, and there was a similar trend in the ZHHXS-HS group on days 1 and 3. However, there were no significant differences among the BC, HS, and ZHHXS-HS groups on days 7 and 28.

UniFrac distance-based PCoA, an unsupervised multivariate statistical method that includes weighted and unweighted distributions, was also used to analyze the gut microbiota composition (**Figures 4**, **5**). The PCoA unweighted distribution showed that the gut microbiota composition in the BC group differed from the HS group along PC2 on days 1 and 7, and along PC1 and PC2 on days 14. However, there were no significant differences between the BC and ZHHXS-HS groups on days 1 and 7 and among the BC, HS, and ZHHXS-HS groups on days 3 and 28. The plot of the weighted PCoA showed comparable changes in the composition of gut microbiota between the BC and HS groups along PC2 on days 1 and 14, PC1 and PC2 on day 3, and no significant difference between the BC and ZHHXS-HS groups on days 1 and 3. There were no differences among the three groups on days 7 and 28.

Key Phylotypes of Hens' Gut Microbiota Changed Due to ZHHXS

Obvious changes in the key phylotypes of gut microbiota at both the phylum and genus levels were found through a taxon-based analysis among the three groups. At the phylum level, Firmicutes, Proteobacteria, and Bacteroidetes were the main groups (**Figure 6**). The relative abundance of Actinobacteria in

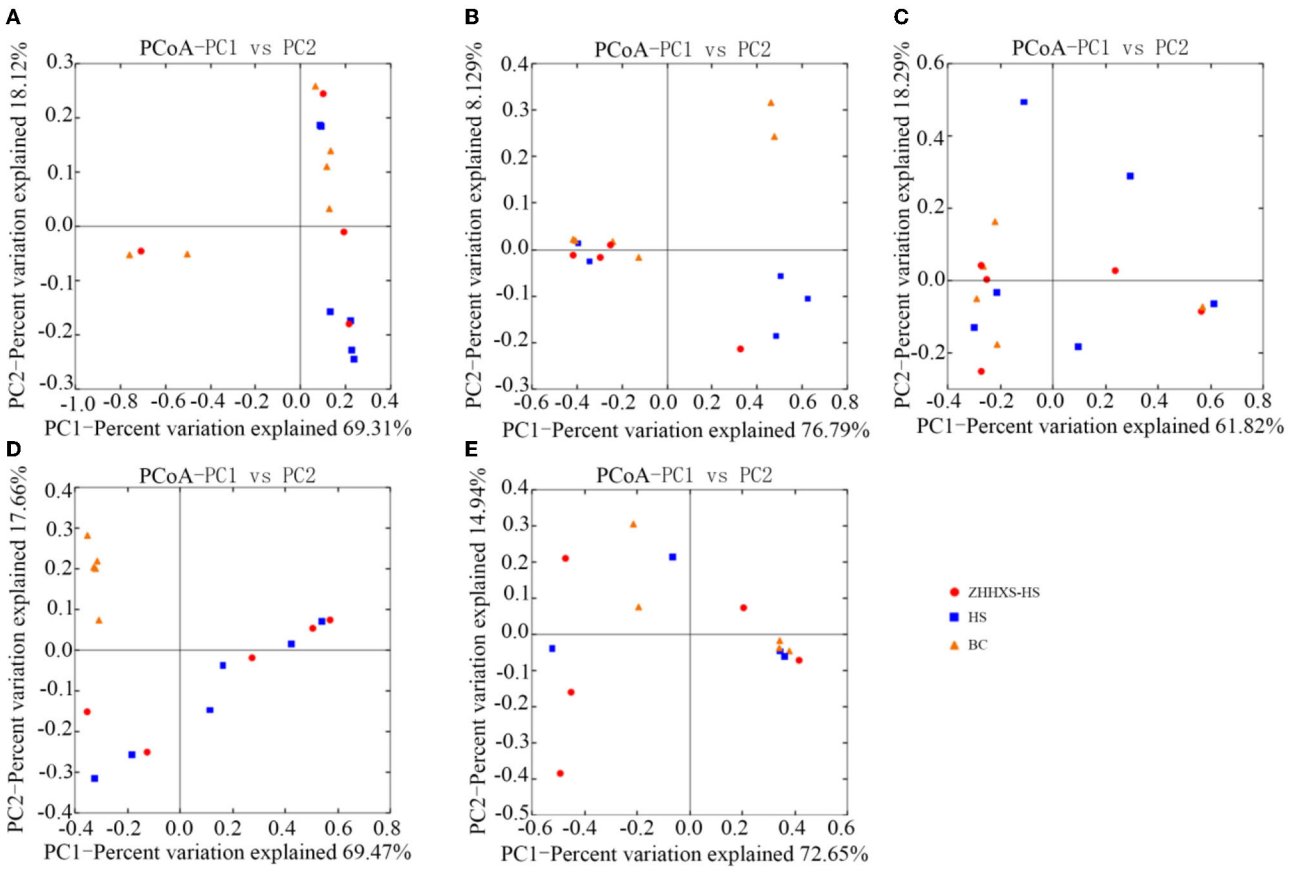


FIGURE 5 | The composition changes of gut microbiota based on Principal coordinate analysis (PCoA) with weight. The PCoA distribution with weight shows on day 1 **(A)**, on day 3 **(B)**, on day 7 **(C)**, on day 14 **(D)**, and on day 28 **(E)** in different groups.

the ZHHXS-HS group was markedly increased compared to the BC group on days 3, 7, and 14 and Bacteroidetes on days 14. There was no distinct difference between the HS and BC groups. The relative abundance of Firmicutes in the HS group differed significantly from the BC group on day 14 and Proteobacteria on day 1. The relative abundance of Firmicutes in the ZHHXS-HS group differed significantly from the BC group.

A taxon-based analysis at the genus level revealed that *Lactobacillus*, *Veillonella*, *Enterococcus*, and *Bacteroides* were the dominant genus (**Figure 7**). The relative abundance of *Bacteroides* and *Enterococcus* changed between the HS and BC groups, and the ZHHXS-HS group had no significant change compared to the HS and BC groups on day 14. The relative abundance of *Lactobacillus* in the BC group significantly increased and *Oscillospira* and *Ruminococcus* decreased compared to the HS and ZHHXS-HS groups at day 14. There were no obvious changes between the HS and ZHHXS-HS groups. There were no marked differences among the three groups on days 1, 3, 7, and 28 in the six genera.

DISCUSSION

This study showed that gut microbiota differed among the ZHHXS-HS, HS, and BC groups. The results indicated that a traditional Chinese herbal formula is credible for heat stress. In agreement with this paper, other studies have reported that traditional Chinese medicine could treat heat stress (26–28). Scutellaria baicalensis, with the functions of clearing away heat and dampness, purging fire, and detoxification, has shown significant effects on the treatment of various diseases, especially hepatitis, diarrhea, vomiting, and high blood pressure (29).

The results of the study of the rarefaction curve showed that the sequencing depth was sufficient to cover the gut microbiota. The results also indicated that the composition of gut microbiota in the feces was markedly different among the ZHHXS-HS, HS, and BC groups. The PCA showed that the composition of gut microbiota in the BC group differed from the HS group and had a similar trend to the ZHHXS-HS group. The PCoA indicated that BC differed from the HS group, but there was no difference from the ZHHXS-HS group in the gut microbiota composition. However, the results of the PCA and PCoA differed at varying times. Other research has indicated that Chinese medicine compounds can change the composition of gut microbiota, although there is little similar research into heat stress (21, 30, 31).

Significant differences were observed at key phylum and genus levels. The relative abundance of Actinobacteria and Bacteroidetes was significantly increased in the ZHHXS group in this study. The results showed that the increased relative

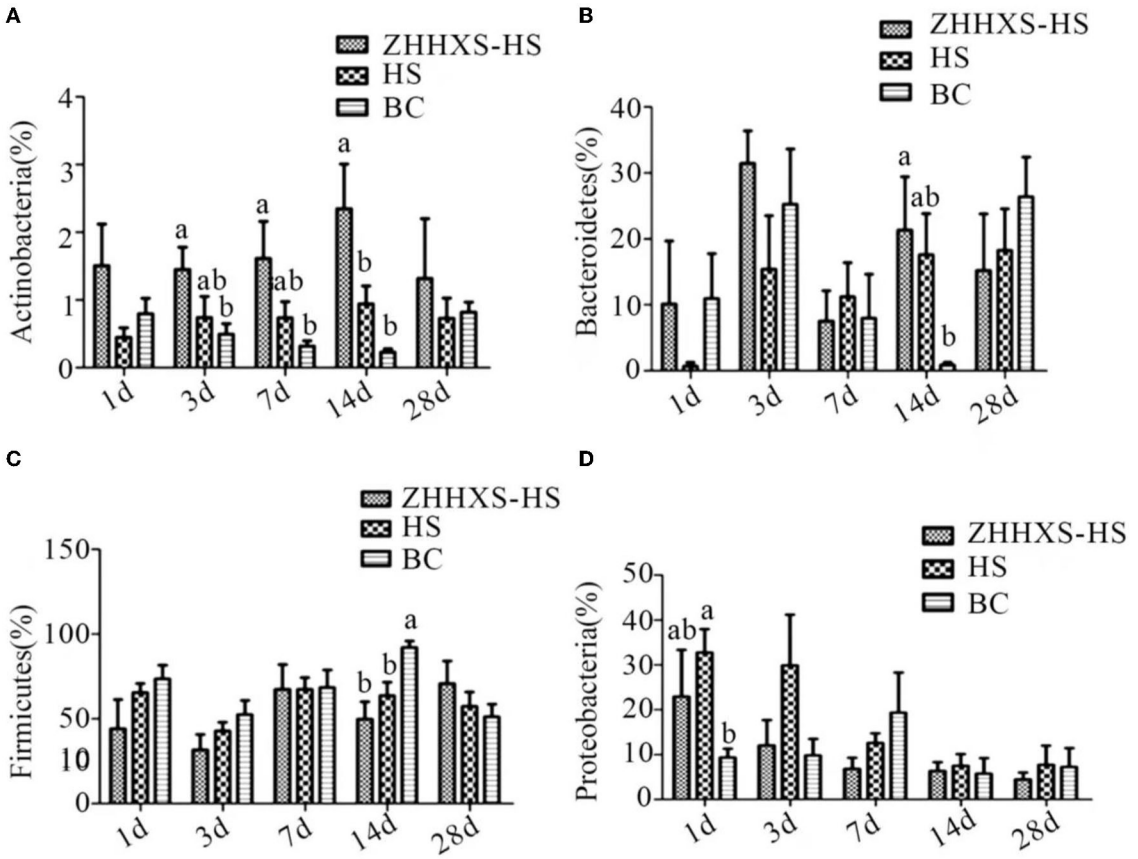


FIGURE 6 | The changes of abundance at the phylum level through taxon-based analysis. **(A–D)** shows the abundance of *Actinobacteria, Bacteroidetes, Firmicutes,* and *Proteobacteria* among different groups across time, respectively. The significance of variance was analyzed by one-way ANOVA. The different letter represents $p < 0.05$ and there was an obvious difference.

abundance of Proteobacteria caused by heat stress was reduced by ZHHXS. Firmicutes did not change with ZHHXS. This result is consistent with a report by Ying Chen that TCM can change the abundance of phylum (31). The relative abundance of *Bacteroides*, a short-chain fatty acid producer that is vital to the growth of gut microbiota (32, 33), increased due to heat stress compared to the BC group, and then decreased by ZHHXS. The changing abundance of *Enterococcus* was stabilized with ZHHXS. The growth promotion in fish and the multiple drug resistance of *Enterococcus* has been documented (34–36). These results further suggest that heat stress using ZHHXS may be mediated by changing the relative abundance of gut microbiota. However, there is no time-dependent change in gut microbiota by ZHHXS, which may be because gut microbiota is susceptible to another factor. Many other studies have shown that TCM can alter the abundance of the different genus to alleviate the severity of the disease (30, 31, 37). Atractylodes macrocephala Koidz. (called Baizhu in China) has long been used to treat gastrointestinal dysfunction. Crude extracts and pure compounds of Atractylodes macrocephala are used to treat gastrointestinal hypofunction and splenic asthenia (38). Pogostemonis Herba is usually used for the treatment of vomiting, abdominal pain, and diarrhea with the function of aromatic damp-resolving (39).

In the current study, the relative abundance of *Lactobacillus*, *Oscillospira*, and *Ruminococcus* was not correlated with ZHHXS and was positively correlated with a lack of treatment, suggesting that *Lactobacillus*, *Oscillospira*, and *Ruminococcus* might be phylotypes associated with the occurrence of heat stress. Heat stress with ZHHXS may be regulated by changing the relative abundance of *Veillonella*, although there was no significant variation among the three groups.

CONCLUSION

This study suggested that the ZHHXS, a Chinese herbal formula, played a vital role in modulating gut microbiota during the treatment of layer hens with heat stress. By comparing the BC group, the ZHHXS-HS group showed that the relative abundance of Bacteroides and Enterococcus shifted to the same tendency. The relative abundance of Actinobacteria and Bacteroidetes became enriching through the ZHHXS on heat stress. So, the

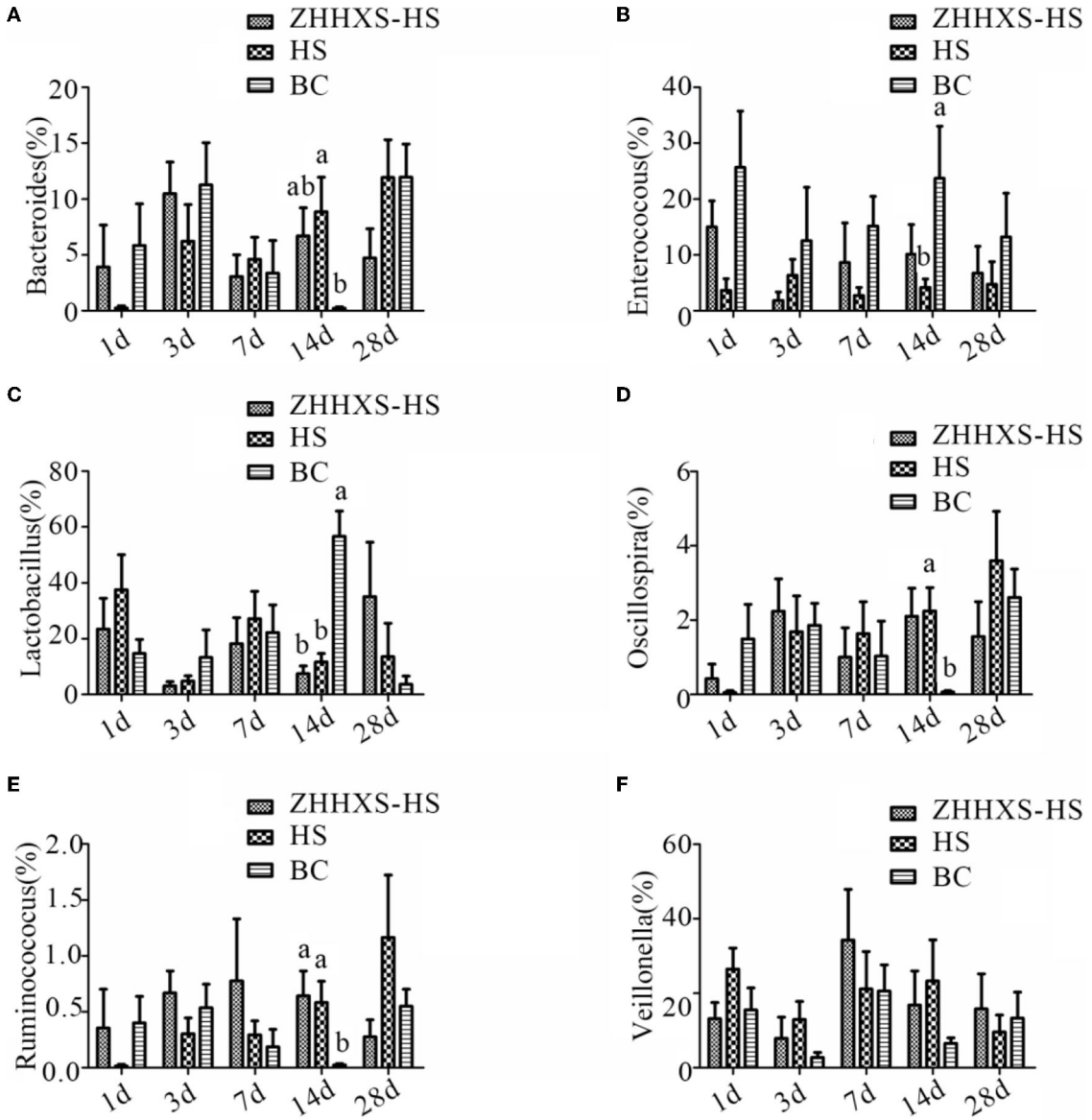


FIGURE 7 | The changes of abundance at the genus level through taxon-based analysis. **(A–F)** shows the abundance of *Bacteroides, Enterococcus, Lactobacillus, Oscillospira, Ruminococcus,* and *Veillonella* among different groups across time, respectively. The significance of variance was analyzed by one-way ANOVA. The different letter represents $p < 0.05$ and there was an obvious difference.

ZHHXS with the functions of clearing away heat and dampness, purging fire, and detoxification can modulate gut microbiota in heat stress. However, the causal relationship between Chinese herbal formula and gut microbiota remains unclear. This study provides evidence that the therapeutic effect of the Chinese herbal formula may function *via* the mediation of gut microbiota.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All experimental procedures used in this study were approved by the Animal Ethics Committee of the South China Agricultural University (Guangzhou, China). The care and use of all animals were performed according to the Guidelines for Animal Experiments of the South China Agricultural University.

AUTHOR CONTRIBUTIONS

DS and CL conceived and designed the experiments. CY, QQ, and LB performed the experiments and collected and analyzed the data. CY and QQ wrote the article, while ZC, JS, and JC revised the article. All authors read and approved the final manuscript.

FUNDING

This study was supported by the Natural Science Foundation of Guangdong Province (2021A1515011010) and the National Natural Science of Foundation (31672594 and 31602096).

REFERENCES

1. St-Pierre NR, Cobanov B, Schnitkey G. Economic losses from heat stress by US livestock industries1. *J Dairy Sci.* (2003) 86:E52–77. doi: 10.3168/jds.S0022-0302(03)74040-5
2. Xu Y, Lai X, Li Z, Zhang X, Luo Q. Effect of chronic heat stress on some physiological and immunological parameters in different breed of broilers. *Poult Sci.* (2018) 97:4073–82. doi: 10.3382/ps/pey256
3. Song ZH, Cheng K, Zheng XC, Ahmad H, Zhang LL, Wang T. Effects of dietary supplementation with enzymatically treated *Artemisia annua* on growth performance, intestinal morphology, digestive enzyme activities, immunity, and antioxidant capacity of heat-stressed broilers. *Poult Sci.* (2018) 97:430–7. doi: 10.3382/ps/pex312
4. Cramer TA, Kim HW, Chao Y, Wang W, Cheng HW, Kim Y. Effects of probiotic (*Bacillus subtilis*) supplementation on meat quality characteristics of breast muscle from broilers exposed to chronic heat stress. *Poult Sci.* (2018) 97:3358–68. doi: 10.3382/ps/pey176
5. Tsiouris V, Georgopoulou I, Batzios C, Pappaioannou N, Ducatelle R, Fortomaris P. Heat stress as a predisposing factor for necrotic enteritis in broiler chicks. *Avian Pathol.* (2018) 47:616–24. doi: 10.1080/03079457.2018.1524574
6. Alhenaky A, Abdelqader A, Abuajamieh M, Al-Fataftah AR. The effect of heat stress on intestinal integrity and Salmonella invasion in broiler birds. *J Therm Biol.* (2017) 70:9–14. doi: 10.1016/j.jtherbio.2017.10.015
7. Qu Q, Li H, Bai L, Zhang S, Sun J, Lv W, et al. Effects of heat stress on gut microbiome in rats. *Indian J Microbiol.* (2021) 61:338–47. doi: 10.1007/s12088-021-00948-0
8. Yin C, Xia B, Tang S, Cao A, Liu L, Zhong R, et al. The effect of exogenous bile acids on antioxidant status and gut microbiota in heat-stressed broiler chickens. *Front Nutr.* (2021) 8:747136. doi: 10.3389/fnut.2021.747136
9. Jadhav NV, Awati B, Kulkarni S, Waghmare PG, Suranagi MD, Ravikanth K, et al. Heat stress amelioration and production performance in layers supplemented with herbal liquid anti-stressor product. *J Vet Med Anim Health.* (2014) 6:69–74. doi: 10.5897/JVMAH2013.0214
10. He J, He Y, Pan D, Cao J, Sun Y, Zeng X. Associations of gut microbiota with heat stress-induced changes of growth, fat deposition, intestinal morphology, and antioxidant capacity in ducks. *Front Microbiol.* (2019) 10:903. doi: 10.3389/fmicb.2019.00903
11. Shi D, Bai L, Qu Q, Zhou S, Yang M, Guo S, et al. Impact of gut microbiota structure in heat-stressed broilers. *Poult Sci.* (2019) 98:2405–13. doi: 10.3382/ps/pez026
12. Wang HY, Qi LW, Wang CZ, Li P. Bioactivity enhancement of herbal supplements by intestinal microbiota focusing on ginsenosides. *Am J Chin Med.* (2011) 39:1103–15. doi: 10.1142/S0192415X11009433
13. Feng Q, Liu W, Baker SS, Li H, Chen C, Liu Q, et al. Multi-targeting therapeutic mechanisms of the Chinese herbal medicine QHD in the treatment of non-alcoholic fatty liver disease. *Oncotarget.* (2017) 8:27820–38. doi: 10.18632/oncotarget.15482
14. Zhao L, Nicholson JK, Lu A, Wang Z, Tang H, Holmes E, et al. Targeting the human genome-microbiome axis for drug discovery: inspirations from global systems biology and traditional Chinese medicine. *J Proteome Res.* (2012) 11:3509–19. doi: 10.1021/pr3001628
15. Xu J, Chen HB, Li SL. Understanding the molecular mechanisms of the interplay between herbal medicines and gut microbiota. *Med Res Rev.* (2017) 37:1140–85. doi: 10.1002/med.21431
16. Cao ZJ, Yu JC, Kang WM, Ma ZQ. [Research advances in the gut microbiota and inflammation in obesity]. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao.* (2013) 35:462–5. doi: 10.3881/j.issn.1000-503X.2013.04.020
17. Guarner F, Malagelada JR. Gut flora in health and disease. *Lancet.* (2003) 361:512–9. doi: 10.1016/S0140-6736(03)12489-0
18. Donaldson GP, Lee SM, Mazmanian SK. Gut biogeography of the bacterial microbiota. *Nat Rev Microbiol.* (2016) 14:20–32. doi: 10.1038/nrmicro3552
19. Liu C, Zhang C, Lv W, Chao L, Li Z, Shi D, et al. Structural modulation of gut microbiota during alleviation of suckling piglets diarrhoea with herbal formula. *Evid Based Complement Alternat Med.* (2017) 2017:8358151. doi: 10.1155/2017/8358151
20. Lv W, Liu C, Ye C, Sun J, Tan X, Zhang C, et al. Structural modulation of gut microbiota during alleviation of antibiotic-associated diarrhea with herbal formula. *Int J Biol Macromol.* (2017) 105:1622–9. doi: 10.1016/j.ijbiomac.2017.02.060
21. Xu J, Lian F, Zhao L, Zhao Y, Chen X, Zhang X, et al. Structural modulation of gut microbiota during alleviation of type 2 diabetes with a Chinese herbal formula. *ISME J.* (2015) 9:552–62. doi: 10.1038/ismej.2014.177
22. Feng W, Liu J, Tan Y, Ao H, Wang J, Peng C. Polysaccharides from Atractylodes macrocephala Koidz. Ameliorate ulcerative colitis via extensive modification of gut microbiota and host metabolism. *Food Res Int.* (2020) 138:109777. doi: 10.1016/j.foodres.2020.109777
23. Li C, He L, Dong H, Jin J. Screening for the anti-inflammatory activity of fractions and compounds from Atractylodes macrocephala Koidz. *J Ethnopharmacol.* (2007) 114:212–17. doi: 10.1016/j.jep.2007.08.002
24. Amin A, Hossen MJ, Fu X, Chou J, Wu J, Wang X, et al. Inhibition of the Akt/NF-κB pathway is involved in the anti-gastritis effects of an ethanolic extract of the rhizome of Atractylodes macrocephala. *J Ethnopharmacol.* (2022) 293:115251. doi: 10.1016/j.jep.2022.115251
25. Cui L, Guan X, Ding W, Luo Y, Wang W, Bu W, et al. *Scutellaria baicalensis* Georgi polysaccharide ameliorates DSS-induced ulcerative colitis by improving intestinal barrier function and modulating gut microbiota. *Int J Biol Macromol.* (2021) 166:1035–45. doi: 10.1016/j.ijbiomac.2020.10.259
26. Dong H, Zhong Y, Liu F, Yang K, Yu J, Xu J. Regulating effects and mechanisms of Chinese medicine decoction on growth and gut hormone expression in heat stressed pigs. *Livest Sci.* (2012) 143:77–84. doi: 10.1016/j.livsci.2011.08.015
27. Song X, Xu J, Wang T, Liu F. Traditional Chinese medicine decoction enhances growth performance and intestinal glucose absorption in heat stressed pigs by up-regulating the expressions of SGLT1 and GLUT2 mRNA. *Livest Sci.* (2010) 128:75–81. doi: 10.1016/j.livsci.2009.11.002
28. Song X, Luo J, Fu D, Zhao X, Bunlue K, Xu Z, et al. Traditional Chinese medicine prescriptions enhance growth performance of heat stressed beef cattle by relieving heat stress responses and increasing apparent nutrient digestibility. *Asian Australas J Anim Sci.* (2014) 27:1513–20. doi: 10.5713/ajas.2014.14058
29. Zhao T, Tang H, Xie L, Zheng Y, Ma Z, Sun Q, et al. *Scutellaria baicalensis* Georgi. (Lamiaceae): a review of its traditional uses, botany, phytochemistry, pharmacology and toxicology. *J Pharm Pharmacol.* (2019) 71:1353–69. doi: 10.1111/jphp.13129
30. Yin X, Peng J, Zhao L, Yu Y, Zhang X, Liu P, et al. Structural changes of gut microbiota in a rat non-alcoholic fatty liver disease model treated with a Chinese herbal formula. *Syst Appl Microbiol.* (2013) 36:188–96. doi: 10.1016/j.syapm.2012.12.009
31. Chen Y, Xiao S, Gong Z, Zhu X, Yang Q, Li Y, et al. Wuji Wan formula ameliorates diarrhea and disordered colonic motility in post-inflammation irritable bowel syndrome rats by modulating the gut microbiota. *Front Microbiol.* (2017) 8:2307. doi: 10.3389/fmicb.2017.02307

32. Jumpertz R, Le DS, Turnbaugh PJ, Trinidad C, Bogardus C, Gordon JI, et al. Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. *Am J Clin Nutr.* (2011) 94:58–65. doi: 10.3945/ajcn.110.010132
33. Mohd SM, Sieo CC, Chong CW, Gan HM, Ho YW. Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. *Gut Pathog.* (2015) 7:4. doi: 10.1186/s13099-015-0051-7
34. Leclercq R. Enterococci acquire new kinds of resistance. *Clin Infect Dis.* (1997) 24(Suppl. 1):S80–4. doi: 10.1093/clinids/24.Supplement_1.S80
35. Ghori I, Tabassum M, Ahmad T, Zuberi A, Imran M. *Geotrichum candidum* enhanced the *Enterococcus faecium* impact in improving physiology, and health of *Labeo rohita* (Hamilton, 1822) by modulating gut microbiome under mimic aquaculture conditions. *Turk J Fish Aquat Sc.* (2018) 18:1255–67. doi: 10.4194/1303-2712-v18_11_02
36. Landman D, Quale JM. Management of infections due to resistant enterococci: a review of therapeutic options. *J Antimicrob Chemother.* (1997) 40:161–70. doi: 10.1093/jac/40.2.161
37. Yu J, Guo J, Tao W, Liu P, Shang E, Zhu Z, et al. Gancao-Gansui combination impacts gut microbiota diversity and related metabolic functions. *J Ethnopharmacol.* (2018) 214:71–82. doi: 10.1016/j.jep.2017.11.031
38. Zhu B, Zhang QL, Hua JW, Cheng WL, Qin LP. The traditional uses, phytochemistry, and pharmacology of Atractylodes macrocephala Koidz.: a review. *J Ethnopharmacol.* (2018) 226:143–67. doi: 10.1016/j.jep.2018.08.023
39. Xu F, Cai W, Ma T, Zeng H, Kuang X, Chen W, et al. Traditional uses, phytochemistry, pharmacology, quality control, industrial application, pharmacokinetics and network pharmacology of pogostemon cablin: a comprehensive review. *Am J Chin Med.* (2022) 50:691–721. doi: 10.1142/S0192415X22500288

Conflict of Interest: LB was employed by Wens Foodstuff Group Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Veterinary Science

TYPE Brief Research Report
PUBLISHED 28 July 2022
DOI 10.3389/fvets.2022.940395

OPEN ACCESS

EDITED BY
Dayou Shi,
South China Agricultural University, China

REVIEWED BY
Panagiotis Tassis,
Aristotle University of Thessaloniki, Greece
Dan Yi,
Wuhan Polytechnic University, China

*CORRESPONDENCE
Ha Hoang Chu
chuhoangha@ibt.ac.vn
Ngoc Bich Pham
pbngoc@ibt.ac.vn

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 10 May 2022
ACCEPTED 04 July 2022
PUBLISHED 28 July 2022

CITATION
Ho TT, Trinh VT, Tran HX, Le PTT, Nguyen TT, Hoang HTT, Pham MD, Conrad U, Pham NB and Chu HH (2022) The immunogenicity of plant-based COE-GCN4pII protein in pigs against the highly virulent porcine epidemic diarrhea virus strain from genotype 2. *Front. Vet. Sci.* 9:940395. doi: 10.3389/fvets.2022.940395

The immunogenicity of plant-based COE-GCN4pII protein in pigs against the highly virulent porcine epidemic diarrhea virus strain from genotype 2

Thuong Thi Ho[1,2], Vy Thai Trinh[1,2], Hanh Xuan Tran[3], Phuong Thu Thi Le[3], Tra Thi Nguyen[1,2], Hang Thu Thi Hoang[2], Minh Dinh Pham[2], Udo Conrad[4], Ngoc Bich Pham[1,2]* and Ha Hoang Chu[1,2]*

[1]Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam, [2]Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam, [3]JSC Central Veterinary NAVETCO, Ho Chi Minh, Vietnam, [4]Department Molecular Genetics, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany

Porcine epidemic diarrhea virus (PEDV) is a serious infectious causative agent in swine, especially in neonatal piglets. PEDV genotype 2 (G2) strains, particularly G2a, were the primary causes of porcine epidemic diarrhea (PED) outbreaks in Vietnam. Here, we produced a plant-based CO-26K-equivalent epitope (COE) variant from a Vietnamese highly virulent PEDV strain belonging to genotype 2a (COE/G2a) and evaluated the protective efficacy of COE/G2a-GCN4pII protein (COE/G2a-pII) in piglets against the highly virulent PEDV G2a strain following passive immunity. The 5-day-old piglets had high levels of PEDV-specific IgG antibodies, COE-IgA specific antibodies, neutralizing antibodies, and IFN-γ responses. After virulent challenge experiments, all of these piglets survived and had normal clinical symptoms, no watery diarrhea in feces, and an increase in their body weight, while all of the negative control piglets died. These results suggest that the COE/G2a-pII protein produced in plants can be developed as a promising vaccine candidate to protect piglets against PEDV G2a infection in Vietnam.

KEYWORDS

COE, GCN4-pII motif, PEDV genotype 2, plant-based vaccine, piglet protection, passive immunity

Introduction

Porcine epidemic diarrhea (PED) is an acute and highly contagious enteric disease in swine, caused by the porcine epidemic diarrhea virus (PEDV) (1). Pigs of all ages can be infected by PEDV, resulting in the rapid spread of acute and severe diarrhea, dehydration, and vomiting. Suckling piglets, especially at the age of under 7 days, are the most severely

affected with mortality reaching nearly 100% (2). Since the first outbreaks in Europe, PEDV has caused severe economic loss to the pig industry worldwide, particularly in Asian countries and the United States (1, 3, 4). In Vietnam, PED was first reported in 2009 and then continuously spread in several pig farms throughout the country, causing remarkable economic damage (5).

Porcine epidemic diarrhea virus is a single-stranded RNA virus and a member of the genus Alphacoronavirus, in the family Coronaviridae (6). PEDV can be clustered into two genogroups (G1 and G2) based on the nucleotide and amino acid sequence of Spike (S), especially the changes in amino acid sequence at the N-terminal of the S1 domain (7, 8). Each genogroup contains subgroups a and b. PEDV strains circulating in the north, central, and southern regions of Vietnam from 2015 to 2016 belonged to G1b, G2a, and G2b (4, 9). The differentiation in genetic information between the emerging PEDV strains and PEDV vaccine strains, especially at the neutralizing epitopes, results in the low effectiveness of the current vaccine in the protection of pigs against PEDV strains in the field (9–11). In Vietnam, PEDV G2 strains were found as the major disease causative agents in PED outbreaks (4). To date, subunit vaccines against Vietnamese PEDV G2 strains are not available. Therefore, the development of a novel vaccine against emerging PEDV strains, especially G2 strains, is urgent to control PED outbreaks in Vietnam (4, 9).

The CO-26K-equivalent epitope (COE) is located in amino acids 499–638 within the Spike (S) protein (12). It is a neutralizing epitope recognized by the monoclonal antibody (6). COE protein is considered an important target for developing subunit vaccines against PEDV (12, 13). The plant-based production systems, especially agroinfiltration, have been widely considered a rapid, convenient, and low-cost method to produce a large number of recombinant proteins within a few days in several plants (14, 15). There have been efforts to produce S1 or COE protein as fusion forms against PEDV in various plants such as lettuce, rice, *Nicotiana benthamiana*, *Nicotiana tabacum*, and maize using stable transformation or transient expression (13, 16–21). In a previous study, we demonstrated that plant-based COE/G1a-pII induced neutralizing antibodies in mice (22). However, the immunogenicity and protectivity of plant-based COE/G1a-pII in piglets against PEDV *via* challenge experiments have still not been evaluated.

This study aims to evaluate whether our previous concept could also be worked on another COE variant from a highly virulent PEDV strain belonging to G2 (COE/G2a-pII), which is the currently circulating strain in Vietnam. Moreover, we analyzed if a two-dose vaccination scheme with COE/G2a-pII could induce protective immune responses in piglets from virulent PEDV strains *via* challenge experiments. As expected, all piglets from sows vaccinated with purified COE/G2a-pII protein survived, while all negative control piglets died after the challenge with the highly virulent PEDV G2a strain. In addition, COE/G2a-pII elicited high levels of neutralizing antibodies, PEDV-specific IgG antibodies, COE-specific IgA antibodies, and IFN-γ in sows and piglets.

Materials and methods

Production and characterization of COE/G2a-PII protein from plants

A DNA sequence encoding for the COE/G2a region from the highly virulent NAVET/PEDV/PS6/2010 strain (NAVETCO, Vietnam) belonging to G2a was codon-optimized for expression in *N. benthamiana*, synthesized, and inserted into a cloning vector pUC57-COE (Genewiz, United States). COE/G2a was amplified and inserted in pRTRA-COE/G1a-GCN4pII-cmyc-his-KDEL (22) at sites *Bam*HI and *psp*OMI. A plant expression vector containing COE/G2a-pII was then constructed according to the previous study (22). In this study, COE/G2a-pII protein was expressed in *N. benthamiana via* the agroinfiltration protocol that was mentioned in the previous study (22) with a modification in the OD_{600} agrobacterium suspension mixture of 0.1. After 3 days of infiltration, leaves were collected and stored at -80°C. The expression of COE/G2a-pII protein in plants was detected by SDS-PAGE and Western blot assay using anti-cmyc-antibody as the primary antibody and goat anti-mouse IgG-HRP (Invitrogen) as the secondary antibody. A signal was detected using the Amersham ECL Prime Western blotting detection reagent. The expression level of COE/G2a-pII protein was measured based on the H5N1-specific ScFv protein amount in a standard curve (23) that was conducted and analyzed using the ImageQuant TL 8.0 software (Cytiva) after the signal was detected using the Amersham™ Imager 680 machine. Leaves were subjected to COE/G2a-pII protein purification using the immobilized metal affinity chromatography (IMAC) protocol that was reported in the previous study with a modification in washing buffer containing 10 mM of imidazole (22).

The molecular weight of COE/G2a-pII protein was characterized by size exclusion chromatography (SEC) following the protocol as described earlier (24). An amount of 500 μg IMAC-purified COE/G2a-pII protein was applied onto a Superose™ 6 increase 10/300GL column (GE Healthcare). A high molecular weight kit (GE Healthcare) containing standard proteins with molecular weights ranging from 75 to 2,000 kDa was used to estimate the molecular weight of COE/G2a-pII protein. The presence of COE/G2a-pII protein in SEC fractions was confirmed by SDS-PAGE and Western blotting assay.

Cells and viruses

Vero cells (ATCC CCL-81) were cultured in Eagle's Minimum Essential Medium (EMEM, Gibco) supplemented

with 10% fetal bovine serum (FBS, Gibco), 50 μg/ml gentamycin at 37° C, and 5% CO_2. The NAVET/PEDV/PS6/2010 strain was isolated from piglet samples infected with PEDV and propagated on Vero cells using the protocol previously described (25). The virus in passage 5 was used for challenge experiments in piglets. There was no change in the nucleotide sequence of the S gene of the NAVET/PEDV/PS6/2010 strain in passage 5 when compared to that of the isolated PEDV strain.

Pig immunization and viral challenge experiments

The pig study was reviewed and approved by the ethical committees of the Institute of Biotechnology (IBT); Vietnam Academic of Science and Technology (VAST), Hanoi, Vietnam; and NAVETCO, Ho Chi Minh City, Vietnam, under decision number 07/2015/HĐ-NĐT. The animal protocols were performed based on the "3Rs" and the European Communities Council Directives of 24 November 1986 (86/609/EEC) guidelines for the care and use of animals. Pigs were raised by NAVETCO, Vietnam, and monitored to minimize the stress, suffering, unnecessary pain, or lasting harm in the experiments by veterinarians.

Before 2 weeks of vaccination, blood samples of pregnant sows were collected for serum neutralizing antibody assays performed at NAVETCO's laboratory. Two pregnant sows with negative results of neutralizing antibodies against PEDV were selected for the vaccination experiments. Either purified COE/G2a-pII protein or PBS was formulated extensively with in-house water in oil adjuvant (NAVETCO, Vietnam) at a ratio of 3:7. The primiparous sow (~80 days of gestation, $n = 1$) was intramuscularly immunized on the neck with either adjuvanted COE/G2a-pII protein (100 μg per dose) or PBS on days 0 and 14 post-immunization (pi). After 21 days of the second immunization, farrowing was induced. Sow blood samples were collected on days 0, 35, and 50 pi. Eight piglets born to one COE/G2a-pII-vaccinated sow and two piglets born to one PBS-vaccinated sow were suckled and kept together with their sows. Each 5-day-old piglet was challenged orally with 1 ml of the highly virulent NAVET/PEDV/PS6/2010 strain (10^3 TCID50). Piglets were monitored daily for clinical signs, diarrhea, weight, and death from days 0 to 14 post-challenge (pc). Clinical scores were modified from a scoring method previously described (26). Clinical scores were defined as follows: healthy = 0; anorectic, depression, vomiting, and emaciated = 1; dead = 2. The body weight of piglets was recorded on days 0 and 10 pc. Fecal scores were evaluated as follows based on a scoring method previously described (27): normal = 0, pasty = 1, semi-solid = 2, watery diarrhea = 3. Blood samples of all the piglets were collected *via* the marginal ear veins in serum separator tubes on days 0 and 10 pc for analysis. All blood samples were centrifuged at 3,000 × *g* for 10 min at 4°C, and the sera were stored at −20° C until analysis.

ELISA

PEDV-specific IgG antibodies in the sera of sows and piglets were assessed using a commercial ELISA kit (INgezim PEDV 11.PED.K.1/5, Eurofins INGENASA) according to the manufacturer's recommendation. The S/P values were calculated using the formula: [(test sample value–negative control value) / (positive control value–negative control values)]. Samples were defined as positive for PEDV-specific IgG if the S/P value is > 0.35. COE-specific IgA antibodies in sow and piglet sera were analyzed using an indirect ELISA using SEC-purified COE/G2a-pII protein (1 μg/ml) as antigen according to the protocol described previously (22).

Virus neutralizing antibody test

The presence of neutralizing antibodies in porcine sera was evaluated using a virus-neutralizing assay according to the protocol previously described (28). PEDV strain NAVET/PEDV/PS6/2010 (200 TCID50/0.1 ml) was performed for the assay. The virus neutralization titer (VN titer) was determined as the highest serum dilution that inhibited the cytopathic effect.

Cytokine assay

The level of interferon-gamma (IFN-γ) in the sera of sows and piglets was detected using a commercial ELISA kit (Porcine IFN-γ ELISA Basis kit, Matech) according to the manufacturer's recommendation. Porcine sera with a dilution of 1:20 were used for the cytokine assay. The concentration of IFN-γ in diluted sera (IFN-γ DL) was calculated based on the standard curve of recombinant porcine IFN- γ standard with the range of 16–1,600 pg/ml. The final concentration of IFN-γ in pig sera was calculated using the formula: IFN-γ DL × 20.

Statistical analysis

All statistical analyses were performed in the Sigma Plot software. The differences between the two groups were compared and shown as mean ± standard deviation (SD) using a Mann–Whitney U test. The significant difference was determined if the p-value was <0.05.

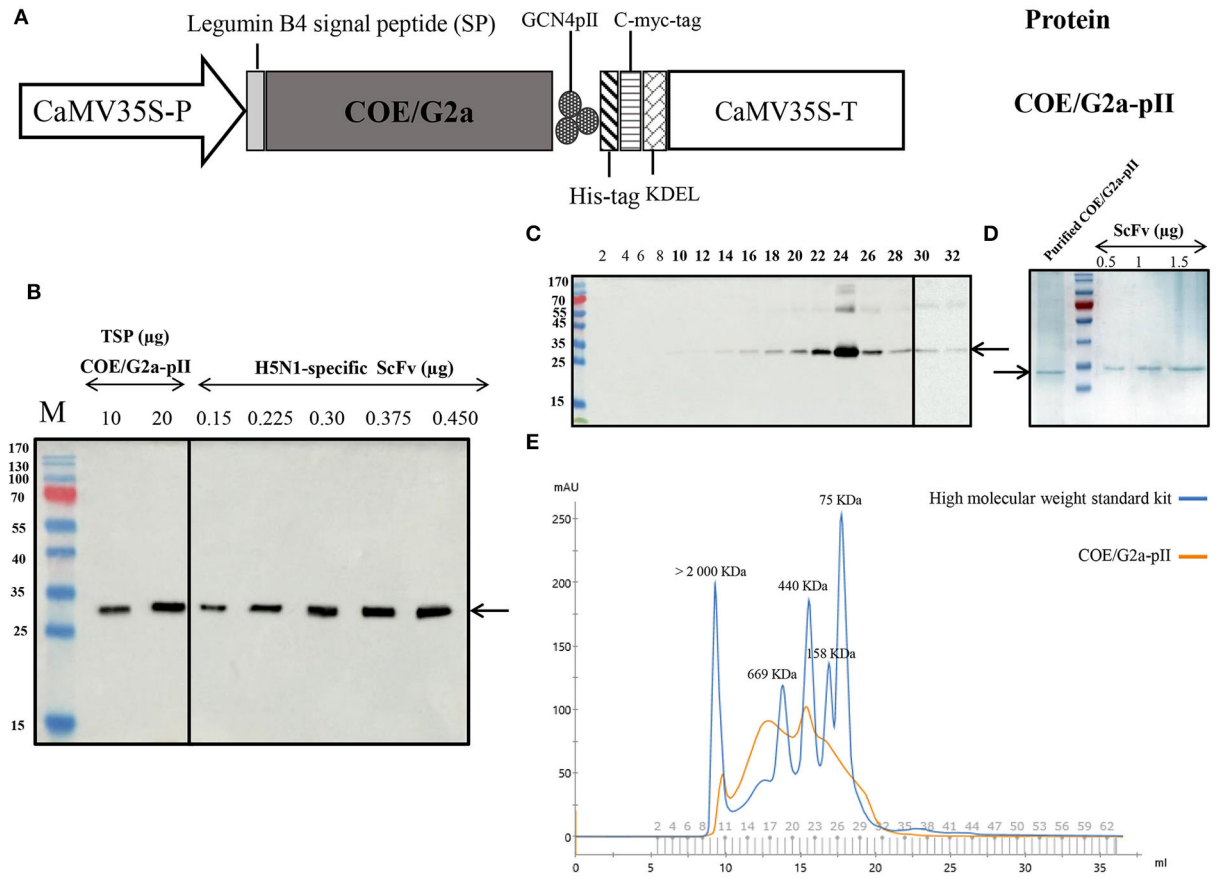


FIGURE 1
Production and characterization of COE/G2a-pII protein from plants. **(A)** Plant expression cassette containing a DNA sequence encoding the highly virulent NAVET/PEDV/PS6/2010 strain (NAVETCO, Vietnam) fused pII motif. CaMV35S-P, cauliflower mosaic virus (CaMV) 35 S promoter; pII, GCN4pII motif; KDEL, endoplasmic reticulum retention; CaMV35S-T, CaMV 35 S terminator. **(B)** Detection of COE/G2a protein expressed in *N. benthamiana* leaves by Western blotting assay. Anti-c-myc monoclonal antibody and HRP conjugated goat anti-mouse IgG were used as primary antibodies and secondary antibodies, respectively. Various amounts of H5N1-specific ScFv antibodies (23) were used to build the standard curve to calculate the accumulation of COE/G2a-pII protein in leaves by ImageQuant TL (Cytiva) after capturing by Amersham™ Imager 680 (Cytiva). **(C)** Detection of SEC-fractions containing COE/G2a-pII protein by Western blotting assay. **(D)** Analysis of IMAC-purified COE/G2a-pII protein by Coomassie Blue staining of SDS-PAGE. **(E)** Characterization of COE/G2a-pII protein by SEC. A standard kit including high molecular weight protein (75–2,000 kDa, GE) was used to estimate the molecular weight of COE/G2a-pII protein.

Results

Production and characterization of COE/G2a-PII

To construct the expression cassette harboring COE/G2a-pII, we replaced the DNA sequence encoding COE from the PEDV DR13 strain (G1a) with that encoding COE/G2a from the highly virulent NAVET/PEDV/PS6/2010 strain (Figure 1A). The expression of COE/G2a-pII protein in *N. benthamiana* leaves was successfully detected by a Western blotting test using anti-cmyc-antibody as the primary antibody (Figure 1B). The accumulation of COE/G2a-pII protein in leaves was calculated based on the standard curve of the H5N1-specific ScFv protein (23). COE/G2a-pII protein accumulated in plant leaves at approximately 118 mg/kg fresh leaves, which accounted for 2.01% of total soluble protein (TSP). COE/G2a-pII protein was purified by IMAC, and then the resulting product was further purified and characterized by SEC (Figures 1C,D). The predicted molecular weight of COE/G2a-pII monomer is approximately 26 kDa; however, N-glycosylation sites in COE protein may influence the PAGE separation resulting in the increase in band size of COE/G2a-pII protein in SDS-PAGE and Western blot of approximately 35 kDa (Figure 1D). The molecular weight of COE/G2a-pII was calculated and characterized by SEC based on the standard kit containing high molecular weight proteins (75 to >2,000 kDa). COE/G2a-pII protein was presented in SEC fractions 10–32 corresponding to the molecular weight of 75 to >2,000 kDa (Figure 1E). COE/G2a-pII protein was mostly detected in SEC fractions 22–26 corresponding to

the molecular weight of approximately 440 kDa. Therefore, COE/G2a-pII is a mixture of multimer forms with a wide range in molecular weight.

Humoral immune responses and cytokine responses in sows

The immunogenicity of IMAC-purified COE/G2a-pII protein was evaluated in pregnant sows. The immunization scheme in pregnant sows is presented in Figure 2A. PEDV-specific IgG responses were detected in the serum of the sow vaccinated with COE/G2a-pII protein with an S/P value of 2.49 on day 35 pi (Figure 2B). There was no increase in PEDV-specific IgG responses in this inoculated sow on day 50 pi compared to that on day 35 pi. In contrast, no PEDV-specific IgG antibody was found in the sow immunized with PBS. The pregnant sow vaccinated with COE/G2a-pII protein developed COE-specific IgA antibodies in serum on day 35 pi with an OD450 mean value of 3.8. Similar to the PEDV-specific IgG responses, the COE-specific IgA antibodies did not increase in the serum of this vaccinated sow on day 50 pi. In contrast, COE-specific IgA antibodies were not detected in the serum of the sow immunized with PBS (Figure 2C). The presence of neutralizing antibodies against PEDV in sow sera was assessed by virus-neutralizing antibody assay. On day 35 pi, the VN titer of 32 was observed in the sow vaccinated with COE/G2a-pII protein. However, the neutralizing antibody response of the COE/G2a-pII-immunized sow was decreased on day 50 pi (Figure 2D). The IFN-γ were found at a high concentration of 932.3 ± 145.2 pg/ml in serum of the sow injected with COE/G2a-pII protein on day 35 pi. Notably, there was a 2-fold increase in IFN-γ level (1796.1 ± 8.6 pg/ml) in this sow on day 50 pi (Figure 2E). IFN-γ was not found in the negative control sow vaccinated with PBS. These data indicate that the COE/G2a-pII protein induces strong humoral immune responses and cytokine responses in the pregnant sow.

Humoral immune responses and cytokine responses in piglets

Since colostrum/milk antibodies from vaccinated mother sows are regularly transferred to piglets *via* suckling, passive immunity to the piglets was assessed by ELISA, virus-neutralizing antibody assay, and cytokine assay. On day 0 pc, PEDV-specific IgG antibodies were detected in the 5-day-old piglets born to sows vaccinated with COE/G2a-pII. The increase in PEDV-specific IgG antibody level was found in piglet sera on day 10 pc (Figure 3A). In addition, the sera of these piglets had high COE-specific IgA antibody responses on day 0 pc. However, there was a slight decrease in COE-specific IgA antibody levels in these piglets on day 10 pc (Figure 3B). Neutralizing antibodies against the highly virulent NAVET/PEDV/PS6/2010 strain were detected in piglets born to sows immunized with COE/G2a-pII protein on day 0 pc. Notably, VN titers found in these piglets on day 10 pc were more than 2-fold higher than those in piglets on day 0 pc (Figure 3C). High levels of IFN-γ were detected in sera of these piglets on day 0 pc with a concentration of 824.5 ± 157.68 pg/ml. Notably, there was a strong increase in IFN-γ levels in piglets born to sows inoculated with COE/G2a-pII protein on day 10 pc (Figure 3D). In contrast, no PEDV-specific IgG, COE-specific IgA, virus-neutralizing antibodies, and cytokine responses were observed in piglets born to sows vaccinated with PBS. These results demonstrate that there was a passive immunity transfer of PEDV-specific IgG, COE-specific-IgA, virus-neutralizing antibodies, and IFN-γ responses from immunized pregnant sows to its piglets *via* sow colostrum/milk.

COE/G2a-PII protein protects piglets against the highly virulent pedv G2a strain

After 1 day of the challenge, all piglets born to sows vaccinated with COE/G2a-pII protein had pasty and semisolid feces (fecal scores of 1–2). However, some piglets in this group had normal feces with scores of 0 starting on day 4 pc. All piglets in this group returned to fecal scores of 0 on day 6 pc. In contrast, piglets delivered from sows immunized with PBS had semisolid feces with scores of 2 on day 1 pc. The feces of these piglets were watery, with scores of 3 beginning at day 2 pc to the later (Figure 4A). Before the challenge, there was no significant difference in the body weight of the two piglet groups. After 10 days of the challenge, there was a 2-fold increase in the body weight of piglets delivered from sow inoculated COE/G2a-pII. In contrast, two piglets delivered from negative sow control had a strong decrease in body weight after the challenge compared to that before the challenge (Figure 4B). All piglets born to sows vaccinated with COE/G2a-pII protein had daily normal clinical scores of around 0 and survived after the challenge (Figures 4C,D). In contrast, the clinical signs of piglets delivered from sows immunized with PBS were around 1 (anorectic, depression, vomiting, and emaciated) beginning at day 2 pc, and the clinical scores of this group increased to 1.5 on day 4 pc (Figure 4C). On day 4 pc, one piglet born to the sow vaccinated with PBS died. Another piglet in this group died on day 14 pc (Figure 4D). Therefore, all piglets in the negative control group died after the viral challenge. The cause of the piglet deaths in the negative group was PEDV infections, verified *via* gross lesions in the small intestine of deceased piglets (data not shown). Taken together, these data indicate that passive immunity provided by a pregnant sow vaccinated with COE/G2a-pII protein could induce protective immune responses in its piglets against a highly virulent PEDV G2a strain.

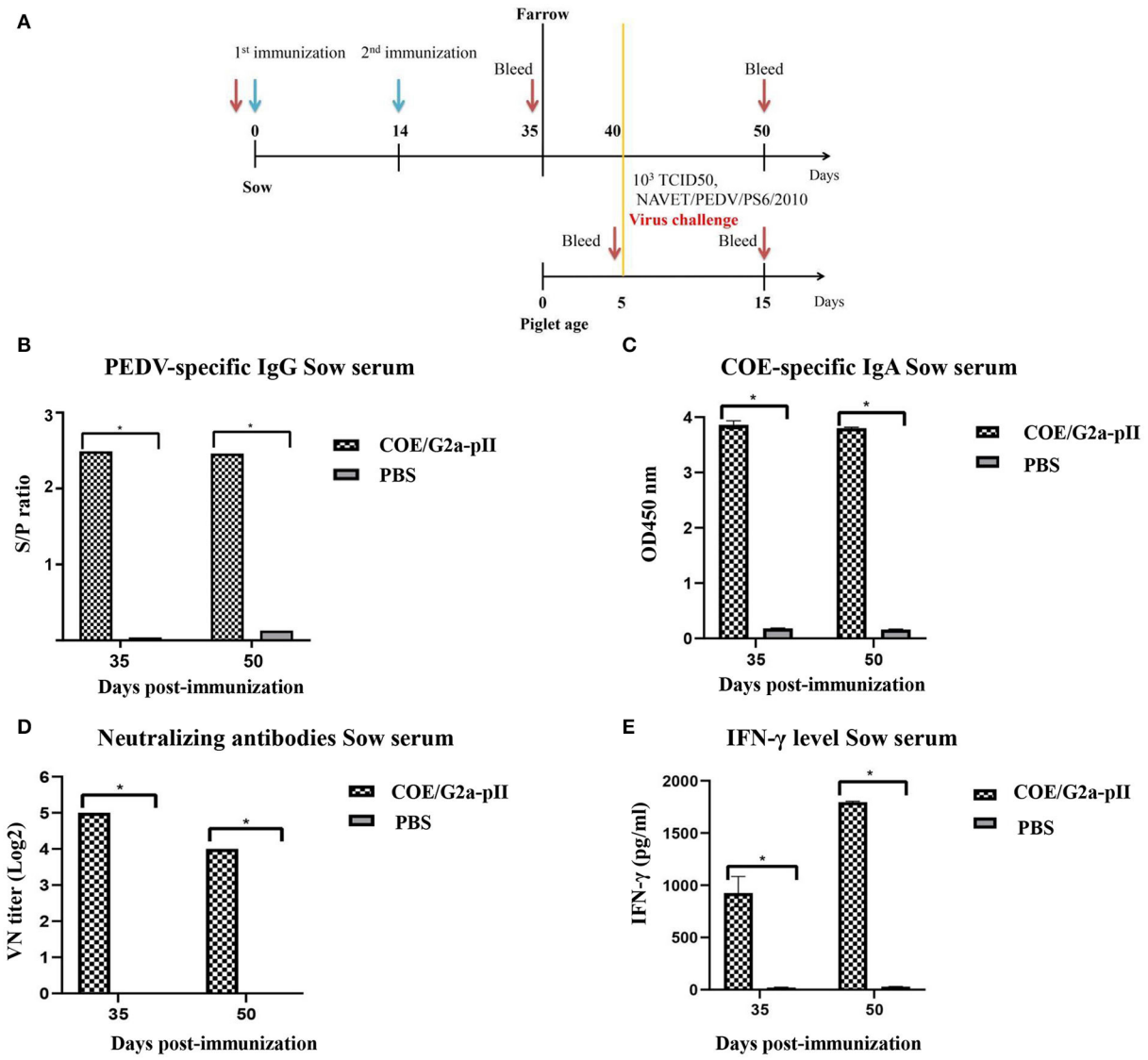


FIGURE 2
Humoral immune responses and cytokine responses in pregnant sows. Data are presented as mean ± standard deviation (SD). * indicates a statistically significant difference ($p < 0.05$). **(A)** Immunization and challenge experiment scheme in pregnant sows (~80 days of gestation, $n = 1$ per group) and piglets ($n = 8$ for COE/G2a-pII group and $n = 2$ for PBS group). The green arrow and red arrow indicate immunization and bleeding time, respectively. **(B)** PEDV-specific IgG antibodies in sow sera vaccinated with COE/G2a-pII or PBS were measured by a commercial ELISA kit. An S/P ratio > 0.35 was defined as positive with PEDV-specific IgG antibody. **(C)** COE-specific IgA antibodies in sow sera were evaluated by an ELISA using SEC-purified COE/G2a-pII as antigen. **(D)** Neutralizing antibodies in sow sera were analyzed by the virus-neutralizing assay. The highly virulent NAVET/PEDV/PS6/2010 strain (200 TCID50/0.1 ml) was used for the assay. A VN titer ≥ 8 was defined as positive with neutralizing antibody against PEDV. **(E)** IFN-γ levels in sow sera were measured based on the standard curve of recombinant porcine IFN- γ standard in a commercial ELISA kit.

Discussion

To the best of our knowledge, this is the first study to evaluate the protective efficacy of a plant COE-pII-based subunit vaccine candidate against a highly virulent PEDV G2a in piglets following the passive immunity. Previous studies indicate that the current commercial PEDV vaccine, which contains G1a PEDV strains, may induce low protective immune responses against PEDV strains in the field (9–11). When collecting all DNA sequences encoding the full S protein of Vietnamese PEDV strains published on NCBI, we obtained 63 nucleotide sequences of full S genes. After building the phylogenetic tree based on full S gene sequences of PEDV PS6, 63 Vietnamese strains, and 62 PEDV strains in the world, then comparing the genotype

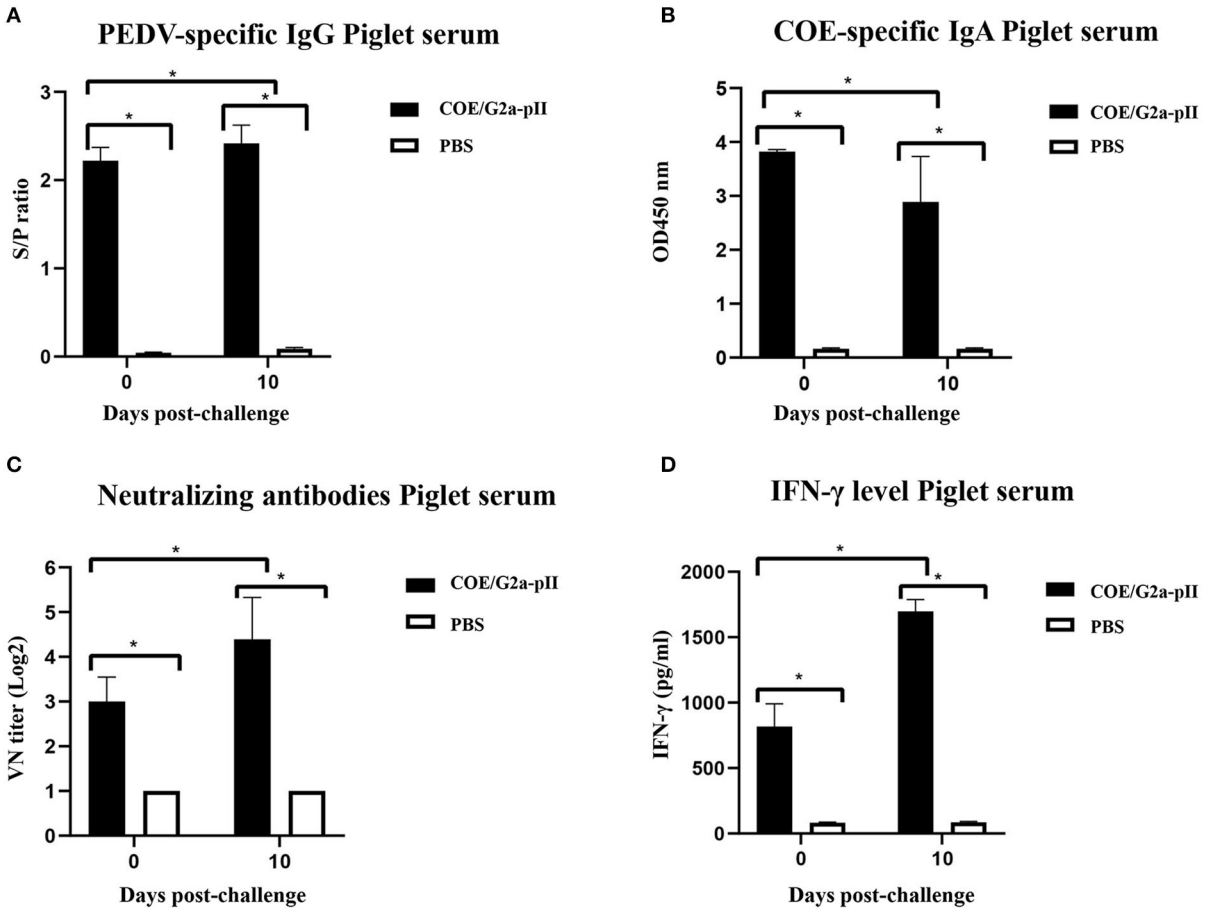


FIGURE 3
Passive transfer of antibodies and cytokine from sows to piglets at the days 0 and 10 pc. Data are presented as mean ± standard deviation (SD). * indicates a statistically significant difference ($p < 0.05$). **(A)** PEDV-specific IgG antibodies in piglet sera from sows ($n = 1$ per group) vaccinated with COE/G2a-pII, or PBS were measured by a commercial ELISA kit. An S/P ratio > 0.35 was defined as positive with a PEDV-specific IgG antibody. **(B)** COE-specific IgA antibodies in piglet sera ($n = 8$ for COE/G2a-pII group and $n = 2$ for PBS group) were evaluated by an ELISA using SEC-purified COE/G2a-pII as antigen. **(C)** Neutralizing antibodies in piglet sera were analyzed by the virus-neutralizing assay. The highly virulent NAVET/PEDV/PS6/2010 strain (200 TCID50/0.1 ml) was used for the assay. A VN titer ≥ 8 was defined as positive with neutralizing antibody against PEDV. **(D)** IFN-γ levels in piglet sera were measured based on the standard curve of recombinant porcine IFN-γ standard in a commercial ELISA kit.

cluster of PEDV strains to previous publications, the Vietnamese PEDV G2a, G2b, and G1b strains were clustered into 35/64 sequences (54.68%), 23/64 sequences (35.93%), 8/64 sequences (12.5%), respectively (data not shown). This finding is also in agreement with the results of previous studies that PEDV strains belonging to the G2 group were detected in most of the PED outbreaks in Vietnam (4). In addition, we also found that PEDV G2a strains were the dominant agents in PED outbreaks in Vietnam. Therefore, the goal of this study was to assess whether immunization with plant-based COE/G2a-pII protein would elicit protective responses in piglets born to immunized sows against highly virulent PEDV G2a strains isolated in Vietnam.

COE/G2a-pII protein was successfully expressed at a high level in plants *via* agroinfiltration. When compared to previous studies, the expression level of COE/G2a-pII protein in *N. benthamiana* is higher than that of COE proteins in previous publications (13, 17, 21, 29). However, the expression level of COE/G2a-pII protein is lower than that of COE/G1a-pII protein in our previous study (234 mg/kg fresh leaves, 4% TSP). This might be explained by the difference in nucleotide sequence and amino acid sequence of the two COE variants. When compared to the native sequence of COE/G1a-pII, the identity in the native nucleotide sequence and amino acid of COE/G2a-pII was 95.7 and 96.4%, respectively (Supplementary Tables 1, 2). After a commercial codon optimization for expression in *N. benthamiana*, the similarity in the nucleotide sequence of two optimized COE variants was 75.5% (Supplementary Table 3). COE/G2a-pII protein is a mixture of multimer forms with a molecular weight of approximately 440 kDa. The size of COE/G2a-pII protein is quite different when compared to our

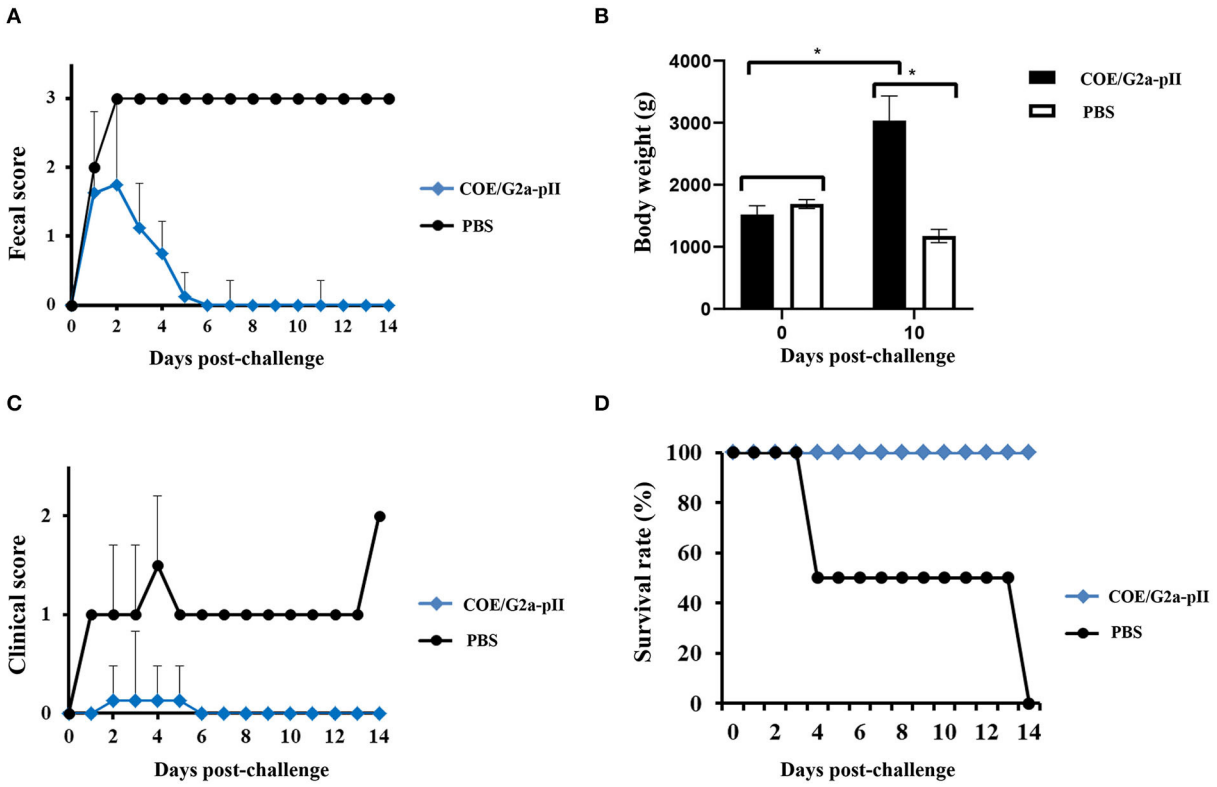


FIGURE 4
Clinical observation and the survival of piglets obtained passive immunity from sow vaccination. The value is presented as the mean ± standard deviation (SD). * means a statistically significant difference ($p < 0.05$). **(A)** Fecal score of piglets ($n = 8$ for COE/G2a-pII group and $n = 2$ for PBS group) was recorded daily after challenge with highly virulent NAVET/PEDV/PS6/2010 strain. **(B)** The body weight of piglets was calculated on the days 0 and 14 pc. **(C)** The clinical scores of piglets were analyzed daily after the challenge. **(D)** After the challenge, the survival rate of piglets was recorded daily.

previous publication that used native PAGE to estimate the size of COE/G1a-pII protein (22). This can be explained by the difference in the capacity of each method used to calculate the size of the protein. When compared to SEC, native PAGE has limitations in the separation of high molecular weight protein. Since high mortality caused by PEDV infection occurs mostly in neonatal piglets at the age of under 1 week, an ideal commercial vaccine against PEDV infection should immunize sows to facilitate lactogenic immunity to be transferred to neonatal piglets following suckling. Results presented here indicate that 100% of 5-days-old-piglets born to COE/G2a-pII-vaccinated sows survived after the challenge with a highly virulent PEDV strain belonging to the G2a group. All piglets born to a PBS-vaccinated sow died. The survival rate obtained in piglets of the PBS group agreed with previous studies in which very high mortality (up to 80%−100%) in suckling piglets infected with PEDV at the age of under 7 days (3, 30). The zero mortality rate in piglets born to COE/G2a-pII-vaccinated sow after the challenge can be partly explained by the strong PEDV-specific IgG, COE-specific IgA, neutralizing antibodies, and IFN-γ responses in sow and transferred passively to piglets. The level of neutralizing antibody responses induced in the COE/G2a-pII-vaccinated sow and its piglets was quite similar to those elicited in sows vaccinated with the inactivated PEDV and their offsprings (31). The decrease in the level of humoral immune responses in the serum of sows vaccinated with COE/G2a-PII protein on day 50 pi might be explained by the transportation of antibodies from the blood to the milk of the sow. The IgA, IgG, and neutralizing antibody response in the serum of piglets on day 10 pc was quite similar to those in the serum of sow on day 50 pi. These findings agreed with those in a previous study in which the level of IgG and IgA in piglets mimicked those in sow sera and colostrums (32). In addition, the humoral immune responses induced in sows and piglets could change at the different time points of sampling. Further study should evaluate humoral immune response in sow and piglet sera at several time points.

Cytokines delivered from maternal responses may play important roles in generating the newborn immune response. IFN-γ is a crucial cytokine that is involved in both innate and adaptive immune responses. It has a critical role in the activation of macrophages and the induction of T helper lymphocyte

differentiation regarding the Th1, consequent aiding cellular responses and enhancing the protective immune response against pathogen infections (33). Therefore, the evaluation of IFN-γ amount is favored to partially characterize the immune response (34). Notably, the level of IFN-γ in sow (day 35 pi) and its offspring (day 0 pc) were detected at a very high concentration of 932.3 ± 145.2 pg/ml and 824.5 ± 157.68 pg/ml, respectively. When compared to the previous studies, the concentration of IFN-γ in sows vaccinated with COE/G2a-pII at farrowing was over 5-fold higher than that in sows vaccinated with killed-PEDV vaccine plus flagellin protein (Vac201-FliC) (31) and over 10-fold higher than that in sows vaccinated with inactivated PEDV harboring IgG-Fc (35). The level of IFN-γ in sow (day 50 pi) and its offspring (day 10 pc) were 2-fold increased in comparison with those before the challenge.

In this study, the amount of COE/G2a-pII protein used to vaccinate sow was 100 μg per dose, which was 4-fold lower than that used in a previous study (36). The pregnant sow in the previous study was vaccinated three times with polyphosphazene in a triple adjuvant combination (TriAdj) with adjuvanted S1 protein (400 μg) (36). After the viral challenge, the survival rate of piglets delivered from the S1-vaccinated sow in the previous study (36) was 87.5%, which was also lower than the survival rate of our piglets born to COE/G2a-pII sow (100%). The lower required dose of plant-based COE/G2a-pII protein for vaccination resulting in the higher protective efficacy against the highly virulent PEDV G2a can be explained by the formation of multimer forms with high molecular weight in the COE/G2a-pII protein. These results indicate that COE/G2a-pII protein produced in plants could be a potential vaccine candidate to prevent PEDV infection. However, our study still has limitations. The number of sows and piglets used in this study was not large as African swine fever outbreaks in Vietnam seriously affected the pig industry when the animal experiment was performed. The results we achieved prompted us to plan further studies involving many more sows and piglets to validate these data. Moreover, the amount of COE/G2a-pII protein per dose and the effect of different adjuvants that improve immune responses will be addressed in further experiments. In addition, the presence or absence of the PEDV in pig feces after the challenge should also be analyzed.

Conclusion

The COE/G2a-pII protein produced in plants induced strong humoral immune responses and IFN-γ responses in the pregnant sow. The sow's immune responses were passively transferred to their piglets following suckling. All piglets born to COE/G2a-pII-vaccinated sows survived and had normal clinical signs after the challenge with the highly virulent PEDV G2a strain. These results suggest that the COE/G2a-pII protein is a potential vaccine candidate to prevent PEDV G2a infection of swine in Vietnam, and these preliminary but important data are the basis for performing more extended studies to develop a plant-based COE/G2a-pII vaccine.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the Institute of Biotechnology (IBT), Vietnam Academic of Science and Technology (VAST), Hanoi, Vietnam, and NAVETCO, Ho Chi Minh City, Vietnam under decision number 07/2015/HĐ-NĐT.

Author contributions

HC, NP, and TH designed the study. TH and VT constructed vectors and performed ELISA IgA and IFN-γ. VT and TN performed transient expression. TH, VT, and TN purified protein. HT and PL performed pig immunization and challenge experiments, performed the virus-neutralizing antibody assay, ELISA IgG, and collected the clinical signs of piglets. TH performed the calculations and data analysis and wrote the manuscript. HC, NP, UC, MP, and HH revised the manuscript. HC and NP hold all the responsibilities related to this manuscript. All authors have read and approved the final manuscript.

Funding

This study was financed by the Ministry of Science and Technology through the project: Development of novel nanoconjugates from nanodiamonds and spike protein of porcine epidemic diarrhea virus toward applications in nanovaccine (code: NĐT/TW/21/09). This study is a part of the thesis results of Ph.D. student TH at GUST, VAST, Hanoi, Vietnam. TH was funded by Vingroup JSC and supported by the Master, Ph.D. Scholarship Programme of Vingroup Innovation Foundation (VINIF), Institute of Big Data (code: VINIF.2021.TS.066).

Acknowledgments

We would like to thank Dr. Hoang Trong Phan and Nhung Hong Nguyen for supporting the vector construction experiment.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.940395/full#supplementary-material

References

1. Song D, Moon H, Kang B. Porcine epidemic diarrhea: a review of current epidemiology and available vaccines. *Clin Exp Vaccine Res.* (2015) 4:166–76. doi: 10.7774/cevr.2015.4.2.166

2. Sun RQ, Cai RJ, Chen YQ, Liang PS, Chen DK, Song CX. Outbreak of porcine epidemic diarrhea in suckling piglets China. *Emerg Infect Dis.* (2012) 18:161–3. doi: 10.3201/eid1801.111259

3. Stevenson GW, Hoang H, Schwartz KJ, Burrough ER, Sun D, Madson D, et al. Emergence of Porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. *J Vet Diagn Invest.* (2013) 25:649–54. doi: 10.1177/1040638713501675

4. Than VT, Choe SE, Vu TTH, Do TD, Nguyen TL, Bui TTN, et al. Genetic characterization of the spike gene of porcine epidemic diarrhea viruses (PEDVs) circulating in Vietnam from 2015 to 2016. *Vet Med Sci.* (2020) 6:535–42. doi: 10.1002/vms3.256

5. Duy DT, Toan NT, Puranaveja S, Thanawongnuwech R. Genetic characterization of porcine epidemic diarrhea virus (PEDV) isolates from Southern Vietnam during 2009–2010 outbreaks. *Thai J Vet Med.* (2011) 41:55–64.

6. Okda FA, Lawson S, Singrey A, Nelson J, Hain KS, Joshi LR, et al. The S2 glycoprotein subunit of porcine epidemic diarrhea virus contains immunodominant neutralizing epitopes. *Virology.* (2017) 509:185—94. doi: 10.1016/j.virol.2017.06.013

7. Lee C. Porcine epidemic diarrhea virus: an emerging and reemerging epizootic swine virus. *Virol J.* (2015) 12:193. doi: 10.1186/s12985-015-0421-2

8. Deng F, Ye G, Liu Q, Navid MT, Zhong X, Li Y, et al. Identification and comparison of receptor binding characteristics of the spike protein of two porcine epidemic diarrhea virus strains. *Viruses.* (2016) 8:55. doi: 10.3390/v8030055

9. Diep NV, Sueyoshi M, Izzati U, Fuke N, Teh APP, Lan NT, et al. Appearance of US-like porcine epidemic diarrhoea virus (PEDV) strains before US outbreaks and genetic heterogeneity of PEDVs collected in Northern Vietnam during 2012–2015. *Transbound Emerg Dis.* (2018) 65: e83–e93. doi: 10.1111/tbed.12681

10. Li W, Li H, Liu Y, Pan Y, Deng F, Song Y, et al. New variants of porcine epidemic diarrhea virus China, 2011. *Emerg Infect Dis.* (2012) 18:1350–3. doi: 10.3201/eid1803.120002

11. Wang X, Chen J, Shi D, Shi H, Zhang X, Yuan J, et al. Immunogenicity and antigenic relationships among spike proteins of porcine epidemic diarrhea virus subtypes G1 and G2. *Arch Virol.* (2016) 161:537–47. doi: 10.1007/s00705-015-2694-6

12. Chang SH, Bae JL, Kang TJ, Kim J, Chung GH, Lim CW, et al. Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. *Mol Cells.* (2002) 14:295–9.

13. Huy NX, Kim SH, Yang MS, Kim TG. Immunogenicity of a neutralizing epitope from porcine epidemic diarrhea virus: M cell targeting ligand fusion protein expressed in transgenic rice calli. *Plant Cell Rep.* (2012) 31:1933—42. doi: 10.1007/s00299-012-1306-0

14. Hajibehzad SS, Honari H, Nasiri J, Mehrizi FA, Alizadeh H. High-level transient expression of the N-terminal domain of IpaD from Shigella dysenteriae in four plant species transformed with different construct configurations. *In Vitro Cell Dev Biol Plant.* (2016) 52:293–302. doi: 10.1007/s11627-016-9760-y

15. Wroblewski T, Tomczak A, Michelmore R. Optimization of Agrobacteriummediated transient assays of gene expression in lettuce, tomato and Arabidopsis. *Plant Biotechnol J.* (2005) 3:259–73. doi: 10.1111/j.1467-7652.2005.00123.x

16. Huy NX, Kim YS, Jun SC, Jin Z, Park SM, Yang MS, et al. Production of a heat-labile enterotoxin B subunit-porcine epidemic diarrhea virus-neutralizing epitope fusion protein in transgenic lettuce (*Lactuca sativa*). *Biotechnol Bioprocess Eng BBE.* (2009) 14:731–7. doi: 10.1007/s12257-009-3012-5

17. Huy NX, Yang MS, Kim TG. Expression of a cholera toxin B subunit-neutralizing epitope of the porcine epidemic diarrhea virus fusion gene in transgenic lettuce (*Lactuca sativa* L). *Mol Biotechnol.* (2011) 48:201–9. doi: 10.1007/s12033-010-9359-1

18. Huy NX, Tien NQD, Kim MY, Kim TG, Jang YS, Yang MS. Immunogenicity of an S1D epitope from porcine epidemic diarrhea virus and cholera toxin B subunit fusion protein transiently expressed in infiltrated Nicotiana benthamiana leaves. *Plant Cell Tissue Organ Cult.* (2016) 127:369–80. doi: 10.1007/s11240-016-1059-5

19. Tien NQ, Huy NX, Kim MY. Improved expression of porcine epidemic diarrhea antigen by fusion with cholera toxin B subunit and chloroplast transformation in Nicotiana tabacum. *Plant Cell Tissue Organ Cult.* (2019) 137:213–23. doi: 10.1007/s11240-019-01562-1

20. Egelkrout E, Hayden C, Fake G, Keener T, Arruda P, Saltzman R, et al. Oral delivery of maize-produced porcine epidemic diarrhea virus spike protein elicits neutralizing antibodies in pigs. *Plant Cell Tissue Organ Cult.* (2020) 142:79–86. doi: 10.1007/s11240-020-01835-0

21. Tien NQ, Yang MS, Jang YS, Kwon TH, Reljic R, Kim MY. Systemic and Oral Immunogenicity of Porcine Epidemic Diarrhea Virus Antigen Fused to Poly-Fc of Immunoglobulin G and Expressed in ΔXT/FT Nicotiana benthamiana Plants. *Front Pharmacol.* (2021) 12:653064. doi: 10.3389/fphar.2021.653064

22. Ho TT, Nguyen GT, Pham NB, Le VP, Trinh TBN, Vu TH, et al. Plant-derived trimeric CO-26K-equivalent epitope induced neutralizing antibodies against porcine epidemic diarrhea virus. *Front Immunol.* (2020) 11:2152. doi: 10.3389/fimmu.2020.02152

23. Pham VD, Hoang H, Phan HT, Conrad U, Chu HH. Production of antibody labeled gold nanoparticles for influenza virus H5N1 diagnosis kit development. *Adv Nat Sci Nanosci Nanotechnol.* (2012) 3:045017. doi: 10.1088/2043-6262/3/4/045017

24. Phan HT, Ho TT, Chu HH, Vu TH, Gresch U, Conrad U. Neutralizing immune responses induced by oligomeric H5N1-hemagglutinins from plants. *Vet Res.* (2017) 48:53. doi: 10.1186/s13567-017-0458-x

25. Hofmann M, Wyler R. Quantitation biological and physicochemical properties of cell culture-adapted porcine epidemic diarrhea coronavirus (PEDV). *Vet Microbiol.* (1989) 20:131–42. doi: 10.1016/0378-1135(89)90036-9

26. Joshi LR, Okda FA, Singrey A, Maggioli MF, Faccin TC, Fernandes MHV, et al. Passive immunity to porcine epidemic diarrhea virus following immunization of pregnant gilts with a recombinant orf virus vector expressing the spike protein. *Arch Virol.* (2018) 163:2327–35. doi: 10.1007/s00705-018-3855-1

27. Annamalai T, Saif LJ, Lu Z, Jung K. Age-dependent variation in innate immune responses to porcine epidemic diarrhea virus infection in suckling versus weaned pigs. *Vet Immunol Immunopathol.* (2015) 168:193–202. doi: 10.1016/j.vetimm.2015.09.006

28. Kusanagi K, Kuwahara H, Katoh T, Nunoya T, Ishikawa Y, Samejima T, et al. Isolation and serial propagation of porcine epidemic diarrhea virus in

cell cultures and partial characterization of the isolate. *J Vet Med Sci.* (1992) 54:313–8. doi: 10.1292/jvms.54.313

29. Kang TJ, Kim YS, Jang YS. Expression of the synthetic neutralizing epitope gene of porcine epidemic diarrhea virus in tobacco plants without nicotine. *Vaccine.* (2005) 23:2294–7. doi: 10.1016/j.vaccine.2005.01.027

30. Carvajal A, Argüello H, Martínez-Lobo FJ et al. Porcine epidemic diarrhoea: new insights into an old disease. *Porc Health Manag.* (2015) 1:12. doi: 10.1186/s40813-015-0007-9

31. Xu X, Du L, Fan B, et al. A flagellin-adjuvanted inactivated porcine epidemic diarrhea virus (PEDV) vaccine provides enhanced immune protection against PEDV challenge in piglets. *Arch Virol.* (2020) 165:1299–309. doi: 10.1007/s00705-020-04567-w

32. Bandrick M, Ariza-Nieto C, Baidoo SK, Molitor TW. Colostral antibody-mediated and cell-mediated immunity contributes to innate and antigen-specific immunity in piglets. *Dev Comp Immunol.* (2014) 43:114–20. doi: 10.1016/j.dci.2013.11.005

33. Kumar V, Abbas AK, Aster JC. *Robbins & Cotran Pathologic Basis of Disease, 10th Edn.* Philadelphia, PA: El Sevier (2020).

34. Navarro E, Mainau E, de Miguel R, Temple D, Salas M, Manteca X. Oral meloxicam administration in sows at farrowing and its effects on piglet immunity transfer and growth. *Front Vet Sci.* (2021) 8:574250. doi: 10.3389/fvets.2021.574250

35. Park JE, Jang H, Kim J-H, Hyun B-H, Shin H-J. Immunization with porcine epidemic diarrhea virus harbouring Fc domain of IgG enhances antibody production in pigs. *Vet Q.* (2020) 40:183–9. doi: 10.1080/01652176.2020.1773006

36. Makadiya N, Brownlie R, van den Hurk J, Berube N, Allan B, Gerdts V, et al. S1 domain of the porcine epidemic diarrhea virus spike protein as a vaccine antigen. *Virol J.* (2016) 13:57. doi: 10.1186/s12985-016-0512-8

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 02 August 2022
DOI 10.3389/fvets.2022.972500

OPEN ACCESS

EDITED BY
Dayou Shi,
South China Agricultural University, China

REVIEWED BY
Xiao Zhang,
Shandong Agricultural University, China
Veronica Risco-Castillo,
INRA École Nationale Vétérinaire d'Alfort (ENVA), France

*CORRESPONDENCE
Xingju Song
songxingju@gxu.edu.cn

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 18 June 2022
ACCEPTED 18 July 2022
PUBLISHED 02 August 2022

CITATION
Jiang Y, Shi Y, Hu D and Song X (2022) The anti-*Toxoplasma* activity of the plant natural phenolic compound piceatannol. *Front. Vet. Sci.* 9:972500. doi: 10.3389/fvets.2022.972500

The anti-*Toxoplasma* activity of the plant natural phenolic compound piceatannol

Yucong Jiang, Yuehong Shi, Dandan Hu and Xingju Song*

College of Animal Science and Technology, Guangxi University, Nanning, China

Toxoplasma gondii is an obligate intracellular protozoan that infects the nucleated cells of warm-blooded animals and causes life-threatening disease in immunocompromised patients. Due to the limited effectiveness and prominent side effects of existing drugs, there is an urgent need to develop new therapeutic options against *T. gondii*. Piceatannol is a natural plant compound with multiple functions such as antibacterial, antileukemic and antiparasitic activities. In the present study, the anti-*T. gondii* activity of piceatannol was evaluated. Piceatannol potently inhibited *Toxoplasma* with a half-maximal effective concentration (EC_{50}) of 28.10 μM. Piceatannol showed a significant inhibitory effect on intracellular proliferation, inhibiting intracellular parasites at a rate of 98.9% when treatment with 100 μM piceatannol. However, the invasion ability of tachyzoites was not affected by piceatannol. By immunofluorescence assay, we noted that the parasite showed abnormalities in cell division after exposure to piceatannol. To determine the *in vivo* effect of piceatannol on acute infection, a model was established by infecting BALB/c mice with the virulent RH strain of *T. gondii*. Mice infected with 500 tachyzoites showed a significant therapeutic effect when treated with 15 mg/kg of piceatannol. These results suggest that piceatannol is a promising drug for the treatment of *T. gondii*.

KEYWORDS

***Toxoplasma gondii*, natural extraction products, piceatannol, antiparasitic, drug discovery**

Introduction

Toxoplasma gondii is a zoonotic parasite that infects almost all warm-blooded animals and humans worldwide. The infection is usually asymptomatic in immunocompetent individuals. However, serious consequences may occur in immunocompromised individuals (1). In patients with HIV or receiving chemotherapy for cancer or organ transplants, active *T. gondii* infections mainly lead to encephalitis, pneumonia or chorioretinitis, and tissue destruction in other organs (2, 3). Women with major infections during pregnancy may result in fetal death, malformation or miscarriage. The main route of infection in humans is through the ingestion of meat containing cysts or food contaminated with oocysts. In addition, people may become infected through blood transfusions, organ transplants and transplacental transmission (4). *T. gondii* infections have caused serious public health problems. One-third of the

world's population is estimated to be infected, and severe outbreaks of toxoplasmosis have occurred in several countries (5, 6).

Currently, clinical treatment of toxoplasmosis relies on chemical drugs. A combination of sulfadiazine and pyrimethamine has become the standard therapy for toxoplasmosis (7). However, this combination still has a high failure rate and is ineffective against chronic infections. Moreover, different severe complications such as teratogenic potential, reversible myelosuppression, neutropenia, thrombocytopenia, hypersensitivity reactions and hepatic necrosis have been reported (8–11). Furthermore, other drugs such as azithromycin, clarithromycin, spiramycin, atovaquone and cotrimoxazole (trimethoprim-sulfamethoxazole) are also commonly used in clinical toxoplasmosis. However, these drugs are less effective than conventional treatment and are often accompanied by severe side effects and incomplete treatment (12–14). For example, spiramycin treatment alone could significant reduction in mother-to-child transmission (MTCT) rates of diagnosed maternal *T. gondii* infection, but if fetal infection is suspected or confirmed pyrimethamine–sulfonamide–folinic acid should be used (15). Thus, the limitations of available treatment options underscore the urgent need for better treatment options for acute and latent toxoplasmosis.

Recently, natural products have been considered as good alternatives for the development of *T. gondii* drugs (16). Various studies have shown that natural extracts/components of plants have inhibitory effects on *T. gondii*. Compounds from antimalarial plants, such as *Artemisia annua*, *Cinnamomum comphora*, *Lippia multiflora* and *Vernonia colorata*, were found to be effective against *T. gondii* (17). Some other plant extracts such as essential oils from *Lavandula angustifolia* and *Pelargonium X,* Vernodalin from *V. colorata*, TAF355 and TAF401 from *Eurycoma longifolia*, and Ginkgolic acids from *Ginkgo biloba* also have a good inhibitory effect on *T. gondi*i (18–21). Resveratrol, a polyphenol family of stilbene molecules from plants, has potent inhibitory activity against several important protozoa, including *Toxoplasma, Leishmania,* and *Amoeba* (22, 23). Piceatannol is a natural analog of resveratrol and is mainly found in passion fruit (*Passiflora edulis* Sims), blueberries, grapes, sugarcane, white tea, and rhubarb (24–26). Piceatannol has been reported to possess antioxidant, anti-proliferative, immune enhancement, anti-inflammatory, anti-thrombotic, anti-cancer, anti-hyperlipidemic and antibacterial activities and is widely used for the prevention/treatment of heart disease, leukemia, cancer, etc. (27). However, it remains unclear whether piceatannol has anti-*T. gondii* effects and the mechanism behind the clearance of intracellular parasites.

In this study, we evaluated the inhibitory effect of piceatannol against *T. gondii*. Our results indicate that piceatannol can potently inhibit *T. gondii* with a high *in vitro* safety index. These results suggest that piceatannol is a promising drug candidate for the treatment of toxoplasmosis.

Results

Piceatannol has a potent anti-*Toxoplasma* activity

The plaque assay was used to comprehensively evaluate the proliferation of RH *T. gondii* tachyzoites treated with piceatannol and DMSO during the entire lytic cycle. The results showed no plaque formation after piceatannol treatment, which differed significantly from the DMSO control group (Figures 1A,B). To determine the effect of different concentrations of piceatannol on tachyzoites, the RH strain of *T. gondii* expressing luciferase (TgRH-Luc) was used, as indicated by the reduced luciferase activity. The parasites in African green monkey kidney (Vero) cells were determined at different piceatannol concentrations using an *in vitro* drug inhibition assay. The results showed a good inhibitory effect of piceatannol on TgRH-Luc in a dose-dependent manner (Figure 1C). The half-maximal effective concentration (EC_{50}) of piceatannol was recorded to be 28.10 μM (95% confidence interval [CI], 25–30 μM). Since piceatannol has an inhibitory effect on the development of *T. gondii*, it is necessary to further investigate whether it acts on intracellular tachyzoites. Intracellular parasites were treated with 100 μM piceatannol. The inhibition rate was 98.9% for the intracellular parasites (Figure 1D). The results indicate that piceatannol has good inhibitory effect on intracellular *T. gondii*.

Inhibition of intracellular proliferation of *T. gondii* by piceatannol

T. gondii tachyzoites in cells involves a complete set of lytic cycles, including invasion, intracellular replication and egress (28). The reduction in plaque formation may be caused by impairment of one or more steps of the lytic cycle. Thus, we next sought to investigate which lytic cycle of *T. gondii* affects piceatannol. We primarily assessed parasite invasion processes, which showed no significant differences between the piceatannol and DMSO treatment groups (Figure 2A). Then, intracellular proliferation was assessed by observing the number of tachyzoites in the vacuoles after piceatannol and DMSO treatment. The results showed that piceatannol potently inhibited the intracellular proliferation of *T. gondii* ($p < 0.001$) (Figure 2B). These results suggest that the reduction in plaque size induced by piceatannol treatment is specifically due to the impairment of intracellular proliferation of the parasites.

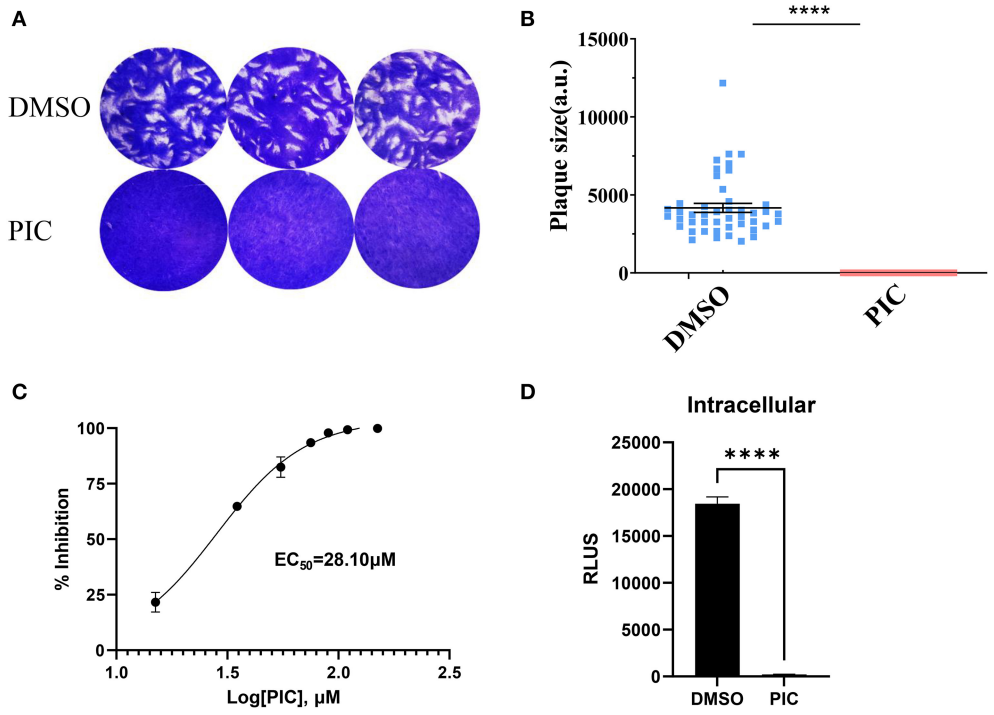


FIGURE 1
Piceatannol displaying a dose-dependent inhibitory effect against TgRH-Luc tachyzoites. **(A)** The Plaque assay for comparing the overall proliferation ability of the parasites after piceatannol and DMSO treatment. TgRH-Luc tachyzoites were added to HFF cells cultured in 12-well plates, with 150 tachyzoites added to each well. Infected cells were treated with DMSO and 100 μM Piceatannol, respectively. Cells were cultured for 7 days followed by fixation with PFA and staining with 0.2% crystal violet. **(B)** Measurement of plaque areas using the pixel point using Pixel in the Photoshop C6S software (Adobe, USA). Data were obtained from three independent experiments. Statistical analysis of the plaque area was performed by Graph Pad Prism using Student's *t* test. Asterisks indicate $p < 0.0001$. **(C)** Inhibition curve of piceatannol on *T. gondii in vitro*. TgRH-Luc was treated with different concentrations of piceatannol ranging from 0 to 150 μM, and relative RLU was detected after 24 h. Percentage proliferation inhibition was calculated as: inhibition rate = $[(RLU_{DMSO} - RLU_{treatment})/RLU_{DMSO}] \times 100$. EC_{50} was calculated using the log (inhibitor) vs. response-variable slope (four parameters) regression equation. The results are shown as the mean ±SEM from three independent experiments. **(D)** Piceatannol inhibition of intracellular *T. gondii*. Freshly released tachyzoites (1×10^5) were allowed to invade the host cells for 3 h at 37° C and 5% CO_2, and then DMEM medium containing piceatannol or DMSO was added after wash three times. The infected cells were cultured for 24 h until fluorescence was detected. Data were obtained from three independent experiments. Statistical analysis of the plaque area was performed by Graph Pad Prism using Student's *t* test. Asterisks indicate $p < 0.0001$.

Treatment with piceatannol reduces parasite virulence in mice

We further examined the effect of piceatannol on parasite proliferation *in vivo*. Two delivery routes (intragastric administration and intraperitoneal injection) were employed independently. In the intragastric administration experiment, 40% of the mice in the DMSO group died on day 7 and the remaining 60% died on day 8 and 9, whereas the death of the mice in the piceatannol-treated group was significantly delayed ($p < 0.0001$), with the last one dying on 15 days post infection (Figure 3A). In the intraperitoneal injection experiment, all mice in the DMSO group died by day 8, whereas death was significantly delayed in the piceatannol-treated group (the last mouse died 15 days after infection) (Figure 3B). These results suggest that piceatannol inhibits the proliferation of *T. gondii in vitro*; however, it does not completely kill the parasite in the mice.

Piceatannol exposure causes abnormal division

Considering the inhibitory effect of piceatannol on parasite proliferation, we sought to determine whether treatment with piceatannol would alter the division of tachyzoites. Immunofluorescence assays were performed to observe the morphology of the parasites after treatment. We noticed that piceatannol treated tachyzoites in a single PV divided asynchronously, and even three daughter cells were found in a single tachyzoite. The number of tachyzoites in untreated *T. gondii* vacuoles was 2, 4, 8, 16, etc. due to their binary fission pattern. However, a large number of abnormal divisions were

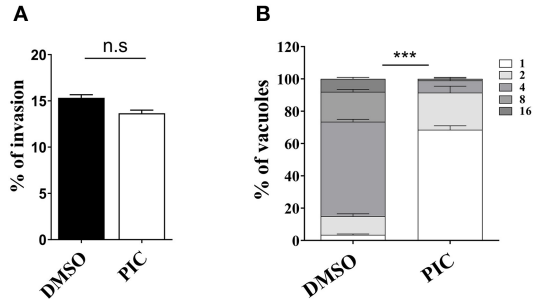


FIGURE 2
Effect of piceatannol on parasite invasion and proliferation. **(A)** Graph of RH invasion suppression efficiency. RH infection was cultured in Vero cells containing 100 μM piceatannol or DMEM and IFA was performed to calculate the rate of RH invasion. Statistical analysis of the plaque area was performed by Graph Pad Prism using Student's *t* test. **(B)** Graph of RH proliferation inhibition efficiency. After infecting Vero cells with RH for 3 h, infected cells were treated with 100 μM piceatannol. The proportion of vacuoles containing 2, 4, 8 and 16 tachyzoites of the RH strain was calculated under fluorescence microscopy and the average value was taken three times. Statistical analysis of the plaque area was performed by GraphPad Prism using two-way ANOVA. n.s, non-significant, ***, $p < 0.001$.

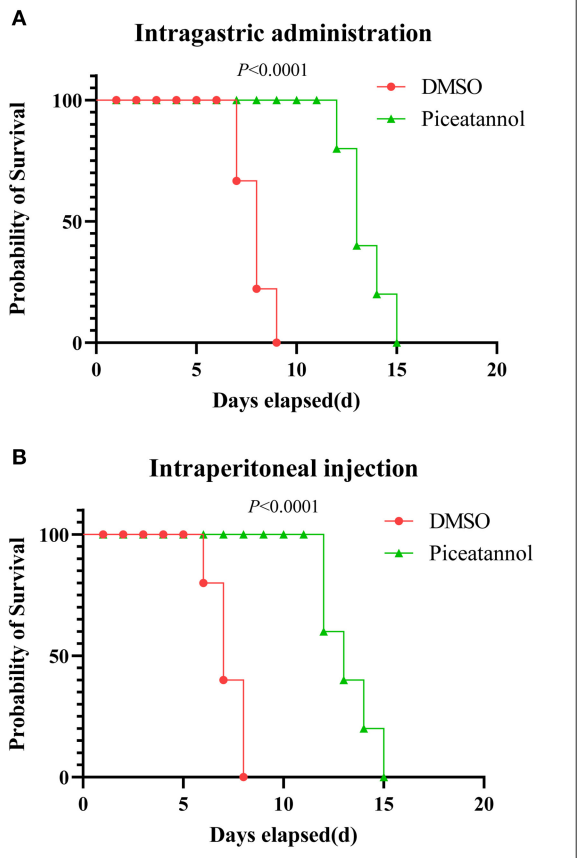


FIGURE 3
Therapeutic effect of piceatannol on *T. gondii* infection in mice. BALB/c mice were infected with 500 tachyzoites by intraperitoneal injection. Then, mice were treated with 15 mg/kg piceatannol or DMSO by intragastric administration **(A)** or intraperitoneal injection **(B)**. The results show a therapeutic effect of piceatannol on mice infected with parasites. Statistical analysis was performed with survival curve of Graph Pad Prism using Log-rank (Mantel-Cox) test (San Diego, CA).

observed after piceatannol treatment (Figure 4A). We found an odd number of parasites (e.g., 3 or 5 tachyzoites) predominantly present in a single vacuole (Figure 4A). We found ∼60% tachyzoites showed abnormal division after piceatannol treatment, while only 5% were found in DMSO treated group (Figure 4B). These results suggested that piceatannol inhibit parasite proliferation by suppressing its cell division.

Piceatannol is not toxic to host cells at antiparasitic concentrations

To assess the cytotoxic effect of piceatannol on host cells, sequentially diluted piceatannol from 20 μM to 500 μM was added to host cells and cell viability was determined using CCK-8 reagent. The results showed that in the therapeutic concentration range, piceatannol was not toxic to cells and that high concentrations of piceatannol had a promotive effect on the proliferation of Vero cells (Figure 5).

Discussion

Toxoplasmosis, as a zoonotic disease, has serious public health implications worldwide and also poses serious hazards to humans and domestic animals. Although most human infections have no complications, they can still be fatal or cause serious problems in fetuses and immunocompromised patients (5). Currently, clinical treatment of toxoplasmosis still relies on chemical drugs. However, this treatment is often accompanied by severe side effects (8–11). Recently, a large number of studies have focused on the use of natural plant extracts for the treatment of toxoplasmosis, which offers the possibility of developing anti-*T. gondii* drugs with high activity, high efficiency, low toxicity, and low cost. Artemisinin and its derivatives have long been discovered and characterized as potent antimalarials, which have revolutionized the treatment of malaria and are being actively studied in *T. gondii* (29, 30). However, drug resistance has limited their widespread use (31–33). Extracts from some antimalarial plants, such as *Glycyrrhiza glabra*, have anti-proliferative effects against *T. gondii* by directly inhibiting the parasite in the cells of infected hosts (34). In addition, the essential oils extracted from *Lavandula angustifolia* and *Pelargonium X. asperum*, the TAF355 and TAF401 from *Eurycoma longifolia* also showed good inhibitory effect on

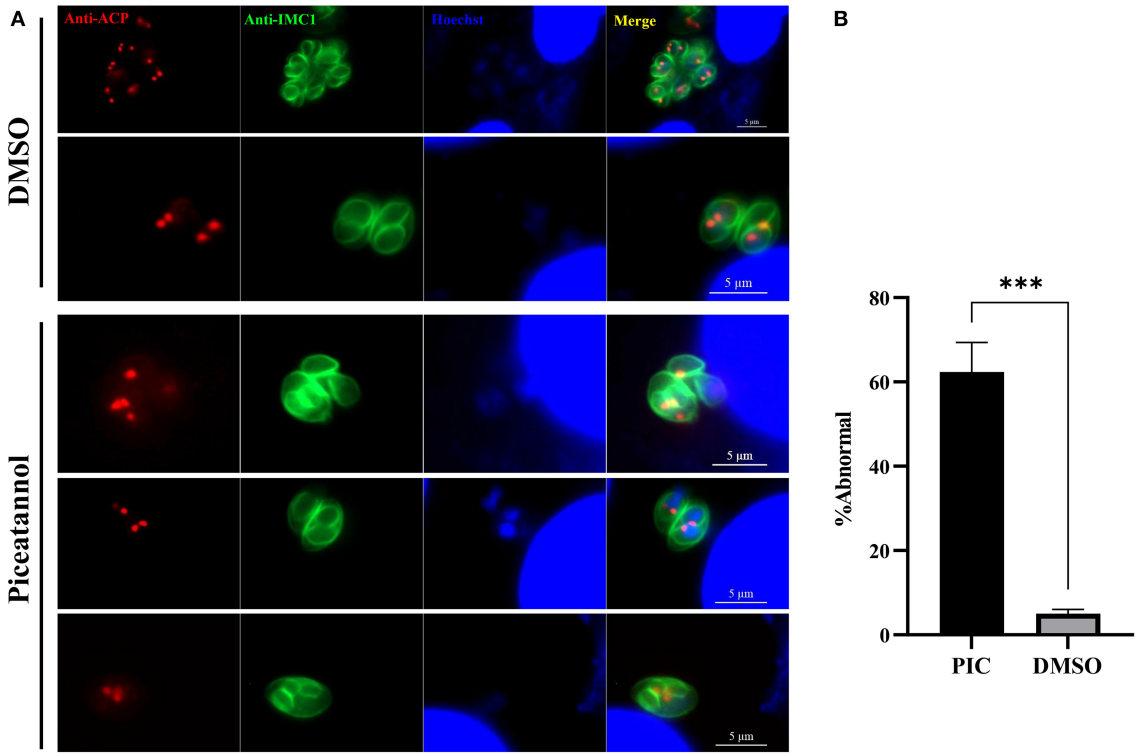


FIGURE 4
Piceatannol exposure resulting in abnormal development of *T. gondii*. Indirect immunofluorescent assay was performed after 100 μM piceatannol treatment to observe the dividing of the parasite. Organelles including IMC, apicoplast and nuclei were stained by using rabbit anti-IMC1 antibody, mouse anti-ACP antibody and Hoechst dye, respectively. **(A)** Abnormal division of *T. gondii* after piceatannol or DMSO treatment. **(B)** The proportion of abnormal divided parasites after piceatannol or DMSO treatment. The proportion of vacuoles containing abnormally splitting tachyzoites was calculated under fluorescence microscopy and the average value was taken three times. Statistical analysis of the plaque area was performed by Graph Pad Prism using Student's *t* test. ***, $p < 0.001$.

T. gondii (19–21). Therefore, the search for anti-*T. gondii* natural products is very promising.

Resveratrol is a natural polyphenolic compound, which can reduce the number of extracellularly grown tachyzoites, probably by disrupting the redox homeostasis of the parasites. Moreover, resveratrol is capable of releasing the burden of cellular stress and promoting apoptosis in *T. gondii* (26). Piceatannol is a natural analog of resveratrol, which found mainly in passion fruit, blueberry, grape, sugarcane, white tea, rhubarb, etc. (35). It has various biological activities such as antioxidant, anti-proliferative, immune enhancement, anti-inflammatory, anti-thrombotic, anti-cancer, anti-hyperlipidemic, and antibacterial (27). In this study, we characterized the anti-*T. gondii* properties of piceatannol. Our results suggest that piceatannol effectively inhibits *T. gondii* intracellularly and extracellularly, with an EC_{50} of 28.10 μM on RH tachyzoites.

In this study, we characterized the anti-*T. gondii* properties of piceatannol. Our results suggest that piceatannol effectively inhibits *T. gondii* intracellularly with an EC_{50} of 28.10 μM on RH tachyzoites. The proliferation of *T. gondii* tachyzoites in cells involves a complete set of lytic cycles, including invasion, intracellular replication and egress (28). To investigate which lytic cycle is affected by piceatannol, we performed invasion and proliferation experiments. The invasion rate of tachyzoites did not change significantly after piceatannol treatment, while proliferation was greatly affected. Thus, it is hypothesized that piceatannol reduces the number of plaques in the plaque assay by inhibiting the proliferation of tachyzoites in the cells. Piceatannol has various biological activities such as antioxidant, anti-proliferative, immune enhancement, anti-inflammatory, anti-thrombotic, anti-cancer, anti-hyperlipidemic, and antibacterial (27). It is a natural analog of resveratrol, which found mainly in passion fruit, blueberry, grape, sugarcane, white tea, rhubarb, etc. (35). Previous research showed that resveratrol can reduce the proliferation of tachyzoites, probably by disrupting the redox homeostasis of the parasites. Moreover, resveratrol is capable of releasing the burden of cellular stress and promoting apoptosis in *T. gondii* (26). Thus, we hypothesize that the mechanism of piceatannol inhibits *T. gondii* development when before and during cell infection might same as resveratrol.

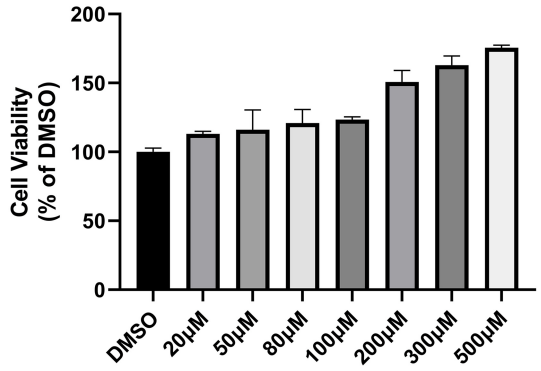


FIGURE 5
Cytotoxicity of piceatannol on Vero cells. Cytotoxicity assay graph. Toxicity of 20–500 μM piceatannol on Vero cells was measured using CCK-8 reagent. DMSO was set as a control. Inhibition rate = (OD_{DMSO}-OD_{PIC})/OD_{DMSO}. Piceatannol was not toxic to Vero cells. This experiment was performed in triplicate.

Further studies showed that treatment with piceatannol resulted in abnormal division of parasites. Since *T. gondii,* as a single-celled eukaryote, undergoes binary division, tachyzoites in parasitophorous vacuoles are usually divided synchronously, the number of tachyzoites in a PV is multiples of 2 (36). Unusually, the piceatannol-treated group showed a large number of abnormal divisions, e. g., asynchronously division and even three daughter cells emerged in a single tachyzoite and 3 or 5 tachyzoites per vacuole were presented. These results suggest that piceatannol may affect *Toxoplasma* division, which may be one of the reasons why piceatannol affects the proliferation of parasites.

Usually, such anti-*Toxoplasma* drugs are toxic to cells. However, in the present study, we found that piceatannol had no toxic effect on cells and it even promoted cell proliferation at higher concentrations. Previous studies have shown that piceatannol enhances the viability of HG-induced H9C2 cardiac myoblasts (37). However, the mechanism of how piceatannol improves cellular activity is unclear and needs to be determined by further studies.

Potent *in vitro* parasite killing and non-toxicity make piceatannol a good anti-*Toxoplasma* candidate. However, piceatannol was not significantly effective in the treatment of mice infected with *T. gondii*. Although it had some effect in delaying the death of mice, it did not prevent the death of mice. We speculate that this may be due to the rapid metabolism in mice. A previous study showed that the blood concentration of piceatannol peaked at 15 min after intragastric administration and decreased significantly after 2 h. Piceatannol was totally metabolized after 4 h of treatment, and a large amount of piceatannol metabolites were found in the urine of rats (38). Therefore, the structure of piceatannol can be further optimized to delay its metabolism *in vivo* and thus improve its therapeutic effect in *T. gondii-infected* mice.

Conclusion

In this study, we characterized the anti-*T. gondii* properties of piceatannol. Piceatannol affected the division and morphology of *T. gondii* and exhibited potent anti-*Toxoplasma* effects *in vitro* without cell toxicity. Our study suggests that piceatannol is a promising drug for the treatment of *T. gondii*.

Materials and methods

Ethics statement

Animal experiments were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Guangxi University.

Parasites, drugs and cell culture

Human foreskin fibroblasts (HFFs) and green monkey kidney (Vero) cells were purchased from the American Type Culture Collection (Manassas, VA, United States) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). In this study, a type I RH strain of *T. gondii* constitutively expressing firefly luciferase (TgRH-Luc) at the UPRT site was used and transferred to Vero cells. Parasites were cultured in DMEM supplemented with 2% fetal bovine serum. The drug monomer piceatannol was purchased from Aladdin Chemical Reagent Company (Aladdin, Shanghai, China).

In vitro inhibition assay

Vero cells were seeded onto 96-well cell plates and cultured at 37°C with 5% CO_2. Cells were then infected with 1×10^5 of TgRH-Luc per well. At 24 h post infection (h.p.i.), 2-fold serial dilutions of piceatannol (final concentration from 57 to 0.22 μM) were added to each parasite-infected well. Equal amounts of DMSO were used as controls. Relative luminescence units (RLU) were detected after 24 h of treatment by a fluorescence microplate reader (Tecan, Infinite M200 PRO, Männedorf, Switzerland) using the Bright-Lumi™ II Firefly Luciferase Assay Kit (Beyotime Biotech, Shanghai, China). Percentage proliferation

inhibition was calculated as: inhibition rate = [(RLU_{DMSO} - $RLU_{treatment}$)/RLU_{DMSO}] × 100%. Samples were run in triplicate and three independent assays were performed. Data are expressed as mean ± standard deviation (SD). Half-maximal effective concentration (EC_{50}) of compounds and 95% confidence interval (CI) were extrapolated using the log (inhibitor) vs. response-variable slope (four parameters) regression equation in GraphPad Prism 8 (GraphPad, La Jolla, CA).

Plaque assays

In order to make a preliminary identification of the anti-*T. gondii* ability of piceatannol, plaque assays were performed as described previously (39). Briefly, purified parasites were used to infect HFF seeded on 12-well-plates (150 tachyzoites/well). HFF cells were treated with 100 μM Piceatannol and DMSO-treated cells were used as a control. After 7 days of undisturbed culture, HFF were fixed with 4% PFA and stained with crystal violet. Cells in the plaque area were counted in pixels using Photoshop C6S software (Adobe, USA), and data from three independent experiments were compiled.

Intracellular inhibition assays

Vero cells growing in 96-well-plates were infected with 1 × 10^5 TgRH-Luc parasites for 3 h and then cultured with piceatannol (100 μM) for 24 h at 37°C with 5% CO_2. Cells and parasites were then lysed, and RLUs were measured as described above.

Intracellular replication assay and invasion assay

An intracellular replication assay was performed to assess the number of parasites per vacuole 24 h after invasion, which is consistent with a previous description (40). Briefly, HFF growing in 12-well-plates seeded on coverslips were inoculated with 1 × 10^5 parasites and cultured continuously for 2 h. They were subsequently treated with 100 μM piceatannol for 24 h, and DMSO-treated cells were used as a control. Then, an indirect immunofluorescent assay was performed using rabbit anti-GAP45 antibodies and Hoechst dye to observe the intracellular replication of tachyzoites. The tachyzoites of each strain in the vacuoles were quantified by counting at least 100 vacuoles using a fluorescence microscope (Zeiss, Germany). For the invasion assay, the percentage of invasion was calculated based on the number of vacuoles per host cell. Three independent experiments were performed.

Effect of piceatannol on *T. gondii* infection in mice

Six-week-old BALB/c mice were infected with 500 tachyzoites by intraperitoneal injection. The parasite-infected mice were divided into four groups of five mice each. After 1 day infection, the mice were treated with 15 mg/kg/d of piceatannol by intragastric administration (group A) and intraperitoneal injection (group B) for 15 days. DMSO was treated as a control group accordingly. Survival was evaluated for up to 15 days.

T. gondii division and organelle observation

1×10^5 tachyzoites were inoculated into HFFs and incubated at 37°C with 5% CO_2 for 3 h waiting for an invasion, and divided into two groups of six wells each, with one group being treated with 100 μM Piceatannol and the other with DMSO as control. After 24 h, IFA was performed to observe the IMC, apicoplast and nuclei of the parasites by using rabbit anti-IMC1 antibody, mouse anti-ACP (acyl carrier protein) antibody and Hoechst dye, respectively.

Cytotoxicity test

Cytotoxicity of piceatannol was evaluated in Vero cell lines using CCK-8 reagent (Beyotime, Shanghai, China). Vero cells (5,000 cells/well) were cultured in 96-well-plates at 37°C and 5% CO_2 for 24 h. Sequentially diluted piceatannol from 20 μM to 500 μM was added to the host cells and incubated for 24 h, and then cell viability was determined using CCK-8 reagent according to the manufacturer's instructions. Absorbance was measured at 450 nm using a Microplate Absorbance Reader (BioRad, Hercules, CA, United States). DMSO was set as a control. Inhibition rate = (OD_{DMSO}-OD_{PIC})/OD_{DMSO}. The cytotoxicity experiment was performed in triplicate.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Guangxi University.

Author contributions

XS conceived and designed the study. YJ performed the experiments. YJ and XS analyzed the data and drafted the manuscript. DH helped in manuscript writing. YS helped in cytotoxicity test. All authors read and approved the final manuscript.

Funding

This work was supported by the Specific Research Project of Guangxi for Research Base and Talents (Grant No. AD22035040), and the Natural Science Foundation of Guangxi Zhuang Autonomous Region (Grant No. 2021GXNSFBA220004).

Acknowledgments

We are grateful to Prof. Qun Liu and Jing Liu (China Agricultural University, China) for providing the HFF cells, Vero cells and TgRH-Luc.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Dubey JP, Brown J, Ternent M, Verma SK, Hill DE, Cerqueira-Cézar CK, et al. Seroepidemiologic study on the prevalence of *Toxoplasma gondii* and *Trichinella spp*. infections in black bears (Ursus americanus) in Pennsylvania, USA. *Vet Parasitol.* (2016) 229:76–80. doi: 10.1016/j.vetpar.2016.09.013

2. Cuomo G, D'Abrosca V, Rizzo V, Nardiello S, La Montagna G, Gaeta GB, et al. Severe polymyositis due to *Toxoplasma gondii* in an adult immunocompetent patient: a case report and review of the literature. *Infection.* (2013) 41:859–62. doi: 10.1007/s15010-013-0427-x

3. Wang ZD, Wang SC, Liu HH, Ma HY, Li ZY, Wei F, et al. Prevalence and burden of *Toxoplasma gondii* infection in HIV-infected people: a systematic review and meta-analysis. *Lancet HIV.* (2017) 4:e177–88. doi: 10.1016/S2352-3018(17)30005-X

4. Opsteegh M, Kortbeek TM, Havelaar AH, van der Giessen JW. Intervention strategies to reduce human *Toxoplasma gondii* disease burden. *Clin Infect Dis.* (2015) 60:101–7. doi: 10.1093/cid/ciu721

5. Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet.* (2004) 363:1965–76. doi: 10.1016/S0140-6736(04)16412-X

6. Hill DE, Chirukandoth S, Dubey JP. Biology and epidemiology of *Toxoplasma gondii* in man and animals. *Anim Health Res Rev.* (2005) 6:41–61. doi: 10.1079/AHR2005100

7. Elsheikha HM, Marra CM, Zhu XQ. Epidemiology, pathophysiology, diagnosis, and management of cerebral toxoplasmosis. *Clin Microbiol Rev.* (2021) 34:17. doi: 10.1128/CMR.00115-19

8. Alday PH, Doggett JS. Drugs in development for toxoplasmosis: advances, challenges, and current status. *Drug Des Devel Ther.* (2017) 11:273–93. doi: 10.2147/DDDT.S60973

9. Ben-Harari RR, Goodwin E, Casoy J. Adverse event profile of pyrimethamine-based therapy in toxoplasmosis: a systematic review. *Drugs R D.* (2017) 17:523–44. doi: 10.1007/s40268-017-0206-8

10. Shammaa AM, Powell TG, Benmerzouga I. Adverse outcomes associated with the treatment of *Toxoplasma* infections. *Sci Rep.* (2021) 11:1035. doi: 10.1038/s41598-020-80569-7

11. Silva MD, Teixeira C, Gomes P, Borges M. Promising drug targets and compounds with anti-*Toxoplasma gondii* activity. *Microorganisms.* (2021) 9:1960. doi: 10.3390/microorganisms9091960

12. Wei HX, Wei SS, Lindsay DS, Peng HJ. A systematic review and meta-analysis of the efficacy of Anti-*Toxoplasma gondii* medicines in humans. *PLoS ONE.* (2015) 10:e0138204. doi: 10.1371/journal.pone.0138204

13. Montazeri M, Sharif M, Sarvi S, Mehrzadi S, Ahmadpour E, Daryani A. A systematic review of *in vitro* and *in vivo* activities of anti-*Toxoplasma* drugs and compounds (2006-2016). *Front Microbiol.* (2017) 8:25. doi: 10.3389/fmicb.2017.00025

14. Dunay IR, Gajurel K, Dhakal R, Liesenfeld O, Montoya JG. Treatment of toxoplasmosis: historical perspective, animal models, and current clinical practice. *Clin Microbiol Rev.* (2018) 31:9. doi: 10.1128/CMR.00057-17

15. Montoya JG, Laessig K, Fazeli MS, Siliman G, Yoon SS, Drake-Shanahan E, et al. A fresh look at the role of spiramycin in preventing a neglected disease: meta-analyses of observational studies. *Eur J Med Res.* (2021) 26, 143. doi: 10.1186/s40001-021-00606-7

16. Cheraghipour K, Masoori L, Ezzatpour B, Roozbehani M, Sheikhian A, Malekara V, et al. The experimental role of medicinal plants in treatment of *Toxoplasma gondii* infection: a systematic review. *Acta Parasitol.* (2021) 66:303–28. doi: 10.1007/s11686-020-00300-4

17. Benoit-Vical F, Santillana-Hayat M, Kone-Bamba D, Mallie M, Derouin F. Anti-*Toxoplasma* activity of vegetal extracts used in West African traditional medicine. *Parasite.* (2000) 7:3–7. doi: 10.1051/parasite/2000071003

18. Chen SX, Wu L, Jiang XG, Feng YY, Cao JP. Anti-*Toxoplasma gondii* activity of GAS *in vitro*. *J Ethnopharmacol.* (2008) 118:503–7. doi: 10.1016/j.jep.2008.05.023

19. Kavitha N, Noordin R, Chan KL, Sasidharan S. *In vitro* anti-*Toxoplasma gondii* activity of root extract/fractions of *Eurycoma longifolia* Jack. *BMC Complement Altern Med.* (2012) 12:91. doi: 10.1186/1472-6882-12-91

20. Huang SY, Yao N, He JK, Pan M, Hou ZF, Fan YM, et al. *In vitro* anti-parasitic activity of *Pelargonium X.* asperum essential oil against *Toxoplasma gondii*. *Front Cell Dev Biol.* (2021) 9:616340. doi: 10.3389/fcell.2021.616340

21. Yao N, He JK, Pan M, Hou ZF, Xu JJ, Yang Y, et al. *In Vitro* evaluation of *Lavandula angustifolia* essential oil on anti-*Toxoplasma* activity. *Front Cell Infect Microbiol.* (2021) 11:755715. doi: 10.3389/fcimb.2021.755715

22. Chan MM. Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin. *Biochem Pharmacol.* (2002) 63:99–104. doi: 10.1016/S0006-2952(01)00886-3

23. Pais-Morales J, Betanzos A, García-Rivera G, Chávez-Munguía B, Shibayama M, Orozco E. Resveratrol induces apoptosis-like death and prevents *in vitro* and *in vivo* virulence of *Entamoeba histolytica*. *PLoS ONE.* (2016) 11:e0146287. doi: 10.1371/journal.pone.0146287

24. Leischner C, Burkard M, Pfeiffer MM, Lauer UM, Busch C, Venturelli S. Nutritional immunology: function of natural killer cells and their modulation by resveratrol for cancer prevention and treatment. *Nutr J.* (2016) 15:47. doi: 10.1186/s12937-016-0167-8

25. Fernandes GFS, Silva GDB, Pavan AR, Chiba DE, Chin CM, Dos Santos JL. Epigenetic regulatory mechanisms induced by resveratrol. *Nutrients.* (2017) 9:1201. doi: 10.3390/nu9111201

26. Chen QW, Dong K, Qin HX, Yang YK, He JL, Li J, et al. Direct and indirect inhibition effects of resveratrol against *Toxoplasma gondii* Tachyzoites *in vitro*. *Antimicrob Agents Chemother.* (2019) 63:2. doi: 10.1128/AAC.01233-18

27. Piotrowska H, Kucinska M, Murias M. Biological activity of piceatannol: leaving the shadow of resveratrol. *Mutat Res.* (2012) 750:60–82. doi: 10.1016/j.mrrev.2011.11.001

28. Blader IJ, Coleman BI, Chen CT, Gubbels MJ. Lytic cycle of *Toxoplasma gondii*: 15 years later. *Annu Rev Microbiol.* (2015) 69:463–85. doi: 10.1146/annurev-micro-091014-104100

29. D'Angelo JG, Bordón C, Posner GH, Yolken R, Jones-Brando L. Artemisinin derivatives inhibit *Toxoplasma gondii in vitro* at multiple steps in the lytic cycle. *J Antimicrob Chemother.* (2009) 63:146–50. doi: 10.1093/jac/dkn451

30. Talman AM, Clain J, Duval R, Ménard R, Ariey F. Artemisinin bioactivity and resistance in malaria parasites. *Trends Parasitol.* (2019) 35:953–63. doi: 10.1016/j.pt.2019.09.005

31. Rosenberg A, Luth MR, Winzeler EA, Behnke M, Sibley LD. Evolution of resistance *in vitro* reveals mechanisms of artemisinin activity in *Toxoplasma gondii*. *Proc Natl Acad Sci U S A.* (2019) 116:26881–91. doi: 10.1073/pnas.1914732116

32. Harding CR, Sidik SM, Petrova B, Gnädig NF, Okombo J, Herneisen AL, et al. Genetic screens reveal a central role for heme metabolism in artemisinin susceptibility. *Nat Commun.* (2020) 11:4813. doi: 10.1038/s41467-020-18624-0

33. Wicht KJ, Mok S, Fidock DA. Molecular mechanisms of drug resistance in *Plasmodium falciparum* malaria. *Annu Rev Microbiol.* (2020) 74:431–54. doi: 10.1146/annurev-micro-020518-115546

34. Pastorino G, Cornara L, Soares S, Rodrigues F, Oliveira M. Liquorice (Glycyrrhiza glabra): a phytochemical and pharmacological review. *Phytother Res.* (2018) 32:2323–39. doi: 10.1002/ptr.6178

35. Inagaki H, Ito R, Setoguchi Y, Oritani Y, Ito T. Administration of piceatannol complexed with α-cyclodextrin improves its absorption in rats. *J Agric Food Chem.* (2016) 64:3557–63. doi: 10.1021/acs.jafc.6b00398

36. Suvorova ES, Francia M, Striepen B, White MW. A novel bipartite centrosome coordinates the apicomplexan cell cycle. *PLoS Biol.* (2015) 13:e1002093. doi: 10.1371/journal.pbio.1002093

37. Zheng J, Ramirez VD. Piceatannol, a stilbene phytochemical, inhibits mitochondrial F0F1-ATPase activity by targeting the F1 complex. *Biochem Biophys Res Commun.* (1999) 261:499–503. doi: 10.1006/bbrc.1999.1063

38. Setoguchi Y, Oritani Y, Ito R, Inagaki H, Maruki-Uchida H, Ichiyanagi T, et al. Absorption and metabolism of piceatannol in rats. *J Agric Food Chem.* (2014) 62:2541–8. doi: 10.1021/jf404694y

39. Shen B, Sibley LD. Toxoplasma aldolase is required for metabolism but dispensable for host-cell invasion. *Proc Natl Acad Sci U S A.* (2014) 111:3567–72. doi: 10.1073/pnas.1315156111

40. Song X, Yang X, Xue Y, Yang C, Wu K, Liu J, et al. Glutaredoxin 1 deficiency leads to microneme protein-mediated growth defects in *Neospora caninum*. *Front Microbiol.* (2020) 11:536044. doi: 10.3389/fmicb.2020.536044

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 26 August 2022
DOI 10.3389/fvets.2022.959449

OPEN ACCESS

EDITED BY
Fazul Nabi,
Lasbela University of Agriculture, Water and Marine Sciences, Pakistan

REVIEWED BY
Ahrar Khan,
Shandong Vocational Animal Science and Veterinary College, China
Yun peng Fan,
Northwest A&F University, China

*CORRESPONDENCE
Yi Wu
wuyi2001cn@163.com;
wuyi2001cn@njau.edu.cn

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 01 June 2022
ACCEPTED 08 August 2022
PUBLISHED 26 August 2022

CITATION
Zhou H, Dai C, Cui X, Zhang T, Che Y, Duan K, Yi L, Nguyen AD, Li N, De Souza C, Wan X, Wu Y, Li K, Liu Y and Wu Y (2022) Immunomodulatory and antioxidant effects of Glycyrrhiza uralensis polysaccharide in Lohmann Brown chickens.
Front. Vet. Sci. 9:959449.
doi: 10.3389/fvets.2022.959449

Immunomodulatory and antioxidant effects of Glycyrrhiza uralensis polysaccharide in Lohmann Brown chickens

Hui Zhou[1,2†], Chen Dai[3†], Xuejie Cui[1,4†], Tao Zhang[5], Yanyun Che[6], Kun Duan[7], Lei Yi[1,8], Audrey D. Nguyen[9], Nannan Li[1], Cristabelle De Souza[10], Xin Wan[1,2], Yu Wu[1,2], Kun Li[1,2], Yuhong Liu[1,4] and Yi Wu[1,2*]

[1]College of Veterinary Medicine, Institute of Traditional Chinese Veterinary Medicine, Nanjing Agricultural University, Nanjing, China, [2]MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China, [3]Experimental Teaching Center of Life Science, College of Life Sciences, Nanjing Agricultural University, Nanjing, China, [4]College of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan, China, [5]Beijing Key Laboratory of Traditional Chinese Veterinary Medicine, Beijing University of Agriculture, Beijing, China, [6]Engineering Laboratory for National Healthcare Theories and Products of Yunnan Province, College of Pharmaceutical Science, Yunnan University of Chinese Medicine, Kunming, China, [7]China Tobacco Henan Industrial Co., Ltd, Zhengzhou, China, [8]Department of Animal Science, Huaihua Polytechnic College, Huaihua, China, [9]Department of Biochemistry and Molecular Medicine, Davis Medical Center, University of California, Sacramento, Sacramento, CA, United States, [10]Department of Pathology, Stanford University, Palo Alto, CA, United States

Glycyrrhiza polysaccharide extract 1 (GPS-1) is a bioactive component isolated from Glycyrrhiza uralensis, also known as Chinese licorice. It appears to be pharmacologically active as an antibacterial, antiviral, and anti-tumor agent. GPS-1 has also been shown to buffer liver health and regulate the immune system. Moreover, GPS-1 is low cost and easy to extract. More study was needed to elucidate the biochemical pathways underlying the immunomodulatory and antioxidant benefits observed in Glycyrrhiza polysaccharide extract 1 (GPS-1). *in vitro* experiments on chicken lymphocytes and dendritic cells (DCs) show that GPS-1 significantly promotes the proliferation of immune cells and is linked to lymphocytes' secretion of IL-12, IFN-γ, and TNF-α by. DC secretion of NO, IL-2, IL-1β, IFN-γ, TNF-α, and IL-12p70 was also increased significantly. Additionally, GPS-1 also displayed a significant antioxidant effect *in vitro*, able to scavenge DPPH, hydrogen peroxide, ABTS, and other free radicals like superoxide anions. Separately, GPS-1 was tested in vivo in combination with the Newcastle disease virus (NDV) – attenuated vaccine. 120 Lohmann Brown chickens were vaccinated, while another 30 became the unvaccinated blank control (BC) group. For three consecutive days 1 mL of GPS-1 was administered at doses of 19.53 μg/mL, 9.77 μg/mL, or 4.88 μg/mL to the ND-vaccinated birds, except for the vaccine control (VC), where $n = 30$ per group. *In vivo* results show that GPS-1 combined with Newcastle disease (ND) vaccine had the best efficacy at significantly increasing chickens' body weight and ND serum antibody titer,

enhancing their secretion of IL-2 and IFN- γ, and promoting the development of immune organs. The results also indicate that GPS-1 was able increase the proliferation of *in vitro* immune cells and elevate their cytokine secretion, which enhances the body's immune response. GPS-1 also clearly has the potential to be used as an immune adjuvant alongside ND vaccination.

KEYWORDS

Glycyrrhiza uralensis polysaccharide, immunomodulatory effects, antioxidant activities, Newcastle disease, chickens

Introduction

Over the past few decades, researchers have isolated various polysaccharides from plants, animals, and microorganisms, and have demonstrated their effective therapeutic activities and relatively low toxicities to mammals. As a result, there is increasing attention to polysaccharides in the field of molecular therapeutics. Prior studies have demonstrated that water-soluble polysaccharides extracted from Chinese herbal medicines provide medicinal benefits such as immune regulation, antioxidation, antitumor activity, liver protection, and anticoagulation (1–3).

Licorice has been used for over 2,000 years in traditional Chinese medicine to treat many human ailments like peptic ulcers, Addison's disease, asthma, cough, sore throat, acne, and boils (4–6). Chinese licorice is still a commonly used medicine in many parts of Asia. It comes from the dried roots and rhizomes of Glycyrrhiza uralensis, and is mainly found in northwest China, including the regions of Xinjiang, Gansu, and Shanxi. Its main active components are triterpenoid saponins and flavonoids, which endue Glycyrrhiza uralensis with potential pharmacological applications as an anti–ulcer, anti–inflammatory, anti–spasmolysis, antioxidant, antiviral, antidepressant, liver protectant, expectorant, and memory enhancement compound (7–10). There is a growing body of evidence that the polysaccharides within many plants like Glycyrrhiza uralensis may prove useful with immunomodulation, lowering glucose, or as a novel antiviral or antitumor agent (11–17). Polysaccharides are a type of sugar moiety with complex molecular structures, created by the condensation and dehydration of several monosaccharide molecules. Glycyrrhiza polysaccharides (GPS) are the main active macromolecules in Glycyrrhiza uralensis (18, 19), and have previously shown promising results as an antibacterial (20), antiviral and antitumor agent (21, 22), a liver protectant (23), and immune regulator (14). In one study that highlighted this immune-regulation potential, Glycyrrhiza polysaccharide was fed to CT-26 tumor-bearing mice and significantly inhibited tumor growth, increased the immune organ index, activated CD4+ and CD8+ immune cells, and increased levels of IL-2, IL-6, and IL-7 cytokines (24). Because many other polysaccharides have been reported have both immunomodulatory and antioxidant effects (25–28), the antioxidant properties of GPS-1 were tested in the present research. In a separate study, Glycyrrhiza polysaccharide was extracted by supercritical CO_2, and an antioxidation experiment was carried out *in vitro* (29). In that investigation Glycyrrhiza polysaccharide was found to possess a notable ability to scavenge OH, O_2, and DPPH. Recently, many studies have suggested that Glycyrrhiza polysaccharides perform a wide range of biological functions. Research reported that dietary supplementation with Glycyrrhiza polysaccharide improves the growth, development, and serum antioxidant levels of chickens raised for meat (30). In addition, Glycyrrhiza polysaccharides also has the ability to improve growth, and the quantities of white blood cell, neutrophils, red blood cells, and platelets, as well as elevate alkaline phosphatase, total protein, globulin, glucose, triglycerides, immunoglobulin A, immunoglobulin G, total antioxidant capacity levels, and immune responses in mice, quails, and weaned piglets (31–33). In this study, we investigated the immune enhancement and antioxidation functions of GPS-1 *in vitro* and *in vivo* (intramuscular injection, unlike previous studies on oral GPS-1). Our aim is to contribute to a deeper understanding of the immunoenhancement and antioxidant activities of GPS-1, and to provide a theoretical basis for its continued development and application as a pharmaceutical.

Materials and methods

Materials and chemicals for *in vitro* experiments

GPS-1 was prepared following previously reported methods (14). The crude polysaccharide was extracted from licorice by the Sevag method, deproteinated by DEAE chromatography column, purified by Sepharose CL−6B and Sephadex G-200 chromatography columns, and lyophilized to get purified GPS-1 extract. RPMI 1640 and DMEM were purchased from GIBCO. The penicillin-streptomycin mixture was sourced from HyClone. Fetal bovine serum was purchased from Tianhang

TABLE 1 A570 value of every polysaccharide groups 1,250–2.44 μg mL^{-1} ($n = 4$).

Concentration	A_{570}
1,250	0.203 ± 0.010^{e}
625	0.347 ± 0.067^{d}
312.5	0.356 ± 0.010^{d}
156.5	0.355 ± 0.014^{d}
78.12	0.386 ± 0.005^{bc}
39.06	0.409 ± 0.013^{ab}
19.53	0.418 ± 0.017^{a}
9.77	0.398 ± 0.038^{ab}
4.88	0.369 ± 0.007^{cd}
2.44	0.344 ± 0.017^{d}
CC	0.365 ± 0.005^{cd}

[a–e]Bars in the histogram with no identical letters indicated a significantly difference ($P < 0.05$). CC (cell control), the same below.

Biotechnology Co., Ltd. Lohmann Brown chickens' recombinant proteins GM-CSF (mGM-CSF) and interleukin 4 (IL-4) were purchased from Peprotech Inc. The MTT solution and heparin was bought from Biofroxx. Phytin (PHA-P) was procured from Solarbio. Human peripheral blood lymphocytes separation medium was purchased from Tianjin Haoyang Biological Products Technology Co., Ltd. Histopak separators were bought from Sigma. IL-1β, Il-12, IL-12P70, IFN-γ, TNF-α, and NO kits were purchased from Nanjing Aoqing Biotechnology Co., Ltd. DPPH was obtained from TCI Chemical Industrial Development Co., Ltd RAW 264.7 cells were purchased from ATCC. ABTS and tromethamine were purchased from Soleil.

Safe concentration of GPS-1 for peripheral lymphocytes

First, the feathers were removed from the blood collection site on the breast and the skin was sterilized with 75% alcohol. Then, a sterile syringe was inserted into the heart to collect 5 mL of blood and immediately mixed with heparin sodium. The fresh blood with anticoagulant was mixed with isotonic saline in a ratio of 1:1. Then, human peripheral blood lymphocyte separation medium was added to the top layer of lymphocytes and centrifuged at 1,500 rpm for 15 min. The second layer of lymphocytes was collected and put into a test tube containing isotonic saline. These lymphocytes in saline were mixed and centrifuged again at 1,500 rpm for 20 min. This procedure was repeated once more to obtain peripheral blood lymphocytes. The lymphocytes were then resuspended to 1×10^6 cells/mL with RPMI 1,640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin, then cultured in 96-well-culture plates at 100 μL per well (34).

A series of GPS-1 concentrations (2.44, 4.88, 9.77, 19.53, 39.06, 78.12, 156.5, 312.5, 625, and 1,259 μg/mL) were added to 96-well-culture plates at 100 μL per well, with 4 wells per concentration. The 96-well-plates were then cultured in a humidified incubator at 37°C and 5% CO_2 for 44 h. Then MTT solution (5 mg/mL, 30 μL/well) was added, and incubation was continued for 4 more h. Afterward, 100 μL DMSO per well was used to dissolve the precipitates while shaking for 5 min. An enzyme-linked immunosorbent assay (ELISA) reader was then used to analyze the absorbance of the solution at a wavelength of 570 nm. At the GPS-1 concentration of 39.06 μg/mL the A570 value is significantly higher than the lymphocyte control group. 39.06 μg/mL was therefore chosen as the maximum safe concentration of GPS-1.

Immune cell proliferation assays

The peripheral blood lymphocytes were prepared according to methods detailed in section Immune cell proliferation assays. The spleen cell suspension was filtered with a 200 mesh cell filter and separated using lymphocyte separation solution. After centrifugation at 2,000 rpm for 10 min, the middle layer containing lymphocytes was isolated and washed twice with PBS to remove impurities. More than 95% of spleen lymphocytes were viable based on trypan blue dye exclusion.

The immature Lohmann Brown chickens bone marrow dendritic cells (chBM-DCs) were obtained from 4-week-old Lohmann Brown chickens following our previously reported method (35). The femurs and tibias were removed and separated from the surrounding muscle tissue under aseptic conditions. Then, the bones were washed three times with 0.01 M PBS (pH 7.2). Clusters of bone containing marrow were collected and centrifuged at 1,500 rpm for 20 min to obtain the precipitate. This precipitate was disaggregated in PBS and centrifuged at 1,500 rpm for 20 min to remove the supernatant solution. The cells were resuspended in PBS, layered onto an equal volume of Histopaque®-1077 solution (Sigma-Aldrich) and centrifuged at 2,500 rpm for 30 min. The immature chBM-DCs cells at the interface were then collected and suspended in complete DMEM cell culture medium (Sigma) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (p/s).

The peripheral blood lymphocytes and chBM-DCs were each adjusted to 5.0×10^6 cells/mL and added into separate 96-well-cell culture plates at 100 μL per well. Based on our determination of the safe concentration of GPS-1 from section Immune cell proliferation assays, GPS-1 was added at doses of 2.44, 4.88, 9.77, 19.53, and 39.06 μg/mL, with four wells per concentration. The cultures control (CC) group and PHA control were also cultured accordingly. All 96-well-plates were then incubated at 37°C and 5% CO_2 for

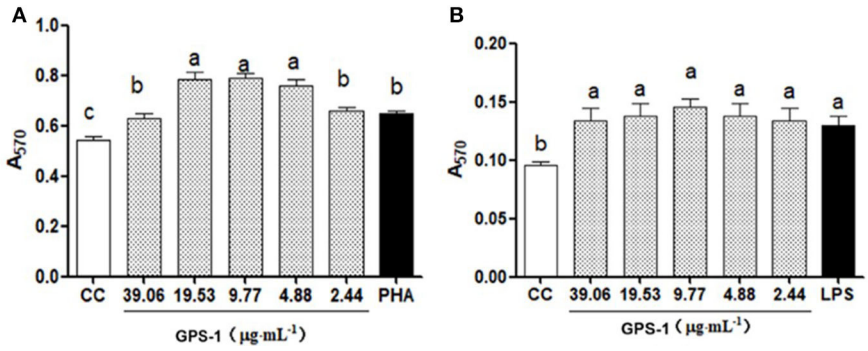


FIGURE 1
Effects of GPS-1 on A570 of chicken peripheral **(A)** and spleen lymphocytes **(B)**. [a–c]Values in the same column with different superscript letters differ significantly at $p < 0.05$.

44 h. After cultivation, the proliferation rate of each well was tested *via* MTT assay following the method in Immune cell proliferation assays.

Supernatant cytokines and NO assay

Supernatant cytokines of peripheral lymphocytes assay

The peripheral blood lymphocytes were cultured in 24-well-cell culture plates with a cell concentration of 1×10^6 cells/mL following a previously published method (36). GPS-1 was added at a range of non-toxic concentrations (2.44, 4.88, 9.77, 19.53, and 39.06 μg/mL) to each cell culture plate, each in triplicate, and incubated at 37°C and 5% CO_2 for 48 h. The culture control (CC) group and PHA control were also incubated with the same conditions ($n = 3$), minus GPS-1 treatment. Then, the supernatants of each group were collected. The concentrations of IL-12, IFN-γ, and TNF-α in the supernatants of each group were measured using ELISA kits.

Supernatant cytokines and NO production of ChBM-DCs precursor cells

The chBM-DC precursor cells were harvested and cultured in 24-well-plates at 37°C and 5% CO_2 for 15 h. After discarding the suspended cells, the adherent cells were incubated in the DMEM complete media (10% FBS, 1% p/s) containing GM-CSF (50 ng/mL) and IL-4 (50 ng/mL) stimulators. On the 3rd and 5th days of incubation, half of the nutrient solution was refreshed. On the 6th day of cultivation, GPS-1 of different concentrations (2.44, 4.88, 9.77, 19.53, and 39.06 μg/mL) was added to all but the CC and LPS groups, then cells were cultivated for another 48 h. The treated cells were divided into two parts. The supernatant of each well was used to measure the concentration of secreted NO in each group (using Griess reagent), and quantify the production of cytokines IL-2, IL-1β, IFN-γ, TNF-α, and IL-12p70 using ELISA kits (37).

Antioxidant activity of GPS-1 *in vitro*

Hydroxyl radical scavenging activity assay

Hydroxyl radical scavenging activity was examined using the salicylic acid trapping method (38). In brief, 2 mL of $FeSO_4$ solution (9 mmol/L), 2 mL of salicylic acid solution (9 mmol/L), and 2 mL of GPS-1 (2.44, 4.88, 9.77, 19.53, or 39.06 μg/mL) were added to each test tube. Then, 2 mL of H_2O_2 solution (8.8 mmol/L) was added to initiate the reaction, which was kept at 37°C in a water bath for 30 min. The absorbance (A1) of each sample was measured at 510 nm. Ascorbic acid was used as a positive control while distilled water was used as blank control (A0). Each group was repeated in triplicate. The scavenging rate (%) was calculated according to the following equation:

$$\text{Scavenging rate (\%)} = \left[1 - \frac{\text{A1}}{\text{A0}}\right] \times 100\%$$

DPPH radical scavenging assay

The DPPH radical scavenging assay was performed according to the previously published description (39). Briefly, 1 mL of DPPH-ethanol solution (1×10^{-4} mol/L) was mixed with 3 mL of each concentration of GPS-1 (2.44, 4.88, 9.77, 19.53, and 39.06 μg/mL), and kept in darkness at 37°C for 30 min. Absorbance (A1) was measured at 517 nm. Ascorbic acid was used as positive control, distilled water was the blank control (A0), and ethanol was used to determine A2. Each group was tested in triplicate. The DPPH radical scavenging effect (%) was calculated as follows:

$$\text{DPPH Scavenging rate } (\%) = \left[1 - \frac{A1 - A2}{A0}\right] \times 100\%$$

ABTS radical scavenging activity assay

ABTS radical scavenging activity was measured according to Roberta Bernini's method (29), with minor modifications. The ABTS radical solution mixture (7 mmol/L of ABTS solution and 2.5 mM of potassium persulfate, 1:1, v/v) was diluted with ethanol to an absorbance (A0) of 0.70 ± 0.02 at 734 nm. Then, 0.2 mL of GPS-1 at different concentrations (2.44, 4.88, 9.77, 19.53, and 39.06 μg/mL) was combined with 0.8 mL of ABTS radical solution mixture, and allowed to react for 6 min in darkness, at room temperature. The absorbance (A1) of each product was measure at 734 nm. BHT was used as the positive control. The ABTS radical scavenging activity of GPS-1 was calculated with the following equation:

$$\text{ABTS radical scavenging activity } (\%) = \left[1 - \frac{A1}{A0}\right] \times 100\%$$

Superoxide anion radical scavenging ability assay

Superoxide anion radical scavenging ability was assayed according to a previously reported method (35, 40). 2 mL of tris-HCl buffer solution (0.05 mol/L, pH 8.2) was mixed with each GSP-1 concentration (2.44, 4.88, 9.77, 19.53, and 39.06 μg/L), 1 mL each. These mixtures were combined with pyrogallol solution (7 mmol/L, 0.6 mL) and kept in a 25°C water bath for 30 min then combined immediately afterward. The absorbance of the combination was detected at 320 nm every 30 s and labeled "A1." The absorbance of the blank control group (A0) was determined using pyrogallol solution (7 mmol/L, 2 mL). The superoxide anion clearance can be calculated as follows:

$$\text{Superoxide anion clearance } (\%) = \left[1 - \frac{A1}{A0}\right] \times 100\%$$

Cell viability assays

RAW 264.7 cells were used to measure cell survival after GPS-1 treatment. Cell density was adjusted to 1×10^6 cells/mL and added into three 96-well-plates at 100 μL per well (41–43).

The first plate was cultured for 30 min at 37°C and 5% CO_2. H_2O_2 was added to all wells except the control group at a range of concentrations (100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000 μM), each in triplicate. The plate was then incubated for another 24 h. Afterward, 30 μL of MTT solution was added to each well, followed by another 4 h of incubation. An ELISA reader was then used to detect absorbances at a 570 nm, allowing us to quantify the damage index of H_2O_2. Each concentration was repeated in triplicate.

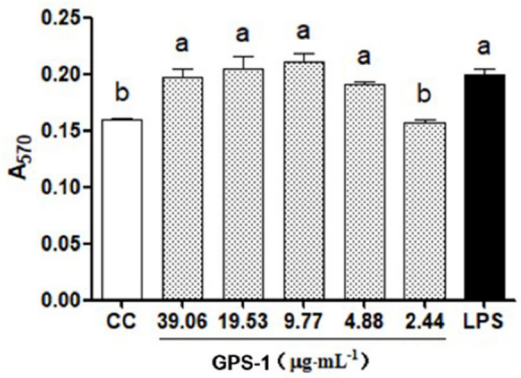


FIGURE 2
GPS-1 effects on the proliferation of chicken dendritic cells (A570). [a,b]Values in the same column with different superscript letters differ significantly at $p < 0.05$.

A second 96-well-plate seeded with RAW 264.7 cells and 0.1 mL of each GPS-1 dose (2.44, 4.88, 9.77, 19.53, 39.06, 78.12, and 156.50 μg/L) was added ($n = 3$ per group). The plate was incubated at 37°C and 5% CO_2 for 24 h. The MTT solution (30 μL) was added into each well, then incubated for another 4 h. After cultivation, the supernatants were discarded, then the precipitates were dissolved in 100 μL DMSO and shaken for 15 min. The absorbance of the solution in each well was detected using an ELISA reader at a wavelength of 570 nm to elucidate the stimulative effects of GPS-1.

Similarly, the third 96-well-plate was treated with different concentrations of GPS-1 (4.88, 9.77 and 19.53 μg/L) and incubated for 24 h. The control group, H_2O_2 model groups, and positive control (25 μg/ml vitamin C) group were also incubated for 24 h. Except for the control group, each group was also treated with 900 μM H_2O_2 for 4 h. An MTT assay was used to analyze the ability of GPS-1 to protect RAW 264.7 cells from oxidative damage due to H_2O_2 exposure.

Materials and chemicals for *in vivo* experiments

The source of GPS-1 is detailed in section Materials and chemicals for *in vitro* experiments, above. RPMI 1640 and DMEM were purchased from GIBCO. Newcastle disease IV vaccine (La Sota Strain) was purchased from Nanjing Qian Yuan Hao Biological Co., Ltd. IL-2, IL-4, and IFN-γ kits and T-AOC, GSH-Px, CAT, Sod and MDA kits were purchased from Nanjing

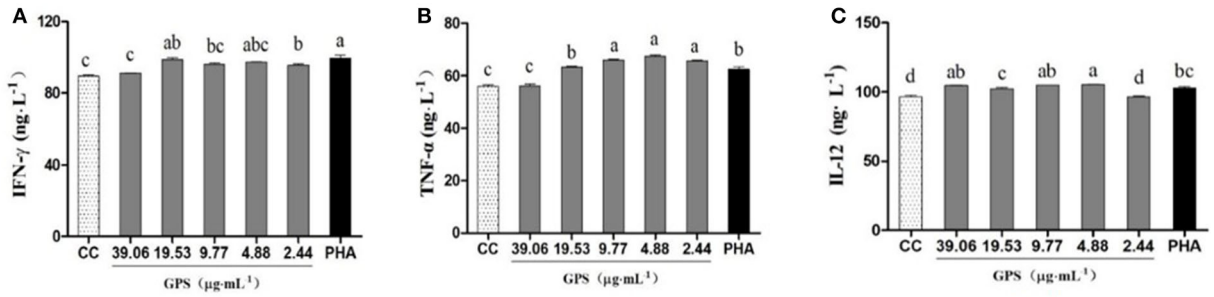


FIGURE 3
Effects of GPS-1 on the secretion of cytokines by lymphocytes. **(A)** IFN-γ; **(B)** TNF-α; **(C)** IL-12. $^{a-d}$Values in the same column with different superscript letters differ significantly at $p < 0.05$.

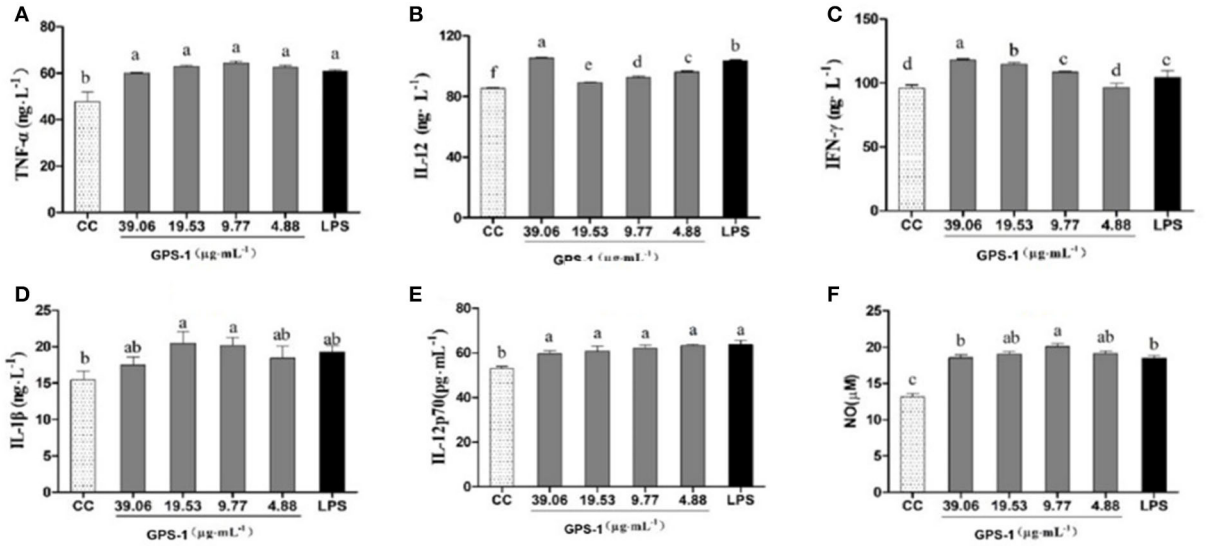


FIGURE 4
Effects of GPS-1 on the secretion of NO **(F)**, and cytokines **(A–E)** by DCs. **(A)** TNF-α; **(B)** IL-12; **(C)** IFN-γ; **(D)** IL-1β; **(E)** IL-12p70; **(F)** NO. $^{a-f}$Values in the same column with different superscript letters differ significantly at $p < 0.05$.

Genscript Biotechnology Co., Ltd. The sodium chloride for injection was purchased from Chenxin Pharmaceutical Co., Ltd.

Animals and conditions

One hundred and fifty unvaccinated, healthy, 1-day-old, male, Lohmann Brown chickens were purchased from Huang Mu Qiao Hatchery in Nanjing Luhe District, China and were housed at the Laboratory Animal Center of Nanjing Agricultural University. Chickens were given a standard diet and reared in standard specific pathogen free (SPF) conditions between 20 and 23°C, with 12–h light/dark cycles. Animal experiments were performed in compliance with the guidelines of the Nanjing Agricultural University Institutional Animal Care and Use Committee (IACUC), detailed in the IACUC-approved protocol (No.:2019BAD22B01).

Animal grouping and vaccination

One hundred and fifty unvaccinated, healthy, 1-day-old, male, Lohmann Brown chickens were randomly divided into five groups: GPS-1 low dose (GPSL) group (1 mg/mL), GPS-1 medium dose (GPSM) group (2 mg/mL), GPS-1 high dose (GPSH) group (4 mg/mL), VC group, and the unvaccinated BC group, with 30 per group. At 13-days-old, all of the Lohmann Brown chickens (except those in the unvaccinated BC group) were immunized with NDV-attenuated vaccine (La Sota; 170902) by intraocular-nasal vaccination, with a booster vaccination at 28 days of

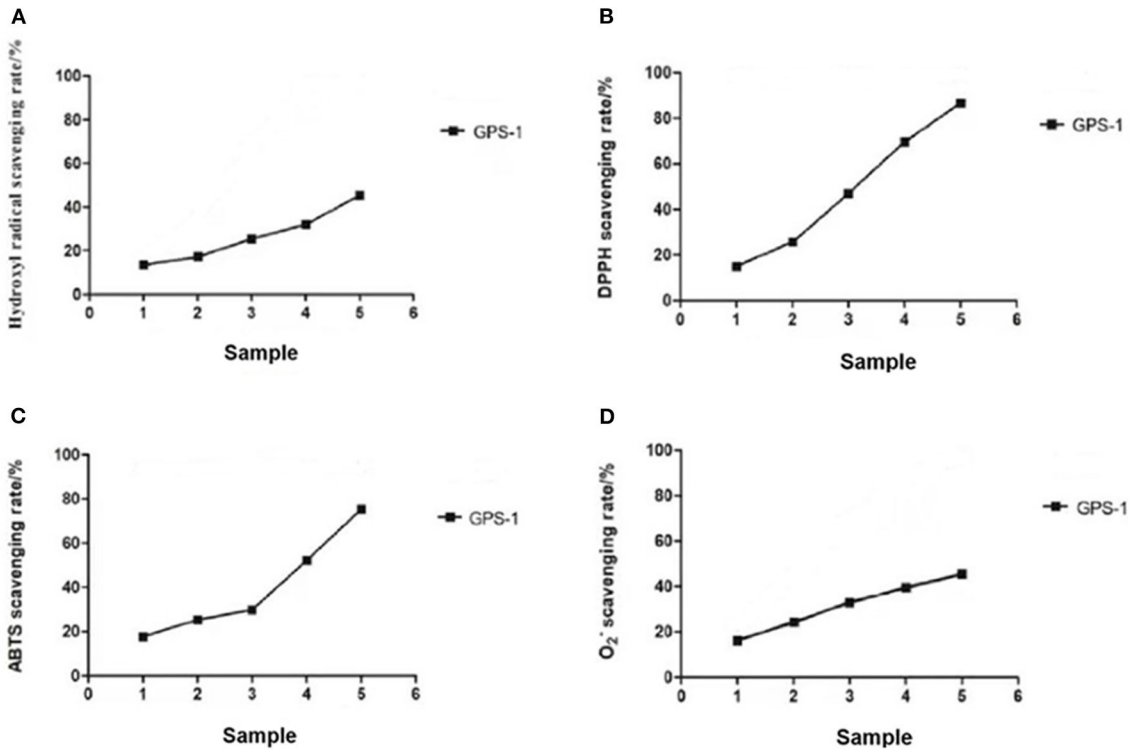


FIGURE 5
The hydroxyl radical-removing rate (%) **(A)** The DPPH radical-removing rate (%) **(B)** The ABTS radical-removing rate (%) **(C)** Superoxide anion radical-scavenging rate (%) **(D)** Sample numbers 1–5 correspond to the concentration of GPS-1 2.44, 4.88, 9.77, 19.53, and 39.06 μg/mL, respectively.

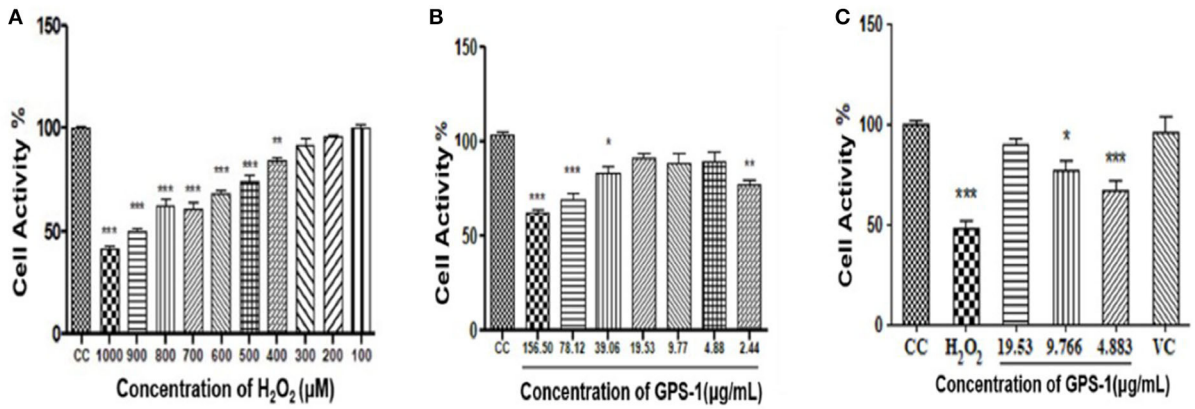


FIGURE 6
Effect of H_2O_2 on the survival rate of RAW 264.7 cells **(A)** Effect of GPS-1 on RAW 264.7 proliferation **(B)** Protective effect of GPS-1 against oxidative damage to RAW 264.7 cells induced by H_2O_2 **(C)** Protective effect of GPS-1 on RAW 264.7 cells exposed to H_2O_2. * stands for $P < 0.05$; ** stands for $P < 0.01$; *** stands for $P < 0.001$.

age. Concurrently with each vaccination, the three GPS-1 medicated groups were injected intramuscularly with 1 mL of GPS-1 at either low, medium, or high dosage for three consecutive days, while the VC group and BC group were treated with the same amount of saline (1 mL), respectively (44).

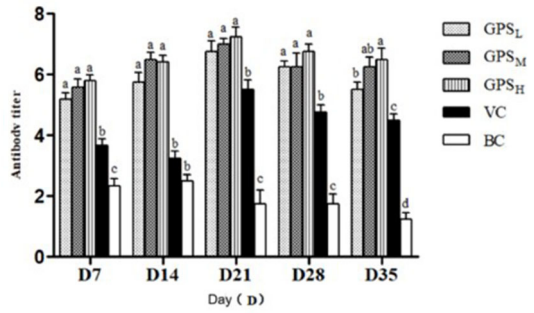


FIGURE 7
Changes in serum antibody titer (Log2). [a–d]Values in the histogram with different letters indicate significant differences.

Serum antibody titer assays

Blood samples were collected from the brachial vein on days 7 (D7), 14 (D14), 21 (D21), 28 (D28), and 35 (D35), after the first vaccination. Serum was tested for antibodies against Newcastle disease (ND) using the HA-HI method, while the IL-2 and IFN-γ levels in serum were determined using ELISA kits.

Morphological changes of immune organs analysis

On the 14th day (D14) after vaccination, the spleen, bursa of Fabricius, and thymus were harvested and fixed in 4% paraformaldehyde solution for 24 h. These immune organs were H&E stained and their morphological changes scrutinized under a light microscope.

Data analysis

Statistical data were analyzed by SPSS 19.0 software and the results were described as the mean ± SE. The least significant difference test was used to determine the significant difference of each mean; $P < 0.05$ was considered statistically significant.

Results

Effects of GPS-1 on immune cell proliferation *in vitro*

As shown in Table 1, there is no significant difference between the A570 values of the 625 μg/mL GPS-1 group and the cell control group. Thus, 625 μg/mL was determined to be the maximum safe concentration. However, the A570 value of the 39.06 μg/mL GPS-1 group was significantly higher than that of the cell control group ($P < 0.05$), so 2.44–39.06 μg/mL were chosen as the concentrations of GPS-1 in cell proliferation tests for the present investigation.

GPS-1 increased lymphocyte proliferation

In this experiment, MTT assays were used to detect the effects of GPS-1 on Lohmann Brown chickens' peripheral blood lymphocytes and splenic lymphocytes *in vitro*. The results in Figure 1 show that GPS-1 at doses of 4.88, 9.77, 19.53, and 39.06 μg/mL can significantly promoted the proliferation of peripheral blood lymphocytes, compared with the phytohemagglutinin (PHA) control group ($P < 0.05$). Within the safe concentration range, GPS-1 could also significantly promote the proliferation of splenic lymphocytes, but there were no significant differences between the doses of GPS-1 and cell control (CC) groups ($P > 0.05$).

GPS-1 induces chBM-DC precursor cell proliferation

The results in Figure 2 show that GPS-1 in the range of 4.88 to 39.06 μg/mL can significantly promote the proliferation of chBM-DCs progenitor cells ($P < 0.05$), but there was no significant difference between the control group and the concentration of 2.44 μg/mL GPS-1 ($P > 0.05$). Thus, the range of concentrations that can effectively enhance the activity of the chBM-DCs progenitor cells is 4.88–39.06 μg/mL.

Effect of GPS-1 on the secretion of cytokines by immune cells

Lymphocyte expression analysis

Data in Figure 3 indicates the levels of IFN-γ and TNF-α were significantly higher in groups treated with GPS-1 doses from 2.44 to 19.53 μg/mL than in the cell control group ($P < 0.05$). Groups given GPS-1 at concentrations of 4.88–39.06 μg/mL also had substantially elevated IL-12, vs. the cell control (CC) group. The most effective GPS-1 dose was 4.88 μg/mL, in which IL-12 was significantly higher than the PHA control group ($P < 0.05$).

chBM-DCs expression analysis

The effects of GPS-1 on the secretion of TNF-α, IL-12, IFN-γ, IL-1 β, and IL-12p70 by chBM-DCs are shown in Figure 4. The secretion of TNF-α (Figure 4A) was significantly higher in groups treated with GPS-1 at concentration of 4.88–39.06 μg/mL than in the CC group ($P < 0.05$), but compare with the LPS group there was not a significant difference ($P > 0.05$). GPS-1 groups in the range of safe concentrations exhibited extremely elevated IL-12 secretion (Figure 4B), a significant improvement over the CC group ($P < 0.05$).

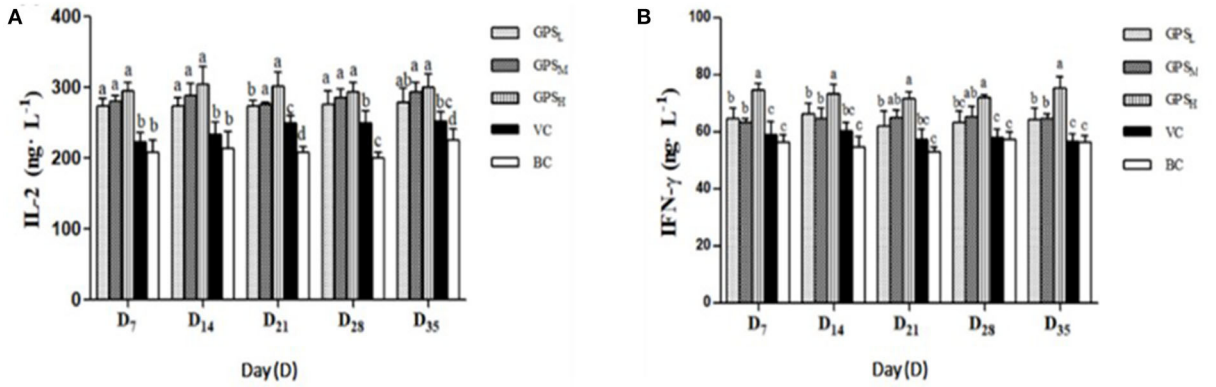


FIGURE 8
The changes of IL-2 **(A)** and **(B)** IFN-γ concentrations in each group (ng·L^{-1}). $^{a-d}$Values in the histogram with different letters indicate significant differences.

Moreover, the IL-12 level in the 39.06 μg/mL GPS-1 group was significantly higher than in the LPS control group. As shown in Figure 4C, the IFN-γ secretion of groups treated with 9.77–39.06 μg/mL GPS-1 was higher than that of the CC group and the difference is significant($P < 0.05$). Additionally, GPS-1 groups at concentrations of 19.53 μg/mL and 39.06 μg/mL have higher IFN-γ secretion than the LPS group and the difference is significant ($P < 0.05$). Compared with the CC group, the secretion of IL-1β (Figure 4D) was higher in GPS-1 groups at the concentrations of 9.77 μg/mL and 19.53 μg/mL ($P < 0.05$). However, the remaining GPS-1 groups and the LPS group showed no significant difference with the CC group ($P > 0.05$). The IL-12p70 secretion (Figure 4E) of all GPS-1 groups and the LPS group was significantly higher than that of the CC group ($P < 0.05$). Moreover, compared with the CC group, GPS-1 at 9.77 μg/mL significantly increased the quantity of NO (Figure 4F) expelled by DCs ($P < 0.05$).

The present results showed that the NO content of endocrine DCs was significantly higher at safe concentrations of GPS-1(4.88, 9.77, 19.53, and 39.06 μg/L) than in the CC control group (Figure 4F). Additionally, the GPS-1 concentration of 9.77 μg/mL produced significantly higher NO than the LPS group. The results indicate that GPS-1 can significantly promote the secretion of NO by dendritic cells and further enhance their immune function.

Antioxidant activity

Hydroxyl radical scavenging activity

In Figure 5A, note the uptick in antioxidant capacity with the corresponding increase in GPS-1 concentration in an apparent dose-dependent relationship. At GPS-1 concentrations between 1 and 5 mg/mL, the scavenging rate rose from 13.47 to 45.29%. Meanwhile, the scavenging rate of ascorbic acid, the positive control (VC), increased from 16.55% at 1 mg/mL to 96.82% at 4.0 mg/mL, where it plateaued. These results show that GPS-1 can scavenge hydroxyl radicals, but is much less effective than ascorbic acid.

DPPH radical scavenging activity

Figure 5B summarizes the scavenging activities of GPS-1. VC (the positive control) exhibited 98.39% antioxidant activity at 1 mg/mL, then plateaued. The antioxidant activity of GPS-1 increased with concentration in a dose-dependent manner. The scavenging rate developed from 15.06% at a GPS-1 concentration of 1 mg/mL to 86.60% at 5 mg/mL, and met its half maximal inhibitory concentration (IC50) at 3.07 mg/mL GPS-1.

ABTS radical scavenging activity

The scavenging effects of GPS-1 and VC on ABTS radicals are examined in Figure 5C. Higher concentrations of GPS-1 improved scavenging activity significantly. The scavenging ratio of GPS-1 rose slowly between 1 and 3 mg/mL, then rose sharply between 3 and 5 mg/mL. The IC50 value of GPS-1 was 3.7 mg/mL. Likewise, VC displayed an excellent ability to scavenge ABTS radicals at 93.53–99.42% at concentrations ranging from 1 to 5 mg/mL.

Superoxide anion radical scavenging ability

The results of the superoxide anion radical scavenging experiment are given in Figure 5D. The antioxidant capacities of GPS-1 and ascorbic acid are both in dose-dependent, linear relationships with their concentrations. The scavenging rate of GPS-1 increased from 16.21% at a GPS-1 concentration of 1

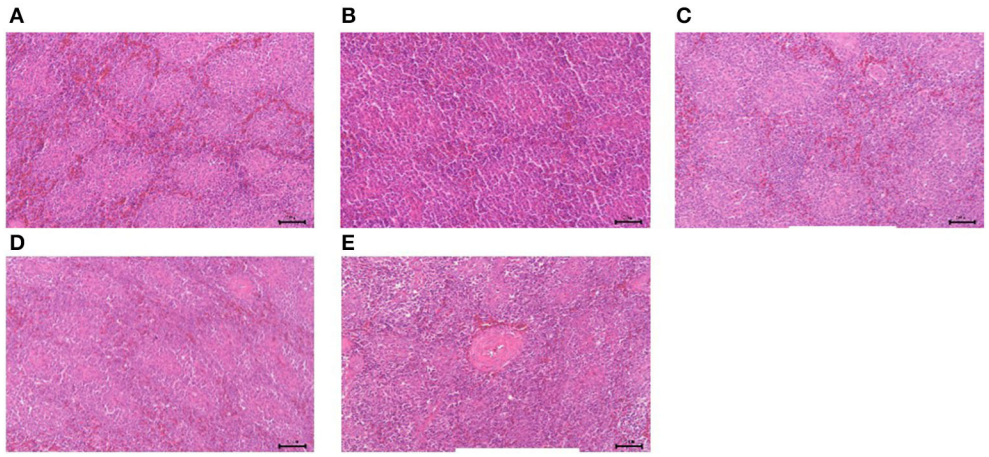


FIGURE 9
Histological changes of the spleen in each group. **(A)** GPS-1L group; **(B)** GPS-1M group; **(C)** GPS-1H group; **(D)** VC group; **(E)** BC group. H&E staining; 200× magnification.

mg/mL to 45.46% at 5 mg/mL, while the scavenging ability of VC grew from 22.87 to 97.58% within the same dosage range.

Effect of GPS-1 on RAW 264.7 cells

As shown in Figure 6A, the survival rate of RAW 264.7 cells declined with the increase of H_2O_2 concentration. When the concentrations of hydrogen peroxide reached 400 μM or above, cell proliferation was profoundly inhibited ($P < 0.001$). The lethality of hydrogen peroxide at 900 μM was 50.35%, which was selected as the treatment dose necessary to induce an oxidative damage model for RAW 264.7 cells.

As seen in Figure 6B, GPS-1 at 2.44 μg/mL and from 39.06 to 156.50 μg/mL significantly inhibited the growth of RAW 264.7 cells compared to the CC group ($P < 0.05$). Therefore, 4.883 μg/mL (GPSL), 9.77 μg/mL (GPSM) and 19.53 μg/mL (GPSH) were selected as the GPS-1 treatment concentrations.

The protective effect of GPS-1 on RAW 264.7 cells exposed to H_2O_2 can be observed in Figure 6C. After hydrogen peroxide treatment, both the high (GPSH,19.53 μg/mL) and middle (GPSM, 9.766 μg/mL) doses of GPS-1 showed significant protective effects on the RAW 264.7 cells exposed to H_2O_2, as compared to the control group ($P < 0.05$). Relative to the CC group, the cell activities of the H_2O_2 and GPSL decreased significantly. GPSHexhibited an excellent 89.84% cell survival rate after hydrogen peroxide damage. The activity of RAW 264.7 cells in the GPSM group (9.77 μg/mL) was only 76.93%, which is lower than that in the cell control (CC) group ($P < 0.05$). However, compared to the hydrogen peroxide group, the cell activity of the GPSM group was significantly increased ($P < 0.01$). The GPS-1 concentration of 4.883 μg/mL also increased cell viability relative to the H_2O_2 group, but not significantly.

Immune enhancement effect of GPS-1 with Newcastle disease vaccine in Lohmann Brown chickens

Changes in serum antibody titer

Variation in serum antibody titers between each group can be seen in Figure 7. On days 7, 21, and 35 after the first ND vaccination, antibody titers from GPS-1 treatment groups were significantly higher than those of VC and BC groups ($P < 0.05$). On days 14 and 28, the antibody titers of the GPSH and GPSM groups were also higher than these control groups ($P < 0.05$). Notably, the difference between the high and medium dose groups (GPSH and GPSM) was not significant ($P > 0.05$). Additionally, even in the lowest dose (GPSL) group the antibody titer was significantly higher than that of both control groups ($P < 0.05$).

The changes of serum IL-2 and IFN-γ concentration

According to Figure 8A, on days 7, 14, and 28 after vaccination, the IL-2 concentrations in the GPS-1 groups were higher than that in the VC and BC groups ($P < 0.05$), with the largest increase seen in the GPSH group. Similarly, on days 7, 14, and 35 the level of IFN-γ in each GPS-1 group was higher than in either the VC or BC groups ($P < 0.05$). Again, GPSH produced the largest response ($P < 0.05$). According to Figure 8B, on days

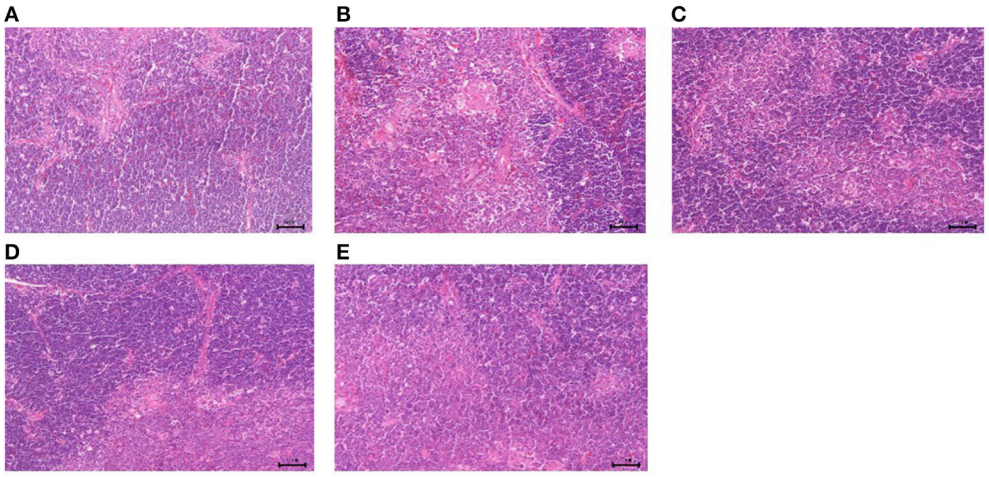


FIGURE 10
Histological changes of the thymus in each group. **(A)** GPS-1L group; **(B)** GPS-1M group; **(C)** GPS-1H group; **(D)** VC group; **(E)** BC group. H&E staining; 200× magnification.

21 and 28, the IFN-γ content of the GPSH and GPSM groups was higher than that of the VC and BC control groups ($P < 0.05$).

Morphological changes of immune organs

The results of GPS-1 treatment on the spleen, thymus, and bursa of Fabricius of are presented in Figures 9–11, respectively. Compared with the VC and BC control groups, there was no observed toxic effect from GPS-1 on the spleen, thymus, or bursa of Fabricius.

Discussion

The growth of chickens is affected by a variety of factors such as stocking density, temperature, pathogenic microorganisms, and immune performance, all of which affect the economic performance of poultry farmers and the prices consumers face in stores (45). Glycyrrhiza polysaccharide (GPS), the most abundant active component of Glycyrrhiza uralensis, has been the subject of a growing body of pharmacological and clinical studies that indicate it can be useful in immune regulation, wound repair, antioxidation, and as an anti-tumor agent (46–48). Due to its extensive biological properties, Glycyrrhiza polysaccharides have attracted increasing attention from the scientific community. Such research will serve as a solid foundation for potential future applications as an immune modulator in poultry. Therefore, we evaluated the effect of Glycyrrhiza polysaccharides on immune cell proliferation *in vitro*, and *in vivo* and antioxidant activity and immune enhancement of chickens given the Newcastle disease (ND) vaccine.

Lymphocyte proliferation is an important indicator of the cellular immune level of the body (49). The *in vitro* test of T and B cell function is short and the conditions are easy to control, so it is suitable for the initial screening of immune-enhancing drugs (50). In this test, the effects of GPS on chickens' peripheral blood lymphocytes and spleen lymphocytes was measured in vitro using MTT assays. The results show that the maximum safe concentration of GPS is 39.06 μg/mL. In the range of 2.44–39.06 μg/mL, GPS can significantly promote the proliferation of T and B lymphocytes. DCs are the most powerful known antigen presenting cells (APCs) (51). DCs can stimulate the activation and proliferation of naive T cells, while macrophages and B cells can only stimulate activated T cells or memory T cells (52). Therefore, DCs are the initiators of specific immune responses. Monocytes, macrophages, and DCs are all derived from myeloid common precursor cells (CMPs). Under the stimulation of GM-CSF and IL-4, CMPs can gradually differentiate into DCs with typical morphological characteristics and functions. The results of this experiment show that GPS in the range of 4.88–39.06 μg/mL can significantly promote the proliferation of chBM-DCs precursor cells, providing an immune enhancement effect. Th1 and Th2 are subsets of helper T cells and are therefore critical for immunomodulation. They can secrete a variety of cytokines and play a major role in the regulation of specific and non-specific immunity. Th1 cells are responsible for cellular immunity, secreting IL-2, TNF- α, IFN- γ, and IFN- α. Th2 cells generally participate in humoral immunity by secreting IL-4, IL-6, and IL-10 (53). IL-12 is a cytokine necessary for activating CD4+ T cells (54). IL-12 and IFN-γ can promote the differentiation of initial Th cells into

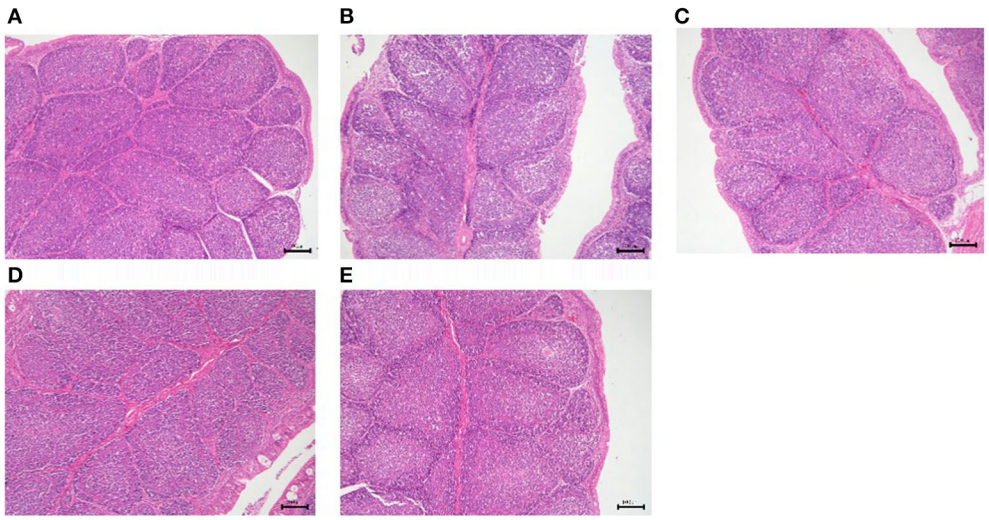


FIGURE 11
Histological changes of the bursa of Fabricius in each group. **(A)** GPS-1L group; **(B)** GPS-1M group; **(C)** GPS-1H group; **(D)** VC group; **(E)** BC group. H&E staining; 200× magnification.

the Th1 subset, and in turn, Th1 can secrete a large amount of IFN-γ, making it vital to the elimination of intracellular pathogens. TNF-α and IL-1 β are essential for the development of inflammation, and are responsible for activating leukocytes and upregulating endothelial cell adhesion molecules (55). IL-12p70 is the most important cytokine for inducing a Th1 response; it triggers NK and T cells to produce IFN-γ and enhances their cytolytic activity, effectively activating anti-tumor effector cells in the tumor environment (56). Our results show that GPS-1enhances immune responses by regulating the Th1-like response induced by lymphocytes and DCs, increasing the cytokines secreted by Th1 (IL-12, TNF- α, and IFN-γ), and promoting the secretion of IL-1 β and IL-12p70 by chBM-DCs.

Nitric oxide (NO) is a highly reactive free radical that acts as a secondary messenger, neurotransmitter, and an effector molecule (57). It has a wide range of physiological functions in the body, such as relaxing vascular smooth muscle, inhibiting platelet aggregation, regulating cerebral blood flow, mediating cytotoxic effects, and regulating the immune system (58). The results of this test show that GPS can significantly enhance the immune function of lymphocytes and DCs, and further promote the secretion of NO by DCs.

Many natural extracts are good sources of antioxidants. Polysaccharides have free radical scavenging abilities that indue them with antioxidant and anti-aging properties (59). With an increased concentration of GPS-1 polysaccharide extract, the clearance rates of OH-, DPPH, ABTS and O^{2-} increased, respectively, reducing the damage to RAW264.7 cells caused by H_2O_2.

Different adjuvants in the ND vaccine can induce different immune responses in Lohmann Brown chickens. Humoral and cellular immune responses play an important role in host defense against ND infection. Antibodies are protective proteins produced by the body in response to an antigen, and antibody titers reflect the state of humoral immunity (60). Antibodies against ND usually appear within 6–10 days after infection and high levels of antibodies are correlated with host protection. Merz et al. reported that humoral and cellular immunity play an important role in host defense against NDV infection (60). This cellular immune response plays a leading role in immune protection against many avian infectious diseases (61). IL-2 and IFN-γ are both secreted by Th1, and possess complementary capabilities. IL-2 plays a key role in T cell differentiation and proliferation (62), while IFN-γ is critical to innate and acquired immunity against viral and intracellular bacterial and protozoan infections (63). Immune organs provide a place for immune cells to differentiate, develop and mature. The increase and decrease of the size of immune organs generally reflects the immune status of organisms. Recent studies have shown that the effects of polysaccharides on the size of the immune organs of chickens are mainly found in the spleen, thymus, and bursa of Fabricius (64). Our results show that GPS-1 can effectively amplify the immune response to Newcastle disease vaccination, cytokine secretion ability, and the development of immune organs in Lohmann Brown chickens.

Conclusion

Based on our findings, we can conclude that GPS-1 can significantly promote the proliferation of immune cells and enhance the secretion of IL-12, IFN-γ, and TNF-α by lymphocytes and the secretion of IL-1 β, IL-12, IL-12P70, IFN-γ, TNF-α, and NO by dendritic cells. Both doses (1, 2, 4mg/ml, 1ml each chickens)of GPS-1 could significantly increase the body weight and serum antibody titer of Lohmann Brown chickens, enhance the secretion of IL-2 and IFN- γ in serum, and promote the development of immune organs. Our experiment also revealed that GPS-1 has a notable ability to scavenge free radicals, among them DPPH, hydrogen peroxide, ABTS, and superoxide anion radicals. Such scavenging abilities may prove useful in providing an immuno-supportive, antioxidant effect. GPS-1 has a clear protective effect against oxidative damage to RAW 264.7 cells. The results of this study indicated that GPS-1 can enhance immune regulation *in vitro* and *in vivo*, and antioxidation *in vitro*. A further study could assess the long-term effects and structure-activity relationships of GPS-1. More investigation into GPS-1 would also help establish a greater degree of accuracy regarding its therapeutic potential.

Data availability statement

The datasets presented in this article are not readily available because no restrictions. Requests to access the datasets should be directed to YiW, wuyi2001cn@163.com.

Ethics statement

The animal study was reviewed and approved by Nanjing Agricultural University No. PZ2020101. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

HZ: writing—original draft preparation and visualization. XC and NL: data curation and visualization. CDa: conceptualization and supervision. TZ: writing—original draft preparation. YC: writing—reviewing and methodology. KD: reviewing and editing. LY: data curation. AN and CDe: writing—reviewing and editing. XW and YuW: data validation. KL and YL: writing—reviewing. YiW: conceptualization, funding acquisition, project administration, and supervision. All authors contributed to the article and approved the submitted version.

Funding

This research was financially supported by the National Natural Science Foundation of China (NSFC, Grant Nos. 31872514 and 32172900), the Open Project Program of Beijing Key Laboratory of Traditional Chinese Veterinary Medicine at Beijing University of Agriculture (No. kf-tcvm202101), Yunnan Provincial Science and Technology Department-Applied Basic Research Joint Special Funds of Yunnan University of Chinese Medicine [2018FF001(-020) and 2019FF002(-012)] and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Acknowledgments

We highly appreciate all other colleagues in the Institute of Traditional Chinese Veterinary Medicine of Nanjing Agricultural University. Additionally, we also appreciate Dr. Wenxia Bai, the chief scientist of Nanjing Superbiotech Co. Ltd., for her excellent technical supports on the histological experiments and data analysis.

Conflict of interest

Author KD was employed by China Tobacco Henan Industrial Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Sun B, Yu S, Zhao D, Guo S, Wang X, Zhao K. Polysaccharides as vaccine adjuvants. *Vaccine.* (2018) 36:5226–34. doi: 10.1016/j.vaccine.2018.07.040

2. Sobhy H, Aboelnaga TR, Behour TS, Razin EA. In vitro trypanocidal activity of essential oils of some plants against Trypanosoma evansi. *Int J Vet Sci.* (2021) 10:191–5. doi: 10.47278/j.ijvs/2021.043

3. Song J, Kim JY, You G, Kang YY, Yang J, Mok H. Formulation of glycyrrhizic acid-based nanocomplexes for enhanced anti-cancer and anti-inflammatory effects of curcumin. *Biotechnol Bioprocess Eng.* (2022) 27:163–70. doi: 10.1007/s12257-021-0198-7

4. Wang C, Chen L, Xu C, Shi J, Chen S, Tan M, et al. A comprehensive review for phytochemical, pharmacological, and biosynthesis studies on Spp. *Am J Chin Med.* (2020) 48:17–45. doi: 10.1142/S0192415X20500020

5. Sun J, Zhang Q, Yang G, Li Y, Fu Y, Zheng Y, et al. The licorice flavonoid isoliquiritigenin attenuates mycobacterium tuberculosis-induced inflammation through notch1/Nf-?b and Mapk signaling pathways. *J Ethnopharmacol.* (2022) 294:115368. doi: 10.1016/j.jep.2022.115368

6. Degla LH, Kuiseu J, Olounlade PA, Attindehou S, Hounzangbe-Adote MS, Edorh PA, et al. Use of medicinal plants as alternative for the control of intestinal parasitosis: assessment and perspectives. *Agrobiol Rec.* (2022) 7:1–9. doi: 10.47278/j.abr/2021.011

7. Matsumoto Y, Matsuura T, Aoyagi H, Matsuda M, Hmwe SS, Date T, et al. Antiviral activity of glycyrrhizin against hepatitis C virus *in vitro*. *PLoS ONE.* (2013) 8:e68992. doi: 10.1371/journal.pone.0068992

8. Sato Y, He J-X, Nagai H, Tani T, Akao T. Isoliquiritigenin, one of the antispasmodic principles of glycyrrhiza ularensis roots, acts in the lower part of intestine. *Biol Pharm Bull.* (2007) 30:145–9. doi: 10.1248/bpb.30.145

9. Yang R, Yuan B-C, Ma Y-S, Zhou S, Liu Y. The anti-inflammatory activity of licorice, a widely used chinese herb. *Pharm Biol.* (2017) 55:5–18. doi: 10.1080/13880209.2016.1225775

10. Yu X, Bao Y, Meng X, Wang S, Li T, Chang X, et al. Multi-pathway integrated adjustment mechanism of licorice flavonoids presenting anti-inflammatory activity. *Oncol Lett.* (2019) 18:4956–63. doi: 10.3892/ol.2019.10793

11. Ao X, Kim IH. Effects of achyranthes bidentata polysaccharides on performance, immunity, antioxidant capacity, and meat quality in pekin ducks. *Poult Sci.* (2020) 99:4884–91. doi: 10.1016/j.psj.2020.06.026

12. Ayeka PA, Bian Y, Mwitari PG, Chu X, Zhang Y, Uzayisenga R, et al. Immunomodulatory and anticancer potential of gan cao (glycyrrhiza uralensis fisch) polysaccharides by Ct-26 colon carcinoma cell growth inhibition and cytokine Il-7 upregulation *in vitro*. *BMC Complement Altern Med.* (2016) 16:206. doi: 10.1186/s12906-016-1171-4

13. Huang G, Mei X, Hu J. The antioxidant activities of natural polysaccharides. *Curr Drug Targets.* (2017) 18:1296–300. doi: 10.2174/1389450118666170123145357

14. Wu Y, Yi L, Li E, Li Y, Lu Y, Wang P, et al. Optimization of glycyrrhiza polysaccharide liposome by response surface methodology and its immune activities. *Int J Biol Macromol.* (2017) 102:68–75. doi: 10.1016/j.ijbiomac.2017.04.006

15. Zhang X, Zhao S, Song X, Jia J, Zhang Z, Zhou H, et al. Inhibition effect of glycyrrhiza polysaccharide (Gcp) on tumor growth through regulation of the gut microbiota composition. *J Pharmacol Sci.* (2018) 137:324–32. doi: 10.1016/j.jphs.2018.03.006

16. Shaukat MB, Abbasi KY, Ahmad MA. Indigenous plants of Pakistan for the treatment of diabetes: a review. *Chin Med.* (2021) 4:44–63. doi: 10.1186/s13020-018-0210-0

17. Ragab EE, Badr H, AbuElkheir A, Enbaawy M. The effectiveness of methanolic extracts of five plants on different salmonella isolates. *J Vet Sci.* (2020) 9:379–84. doi: 10.37422/ijvs/20.041

18. Maria Pia GD, Sara F, Mario F, Lorenza S. Biological effects of licochalcones. *Mini Rev Med Chem.* (2019) 19:647–56. doi: 10.2174/1389557518666180601095420

19. Wang X, Zhang H, Chen L, Shan L, Fan G, Gao X. Liquorice, a unique "guide drug" of traditional Chinese medicine: a review of its role in drug interactions. *J Ethnopharmacol.* (2013) 150:781–90. doi: 10.1016/j.jep.2013.09.055

20. Cai Z, Dai Q, Guo Y, Wei Y, Wu M, Zhang H. Glycyrrhiza polysaccharide-mediated synthesis of silver nanoparticles and their use for the preparation of nanocomposite curdlan antibacterial film. *Int J Biol Macromol.* (2019) 141:422–30. doi: 10.1016/j.ijbiomac.2019.09.018

21. Wang ZY, Nixon DW. Licorice and cancer. *Nutr Cancer.* (2001) 39:1–11. doi: 10.1207/S15327914nc391_1

22. Chen J, Jin X, Chen J, Liu C. Glycyrrhiza polysaccharide induces apoptosis and inhibits proliferation of human hepatocellular carcinoma cells by blocking Pi3k/Akt signal pathway. *Tumour Biol.* (2013) 34:1381–9. doi: 10.1007/s13277-013-0746-7

23. Du J, Cao L, Jia R, Yin G. Hepatoprotective and antioxidant effects of dietary glycyrrhiza polysaccharide against tcdd-induced hepatic injury and Rt-Pcr quantification of Ahr2, Arnt2, Cyp1a Mrna in jian carp (cyprinus carpio var. jian). *J Environ Sci.* (2017) 51:181–90. doi: 10.1016/j.jes.2016.06.026

24. Ayeka PA, Bian Y, Githaiga PM, Zhao Y. The immunomodulatory activities of licorice polysaccharides (glycyrrhiza uralensis fisch) in Ct 26 tumor-bearing mice. *BMC Complement Altern Med.* (2017) 17:536. doi: 10.1186/s12906-017-2030-7

25. Liu J-Y, Feng C-P, Li X, Chang M-C, Meng J-L, Xu L-J. Immunomodulatory and antioxidative activity of cordyceps militaris polysaccharides in mice. *Int J Biol Macromol.* (2016) 86:594–8. doi: 10.1016/j.ijbiomac.2016.02.009

26. Li W-J, Li L, Zhen W-Y, Wang L-F, Pan M, Lv J-Q, et al. Ganoderma atrum polysaccharide ameliorates ros generation and apoptosis in spleen and thymus of immunosuppressed mice. *Food Chem Toxicol.* (2017) 99:199–208. doi: 10.1016/j.fct.2016.11.033

27. Zhang Y, Zeng Y, Cui Y, Liu H, Dong C, Sun Y. Structural characterization, antioxidant and immunomodulatory activities of a neutral polysaccharide from cordyceps militaris cultivated on hull-less barley. *Carbohydr Polym.* (2020) 235:115969. doi: 10.1016/j.carbpol.2020.115969

28. Liang Q, Zhao Q, Hao X, Wang J, Ma C, Xi X, et al. The effect of polysaccharide on immunization analyzed by intestinal flora and proteomics. *Front Nutr.* (2022) 9:841230. doi: 10.3389/fnut.2022.841230

29. Wu Y-T, Huo Y-F, Xu L, Xu Y-Y, Wang X-L, Zhou T. Purification, characterization and antioxidant activity of polysaccharides from porphyra haitanensis. *Int J Biol Macromol.* (2020) 165:2116–25. doi: 10.1016/j.ijbiomac.2020.10.053

30. Qiao Y, Liu C, Guo Y, Zhang W, Guo W, Oleksandr K, et al. Polysaccharides derived from astragalus membranaceus and glycyrrhiza uralensis improve growth performance of broilers by enhancing intestinal health and modulating gut microbiota. *Poult Sci.* (2022) 101:101905. doi: 10.1016/j.psj.2022.101905

31. Chen J, Zhu X-Q, Yang L, Luo Y, Wang M-Y, Liu X-T, et al. Effect of glycyrrhiza uralensis fisch polysaccharide on growth performance and immunologic function in mice in ural city, Xinjiang. *Asian Pac J Trop Med.* (2016) 9:1078–83. doi: 10.1016/j.apjtm.2016.08.004

32. Li C, Zhao P, Shao Q, Chen W, Huang S, Wang X, et al. Effects of dietary glycyrrhiza polysaccharide on growth performance, blood parameters and immunity in weaned piglets. *J Anim Physiol Anim Nutr.* (2022) 1–11. doi: 10.1111/jpn.13692. [Epub ahead of print].

33. Zhang C, Li CX, Shao Q, Chen WB, Ma L, Xu WH, et al. Effects of glycyrrhiza polysaccharide in diet on growth performance, serum antioxidant capacity, and biochemistry of broilers. *Poult Sci.* (2021) 100:100927. doi: 10.1016/j.psj.2020.12.025

34. Wang Y, Zhang X, Ma X, Zhang K, Li S, Wang X, et al. Study on the kinetic model, thermodynamic and physicochemical properties of glycyrrhiza polysaccharide by ultrasonic assisted extraction. *Ultrason Sonochem.* (2019) 51:249–57. doi: 10.1016/j.ultsonch.2018.10.012

35. Wu L, Fan Y, Fan C, Yu Y, Sun L, Jin Y, et al. Licocoumarone isolated from glycyrrhiza uralensis selectively alters lps-induced inflammatory responses in raw 2647 macrophages. *Eur J Pharmacol.* (2017) 801:46–53. doi: 10.1016/j.ejphar.2017.02.049

36. Kamble NM, Jawale CV, Lee JH. Interaction of a live attenuated salmonella gallinarum vaccine candidate with chicken bone marrow-derived dendritic cells. *Avian Pathol.* (2016) 45:235–43. doi: 10.1080/03079457.2016.1144919

37. He X, Li X, Liu B, Xu L, Zhao H, Lu A. Down-regulation of treg cells and up-regulation of Th1/Th2 cytokine ratio were induced by polysaccharide from radix glycyrrhizae in H22 hepatocarcinoma bearing mice. *Molecules.* (2011) 16:8343–52. doi: 10.3390/molecules16108343

38. Kungel PTAN, Correa VG, Corrêa RCG, Peralta RA, Soković M, Calhelha RC, et al. Antioxidant and antimicrobial activities of a purified polysaccharide from yerba mate (ilex paraguariensis). *Int J Biol Macromol.* (2018) 114:1161–7. doi: 10.1016/j.ijbiomac.2018.04.020

39. Bernini R, Barontini M, Cis V, Carastro I, Tofani D, Chiodo RA, et al. Synthesis and evaluation of the antioxidant activity of lipophilic phenethyl trifluoroacetate esters by *in vitro* abts, dpph and in cell-culture dcf assays. *Molecules.* (2018) 23:208. doi: 10.3390/molecules23010208

40. Sun YX, Liu JC, Kennedy J. Purification, composition analysis and antioxidant activity of different polysaccharide conjugates (apps) from the fruiting bodies of auricularia polytricha. *Carbohydr Polym*. (2010) 82:299–304. doi: 10.1016/j.carbpol.2010.04.056

41. Li J-W, Liu Y, Li B-H, Wang Y-Y, Wang H, Zhou C-L. A polysaccharide purified from radix adenophorae promotes cell activation and pro-inflammatory cytokine production in murine raw2647 macrophages. *Chin J Nat Med*. (2016) 14:370–6. doi: 10.3724/SP.J.1009.2016.00370

42. Wu L, Liu Y-J. Development of dendritic-cell lineages. *Immunity*. (2007) 26:741–50. doi: 10.1016/j.immuni.2007.06.006

43. Sanjeewa KKA, Fernando IPS, Kim E-A, Ahn G, Jee Y, Jeon Y-J. Anti-inflammatory activity of a sulfated polysaccharide isolated from an enzymatic digest of brown seaweed in raw 264.7 cells. *Nutr Res Pract*. (2017) 11:3. doi: 10.4162/nrp.2017.11.1.3

44. Zhang P, Ding R, Jiang S, Ji L, Pan M, Liu L, et al. The adjuvanticity of ganoderma lucidum polysaccharide for newcastle disease vaccine. *Int J Biol Macromol*. (2014) 65:431–5. doi: 10.1016/j.ijbiomac.2014.01.067

45. Weimer SL, Robison CI, Tempelman RJ, Jones DR, Karcher DM. Laying hen production and welfare in enriched colony cages at different stocking densities. *Poult Sci*. (2019) 98:3578–86. doi: 10.3382/ps/pez107

46. Lian K-X, Zhu X-Q, Chen J, Liu G, Gu X-L. Selenylation modification: enhancement of the antioxidant activity of a glycyrrhiza uralensis polysaccharide. *Glycoconj J*. (2018) 35:243–53. doi: 10.1007/s10719-018-9817-8

47. Hao B, Wang X, Ma X, Jin Y, Fan W, Laba C, et al. Preparation of complex microcapsules of soluble polysaccharide from glycyrrhiza uralensis and its application in wound repair and scar inhibition. *Int J Biol Macromol*. (2020) 156:906–17. doi: 10.1016/j.ijbiomac.2020.03.121

48. Aipire A, Mahabati M, Cai S, Wei X, Yuan P, Aimaier A, et al. The immunostimulatory activity of polysaccharides from *Glycyrrhiza uralensis*. *PeerJ*. (2020) 8:e8294. doi: 10.7717/peerj.8294

49. Chen X, Sheng Z, Qiu S, Yang H, Jia J, Wang J, et al. Purification, characterization and *in vitro* and *in vivo* immune enhancement of polysaccharides from mulberry leaves. *PLoS ONE*. (2019) 14:e0208611. doi: 10.1371/journal.pone.0208611

50. Fan Y, Ma L, Zhang W, Xu Y, Suolangzhaxi, Zhi X, et al. Microemulsion can improve the immune-enhancing activity of propolis flavonoid on immunosuppression and immune response. *Int J Biol Macromol*. (2014) 63:126–32. doi: 10.1016/j.ijbiomac.2013.09.039

51. Qin T, Ren Z, Yi L, Liu X, Luo Y, Long Y, et al. Immunological modulation effects of an acid epimedium polysaccharide on immune response in chickens. *Int Immunopharmacol*. (2019) 70:56–66. doi: 10.1016/j.intimp.2019.02.009

52. Zamorina SA, Litvinova LS, Yurova KA, Khaziakhmatova OG, Timganova VP, Bochkova MS, et al. The role of human chorionic gonadotropin in regulation of naïve and memory T cells activity *in vitro*. *Int Immunopharmacol*. (2018) 54:33–8. doi: 10.1016/j.intimp.2017.10.029

53. Oreja-Guevara C, Ramos-Cejudo J, Aroeira LS, Chamorro B, Diez-Tejedor E. Th1/Th2 cytokine profile in relapsing-remitting multiple sclerosis patients treated with glatiramer acetate or natalizumab. *BMC Neurol*. (2012) 12:95. doi: 10.1186/1471-2377-12-95

54. Meng J, Hu X, Shan F, Hua H, Lu C, Wang E, et al. Analysis of maturation of murine dendritic cells (Dcs) induced by purified ganoderma lucidum polysaccharides (Glps). *Int J Biol Macromol*. (2011) 49:693–9. doi: 10.1016/j.ijbiomac.2011.06.029

55. Kim HS, Shin BR, Lee HK, Park YS, Liu Q, Kim SY, et al. Dendritic cell activation by polysaccharide isolated from angelica dahurica. *Food Chem Toxicol*. (2013) 55:241–7. doi: 10.1016/j.fct.2012.12.007

56. Huang D, Nie S, Jiang L, Xie M. A novel polysaccharide from the seeds of plantago asiatica L. induces dendritic cells maturation through toll-like receptor 4. *Int Immunopharmacol*. (2014) 18:236–43. doi: 10.1016/j.intimp.2013.11.024

57. Ju J, Hou R, Zhang P. D-allose alleviates ischemia/reperfusion (I/R) injury in skin flap via Mkp-1. *Mol Med*. (2020) 26:21. doi: 10.1186/s10020-020-0138-6

58. Navasardyan I, Bonavida B. Regulation of T cells in cancer by nitric oxide. *Cells*. (2021) 10:2655. doi: 10.3390/cells10102655

59. Yu Y, Shen M, Song Q, Xie J. Biological activities and pharmaceutical applications of polysaccharide from natural resources: a review. *Carbohydr Polym*. (2018) 183:91–101. doi: 10.1016/j.carbpol.2017.12.009

60. Merz DC, Scheid A, Choppin PW. Immunological studies of the functions of paramyxovirus glycoproteins. *Virology*. (1981) 109:94–105. doi: 10.1016/0042-6822(81)90474-8

61. Lillehoj HS, Trout JM. Coccidia: a review of recent advances on immunity and vaccine development. *Avian Pathol*. (1993) 22:3-31. doi: 10.1080/03079459308418897

62. Assen FP, Sixt M. The dynamic cytokine niche. *Immunity*. (2017) 46:519–20. doi: 10.1016/j.immuni.2017.04.006

63. Qiu Y, Yang H. Effects of intraepithelial lymphocyte-derived cytokines on intestinal mucosal barrier function. *J Interferon Cytokine Res*. (2013) 33:551–62. doi: 10.1089/jir.2012.0162

64. Ren Z, Yu R, Meng Z, Sun M, Huang Y, Xu T, et al. Spiky titanium dioxide nanoparticles-loaded plantaginis semen polysaccharide as an adjuvant to enhance immune responses. *Int J Biol Macromol*. (2021) 191:1096–104. doi: 10.1016/j.ijbiomac.2021.09.184

10.3389/fvets.2022.959449

Nomenclature

GPS-1, Glycyrrhiza polysaccharide extract 1; ND, Newcastle disease; NDV, Newcastle disease vaccine; PHA-P, Phytin; mGM-CSF, Chicken recombinant proteins GM-CSF; il-4, Interleukin 4; chBM-DCs, immature chicken bone marrow dendritic cells.

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 02 September 2022
DOI 10.3389/fvets.2022.971058

OPEN ACCESS

EDITED BY
Yi Wu,
Nanjing Agricultural University, China

REVIEWED BY
Guangliang Shi,
Northeast Agricultural University, China
Ruonan Bo,
Yangzhou University, China
Wuren Ma,
Northwest A&F University, China

*CORRESPONDENCE
Dayou Shi
shidayou@scau.edu.cn

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 16 June 2022
ACCEPTED 12 August 2022
PUBLISHED 02 September 2022

CITATION
Li S, Lin R, Chen J, Hussain R, Zhang S, Su Y, Chan Y, Ghaffar A and Shi D (2022) Integrated gut microbiota and metabolomic analysis reveals immunomodulatory effects of Echinacea extract and Astragalus polysaccharides. *Front. Vet. Sci.* 9:971058. doi: 10.3389/fvets.2022.971058

Integrated gut microbiota and metabolomic analysis reveals immunomodulatory effects of Echinacea extract and Astragalus polysaccharides

Shaochuan Li[1†], Renzhao Lin[1†], Jiaxin Chen[1], Riaz Hussain[2], Shiwei Zhang[1], Yalin Su[1], Yanzi Chan[1], Abdul Ghaffar[2] and Dayou Shi[1*]

[1]College of Veterinary Medicine, South China Agricultural University, Guangzhou, China, [2]The Islamia University of Bahawalpur, Bahawalpur, Pakistan

Immunosuppression in different animals increases the susceptibility of various infections caused by pathogenic microorganisms leading to increase risks posed by antibiotics in different animal farming sectors. Therefore, investigation of the interactions between natural medicines and the intestinal environmental ecosystem is of vital importance and crucial. This study for the first time investigated the effects of Echinacea Extract (EE) and Astragalus polysaccharide (APS) on the gut using 16S rRNA and metabolomic analysis approaches in immunosuppressed broiler chickens. There were four groups divided into control (C), immunosuppression (IS), EE, and APS groups. Sequencing of gut microbes showed that immunosuppression decreased the relative abundance of *Anaerofustis*, *Anaeroplasma*, *Anaerotroncus*, and *Lachnospira* in the gut while increasing that of *c_115* and *Holdemania*. However, EE and APS diminished the effects on the immunosuppression on the microbiota. The results revealed up-regulation of the relative abundance of *Enterococcus* in broiler chickens. In addition, EE reduced the relative abundance of *Ruminococcus* and *Blautia*. The results on metabolomic analysis revealed that immunosuppression mainly affects cyanuric acid metabolism, starch and sucrose metabolism while interconversion of pentose and glucuronide. EE and APS, on the other hand mainly impact butyrate metabolism, alanine, aspartate and glutamate metabolism while the interconversion of pentose and glucuronide, and D-glutamine and D-glutamate metabolism. Results regarding correlation analysis revealed significantly metabolic pathways including TCA cycle, butyrate metabolism, glyoxylate and dicarboxylate metabolism, propionate metabolism, alanine, aspartate and glutamate metabolism associated with *Ruminococcus* and *Blautia*. Both EE and APS can antagonize the effects of immunosuppression by modulating the disrupted gut microbiota. Nevertheless, EE might have a

bidirectional regulatory functions on the intestinal health and further studies are needed to know the exact and relevant mechanisms of action regarding the effects of EE and APS.

KEYWORDS

gut microbiota, metabolomic analysis, immunosuppression, Echinacea extract, Astragalus polysaccharides

Introduction

Immunosuppression is a condition in which the immune response of the body is suppressed due to various reasons resulting in low immunity and increased vulnerability to various diseases. In poultry farming, immunosuppressive diseases in broiler birds are caused by different disorders in the immune responses from the body affecting the abnormal daily feed intake, feed conversion ratio, body weight growth, poor egg production and mortality leading to irreparable economic losses (1–3). In recent years, researchers have carried out a lot of research on the mechanisms and pharmacological control of immunosuppressive diseases in chickens. Since the blind use of antibiotics in poultry and livestock farming has led to development of drug resistance in animals and occurring of different drug residues in food animals. Recently different studies have been planned to focus on the safe and more stable herbs or immunomodulators derived from natural plants (4).

Echinacea was officially used to assist in the treatment of colds, infections, poisonous insects and snake bites in the United States as early as 1887 (5). It has been recorded that Echinacea can proliferate the NK cells of the organism mainly by inhibiting the activity of 5-lipoxygenase and cyclooxygenase (6). It also increases the phagocytic index of granulocytes and enhances the immunity responses of the body *via* production of cytokines such as TNF-α, IFN-β-2, IL-1, and IL-6 by stimulating the macrophages and splenocytes (7, 8).

Astragalus polysaccharides (APS) is an ideal immune promoter that regulates the immune response of different organisms mainly by affecting humoral and cellular immunity. In viral infections APS effectively increases the expression of cytokines such as IL-2, IL-4, IL-6, IL-10, and IL-12 (9). Moreover, it also enhances the phagocytic index of macrophages, stimulates T-cell proliferation and antigen presentation, upregulates levels of immune-related factors, and their ability to synthesize NO by promoting the growth of cells and increasing resistance to viruses (10). APS also has some immunomodulatory capacity. During immune stress, APS reduces transcription of *TLR4* and *NF-κB* genes and inhibits the expression of pro-inflammatory cytokines (11). Furthermore, it affects the morphological development of the jejunum and promotes the synthesis and secretion of specific antibodies following immunization (12). It effectively enhances the efficacy of the vaccine when used as an adjuvant for vaccine during immunization.

The intestinal flora is involved in the digestion and absorption of food, the immune response, growth and development processes, and physiological and structural changes in poultry (13). Research has confirmed that the gut flora is closely linked to host immunity (14) and substance metabolism (15). The causes of lower immunity are different including abnormalities in autoimmunity due to viral or parasitic infections or long-term use or abuse of antibiotics, disruption of the dynamic micro-ecological balance between host and flora, and changes in the number, type, proportion and function of intestinal flora (16). It is recorded that changes in the normal gut flora led to decrease in the productive performance of the organism and even a decrease in immunity, resulting in increased morbidity and mortality, which may cause huge economic losses for the farming industry (17).

The gut microbiota is involved in host immunity in a variety of ways. Most notably through the interactions between immune effector cells and metabolites produced by the gut microbiota during metabolism, which can induce the maturation of immune cells and can activate memory cells. The intestinal epithelium acts as the first barrier for the variety of infectious agents (18) and can possess a wealth of ways of interacting with bacterial metabolites. Metabolites of the intestinal flora are involved in the immune process, dominated by short-chain fatty acids (SCFA), secondary bile acids and tryptophan metabolites (19, 20). Among them, SCFA is a key metabolite in maintaining intestinal homeostasis. It serves as an important source of energy for intestinal epithelial cells and can influence the immune function of the intestinal mucosa by regulating the pH of the intestinal environment (21). The metabolic activity of the microbiota on the intestinal contents has been reported to extensively induce the activation of immune effector cells, thereby enhancing the immune response (22).

Gas chromatograph mass spectrometer (GC-MS) untargeted metabolomics and 16S rDNA intestinal flora sequencing analysis have been used to detect the changes in metabolites and microbiota in the cecum. Therefore, this experimental study was designed and executed to investigate the integrated analysis of the possible effects of Echinacea extract (EE) and APS on the

gut microbiota and metabolites of immunosuppressed chickens, and the mechanisms and pathways by which they exert their immunomodulatory effects.

Materials and methods

Experimental animals and diets

All the experiments were approved by the Animal Ethics Committee of South China Agricultural University (License Number: 2017A087) and were conducted following the ethical code of conduct for animal care and use. A total of one hundred fast-growing broilers having 4 days of age (male and female) were (purchased from Guangdong Zhiwei Agricultural Technology Co., Ltd.) and were randomly divided into four groups. Both groups C and IS were given ordinary drinking water; group EE: 4 g of EE (purchased from Guangzhou Huanong University Experimental Veterinary Drug Co., Ltd.) per liter of water; group APS: 1.2 g of APS (purchased from Beijing Shengtaier Biotechnology Co., Ltd.) per liter of water. The chickens in all four groups were fed the normal basal diet and fresh water *ad libitum* during the trial.

Establishment of the immunosuppression model

The birds kept in group C were injected 0.5 mL of normal saline in the pectoral muscle and the birds of other groups (IS, EE, APS) were injected with cyclophosphamide in the pectoral muscle at a dose of 100 mg/kg. Once daily for 3 days.

16S-rRNA analysis of gut microbiota

Cecal contents on days 7 and 14 were collected aseptically from the birds and then aliquot at 0.5 g/tube into 2 mL centrifuge tubes. After collection all the samples were immediately frozen in liquid nitrogen and transferred to laboratory and stored at −80°C. Total microbiome DNA was extracted from the gut contents and the target fragments were purified by PCR. Their amplification products were obtained and quantified by fluorescence. On-board libraries were constructed in the TruSeq Nano DNA LT Library Prep Kit, sequenced and the raw data were obtained for further process.

For continued expansion of the sequencing volume, sample size, was first predicted and measured by plotting sparsity and species accumulation curves (23). The top 10 species with the greatest abundance in each group were selected from the taxonomic levels of the phylum and genus based on the results of species annotation. The cumulative bar chart of species relative abundance was generated to visualize the species with greater relative abundance in different taxonomic ranks and their proportions. Alpha diversity reflects the richness of a sample community through metric indices such as Chao1, Shannon, and Simpson. The analysis of beta diversity was performed using multidimensional scalar analysis NMDS. Its results and purposes are to test for similarity and similarity in community structure from different samples (24, 25). RStudio 0.99.447 software (Developed by JJ Allaire Company) was used to count the distribution of sequence lengths contained in the samples, QIIME software (Quantitative Insights Into Microbial Ecology, v1.8.0, http://qiime.org/) (26) was used to classify OTUs, and rare OTUs were removed, and then the identification results were represented by histograms to determine the differences.

GC-MS metabolomics analysis

A total of 40 mg of intestinal contents was added to 500 μL of pre-cooled water containing 10 μg/mL of demethyl leucine. Samples were sonicated in an ice water bath for 10 min and placed overnight at −20°C. Then the samples were centrifuged at 14,000 g for 15 min at 4°C. Supernatant was removed and extraction of the residue was done. The two supernatants were combined and 100 μL of the supernatant from the extract was obtained and 50 μg/mL norleucine added. Ten microliter of valine was evaporated and 30 μL of methoxypyridine hydrochloride was added to the dry matter and then incubated at 37°C for 90 min. After that 30 μL of BSTFA containing 1% TMCS was added, vortexed for 30 s, and then derivatized. Detection and analysis were performed using a gas chromatograph-mass spectrometer. The raw GC-MS data were processed using excel with reference to literature methods (27). The raw data from GC-MS was automatically deconvoluted using AMDIS software and matched against a self-built standard database (including retention times and mass spectra), the Golm metabolome database, and the Agilent Fiehn GC/MS metabolomics RTL library.

The data files were imported into SIMCA software (version 14.1) for multidimensional statistical analysis such as PCA and PLS-DA. The data were first formatted with the default mean-centered and UV (unit variance) before analysis. Then the optimal principal component scores were automatically calculated and the optimal model was built. To avoid model overfitting, the optimal number of principal components is calculated using the default 7-round cross-validation (7-round cross-validation) of SIMCA software. The model quality evaluation parameters are R2X or R2Y and Q2 values, which are used to evaluate the robustness of the pattern recognition model, where R2X (PCA) or R2Y (PLS-DA) indicates the proportion of data variance that can be explained by the current model, i.e., the explanation rate, which indicates the goodness of fit of the model. Q2 indicates the proportion of data variance that can be predicted by the current model, i.e., the prediction rate, which indicates the predictive power of the current model.

The combination of a VIP value (Variable importance in the projection) >1 and a *p*-value < 0.05 for unidimensional statistical analysis was used to screen for differential metabolites. Fold change (Log2FC) was calculated as the logarithm (with a base of 2) of the ratio of the mean values of the data from group 1 and group 2, with a positive value indicating that the substance was at a higher level in group 1 than in group 2 and a negative value indicating the opposite.

Analysis of the link between flora and metabolic processes

After sequencing and analysis of the intestinal flora, flora with significant differences were selected. The metabolites of these flora were collected through literature review and database searches. Using the online mapping software Bioladder, the data for the differential metabolites were transformed into volcano plots for display, and metabolites with significant differences were identified in the plots using $P < 0.05$ and Log2FC absolute values ≥1 as screening criteria. Pathway analysis of the differential metabolites was performed using the online software MetaboAnalyst (version 4.0 http://www.metaboanalyst.ca/) according to the metabolites with significant differences. The results of both were correlated to analyze the relationship between the gut microbiota and metabolic activity in the gut.

Results

Diversity analysis

The results on diversity analysis showed that the number of OTUs in the broilers gut microbiota tested in the samples were very rich. The OTU division and classification status also differed between the groups. At day 7 of the trial, no significant differences were found in the α-diversity indices of Ace, Chao1,

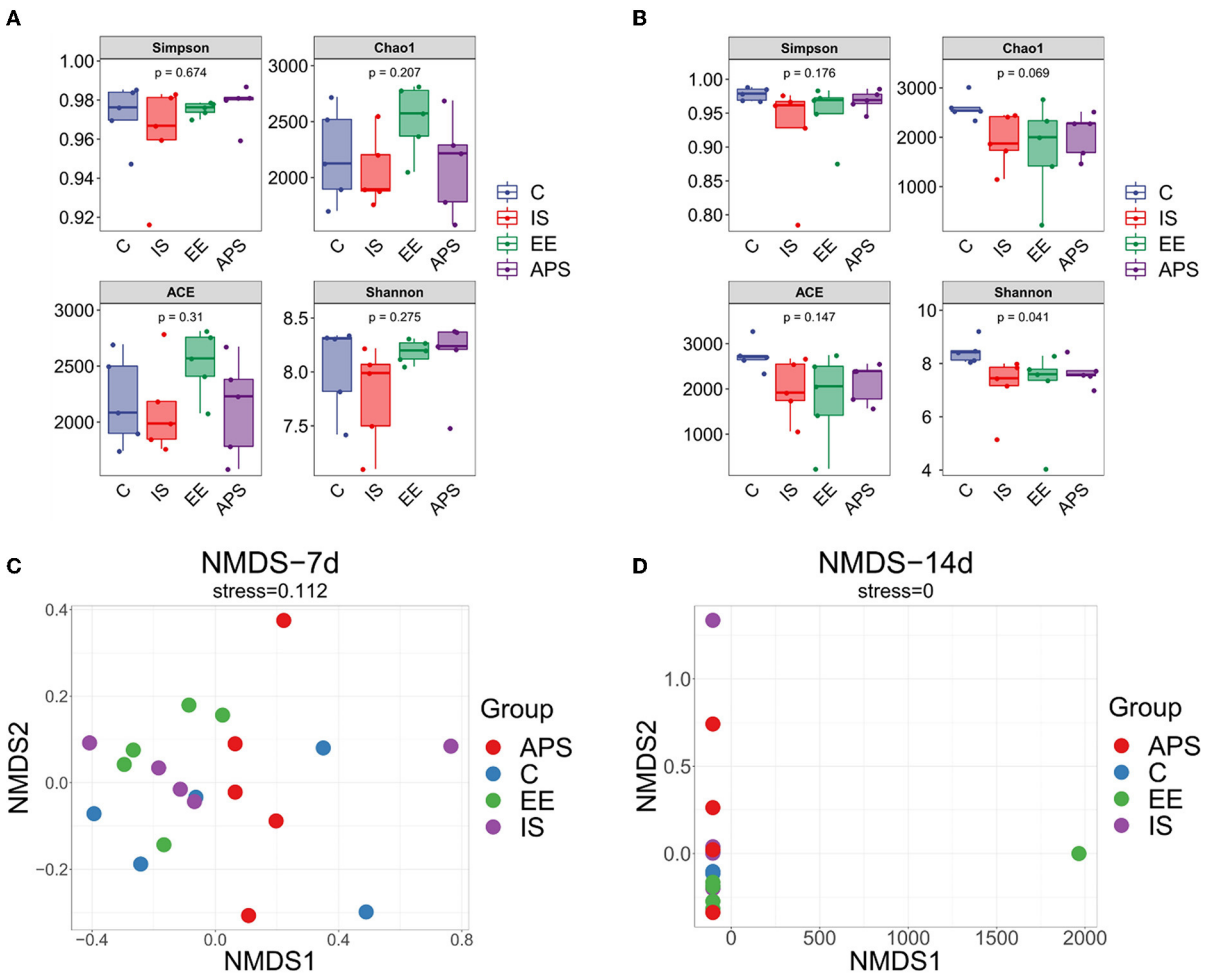


FIGURE 1
Diversity analysis result. Alpha diversity index difference box plot **(A)** 7d; **(B)** 14d. NMDS analysis plots **(C)** 7d; **(D)** 14d ($N = 5$).

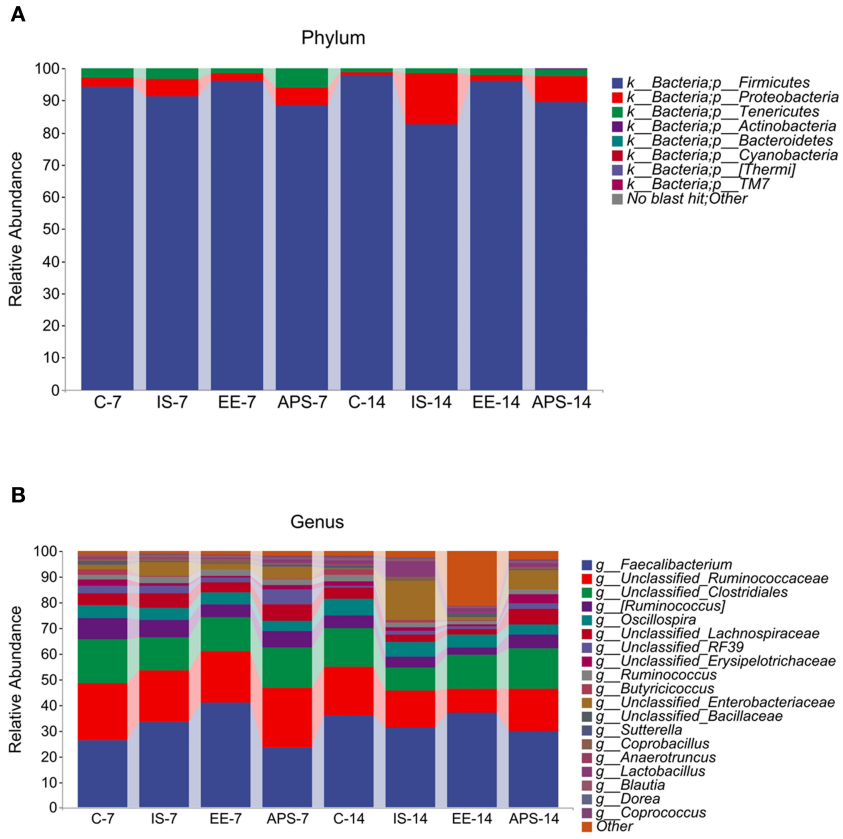


FIGURE 2
Map of taxonomic composition and abundance distribution of communities at the phylum and genus level. **(A)** Phylum; **(B)** Genus. The horizontal coordinates are arranged according to groups at different times with each bar representing a group and colors distinguishing each taxonomic unit. The vertical coordinates represent the relative abundance of each taxonomic unit. The longer the bar, the higher the relative abundance of that taxonomic unit in the corresponding sample ($N = 5$).

Shannon, and Simpson (Figure 1A, $P > 0.05$). On day 14 of trial, except for the Shannon index ($P < 0.05$), there was no significant difference in other indices (Figure 1B, $P > 0.05$). Results showed that under the influence of immunosuppression, the species community diversity of the gut microbiota was affected. The samples were significantly dispersed (Figure 1C) but the groups gradually approached over time (Figure 1D) after the cessation of immunosuppression. The results showed that the impact of immunosuppression on the gut microbiota of chickens could be gradually alleviated over time, and the species community diversity in each group showed a recovery trend, among which the species abundance diversity recovered more significantly.

Statistics on the number of microbial taxa at each taxonomic level

The taxonomic test results showed that six major taxa were detected at the phylum level, including *Firmicutes*, *Proteobacteria*, *Tenericutes*, *Bacteroidetes*, *Actinobacteria* and *Cyanobacteria* (Figure 2A), while at the genus level for *Faecalibacterium*, *Oscillospira*, *Ruminococcus*, *Lactobacillus*, *Mollicutes_RF39*, *Enterococcus*, *Anaerotruncus,* and *Sutterella* (Figure 2B). Over the course of the experiment, the relative abundance of Firmicutes was similar in group C (94.37% at 7 days and 97.83% at 14 days) and group EE (95.98% at 7 days and 96.22% at 14 days), which had a higher percentage of Firmicutes than the IS and APS groups. The IS group reduced the Firmicutes. The IS group decreased the relative abundance of Firmicutes from 91.29 to 82.65% in the experiment, while all other groups showed an increasing trend. In contrast, the relative abundance of Proteobacteria was higher in the IS group, increasing from 5.43 to 15.75%, and similarly, the APS group showed an increasing trend (from 5.51 to 8.19%). The other two groups showed a decreasing trend, accounting for only 1.08% (C) and 1.66% (EE) at 14 days (Figure 2A). At the genus level, the relative abundance of Enterobacteriaceae changed significantly in the IS group, increasing from 5.37 to 15.48%, which was significantly higher than the content share of other groups. At the genus level, the relative abundance of Enterobacteriaceae

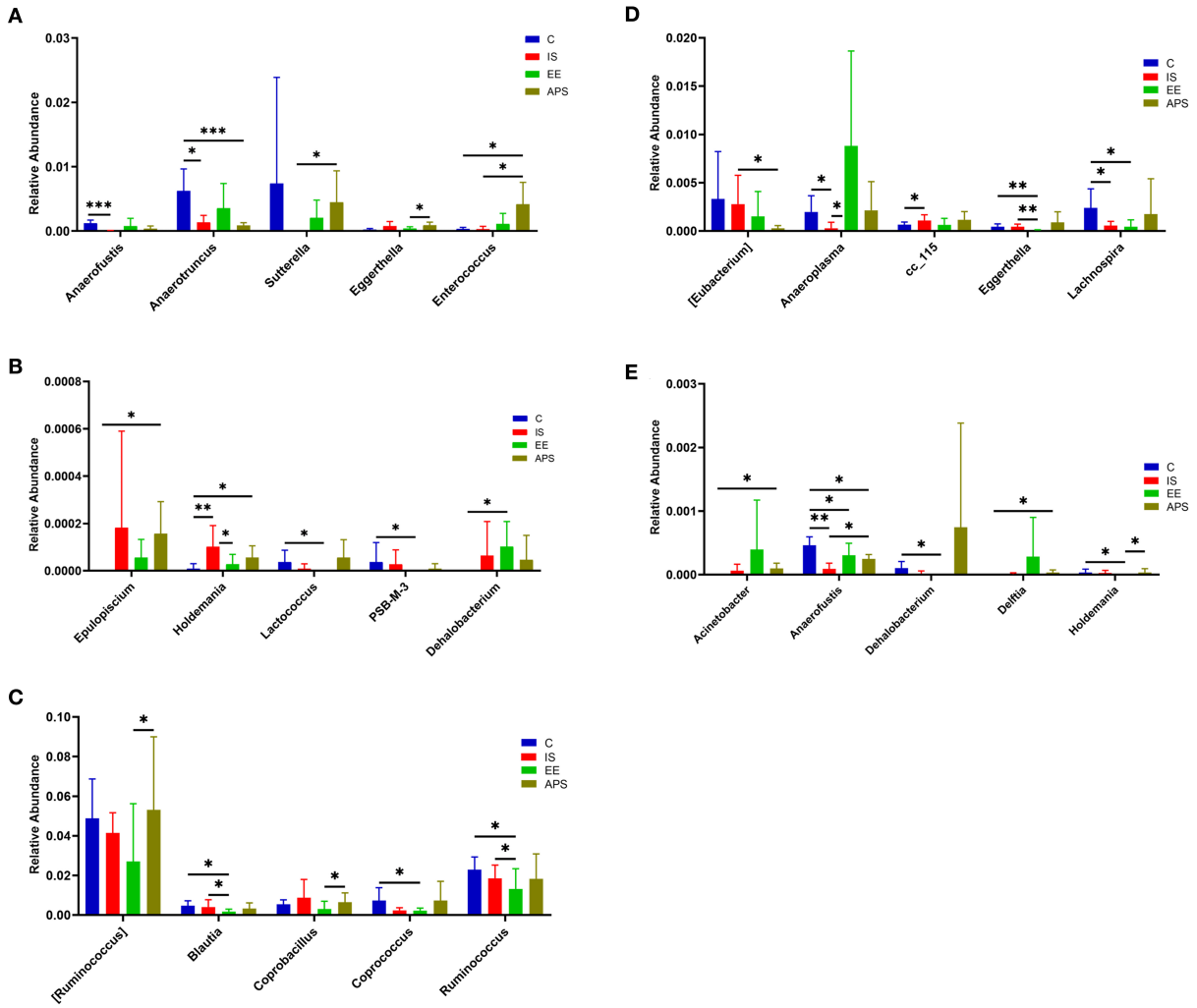


FIGURE 3
(A,B) Represent the abundance distribution of the top 10 taxa with the most significant differences between groups at 7 days; **(C–E)** represent the top 15 taxa with the most significant differences at 14 days Abundance distribution of the taxa (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) ($N = 5$).

varied significantly in the IS group, increasing from 5.37 to 15.48%, which was remarkably higher than the content share of the other groups, of which the APS group showed an increasing trend (4.91–7.44%). On the contrary, the other two groups showed a decreasing trend (Figure 2B).

Analysis of differences in taxonomic composition between groups

After 7 days, compared with group C, the abundance of *Anaerotruncus* ($P < 0.05$) and *Anaerofustis* ($P < 0.001$) in IS group were significantly decreased, and *Holdemania* was significantly increased ($P < 0.01$). Compared with IS group, *Holdemania* in EE group was significantly decreased ($P < 0.05$); compared with IS group, *Enterococcus* and *Sutterella* were significantly increased in APS group ($P < 0.05$; Figures 3A,B; Table 1). At day 14 of the trial, compared with the C group, the abundance of *Anaerofustis* ($P < 0.01$), *Anaeroplasma* ($P < 0.05$) and *Lachnospira* ($P < 0.05$) in the IS group was significantly reduced, and *cc_115* was significantly increased ($P < 0.05$); Compared with the IS group, the *Anaeroplasma* in the EE group was significantly increased ($P < 0.05$). Compared with the IS group, the *Anaerofustis* ($P < 0.05$) in the EE group was significantly increased, and *[Eubactreium]* ($P < 0.05$) was significantly less in the IS group (Figures 3C–E; Table 1).

Principal components analysis

Principal component analysis was performed on the intestinal contents of each group at day 7 and the cumulative interpretation rate of the model was $R^2X = 0.585$. Current PCA

TABLE 1 Summary of genera with significant variation during the trial.

Genus	IS vs. C	EE vs. IS	APS vs. IS
Anaerofustis	↓***	↑	↑*
Anaeroplasma	↓*	↑*	↑
Anaerotruncus	↓*	↑	↓
Blautia	↓	↓*	↓
cc_115	↑*	↓	-
Eggerthella	↑	↓**	↑
Enterococcus	-	↑	↑*
Holdemania	↑**	↓*	↓
Lachnospira	↓*	-	↑
Ruminococcus	↓	↓*	↑

The table is not divided by time periods and only based on the results of the trial process. "↑" indicates that the abundance of the genus increases, "↓" indicates a decrease, and "-" indicates that it cannot be determined.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

models can be reliably used to describe metabolic differences between samples. There was separation between group C and the other three groups.

Principal component analysis was performed on the intestinal contents of each group at 14 days, and the cumulative interpretation rate of the model was $R^2X = 0.604$. Current PCA models can be reliably used to describe metabolic differences between samples. There was a significant separation between group C and the other three groups.

Partial least squares discrimination analysis

A PLS-DA model with 2 effective principal components was developed for the content at day 7 in treatment group, $R^2X = 0.338$, $R^2Y = 0.445$, and $Q^2 = 0.174$, and the PLS-DA score plot showed a significant separation between at day 7 in group C and the other 3 sample groups. A PLS-DA model with 2 effective principal components was developed at day 14 of treatment group of contents, $R^2X = 0.288$, $R^2Y = 0.527$, and $Q^2 = 0.268$, and the PLS-DA score plot showed a trend toward separation between the four sample groups.

Differential analysis of metabolites and metabolic pathways

At day 7, a total of 28 different substances were screened (Table 2). At 14 days, a total of 22 different substances were screened (Table 3). Compared with group C, the different metabolic pathways of intestinal contents in group IS at day 7 were Cyanoamino acid metabolism, Cysteine and methionine metabolism, Starch and sucrose metabolism, and Glycerolipid metabolism. At 14 days, it is Starch and sucrose metabolism, Pentose and glucuronate interconversions, Galactose metabolism, D-Glutamine and D-glutamate metabolism, Ascorbate and aldarate metabolism, Pantothenate and CoA biosynthesis, Butanoate (Figure 4A). At 7 days, compared with IS, the different metabolic pathways of intestinal contents in EE were Citrate cycle (TCA cycle), Butanoate metabolism, Glyoxylate and dicarboxylate metabolism, Propanoate metabolism, Alanine, aspartate and glutamate metabolism. At 14 days, it is Pentose and glucuronate interconversions, D-Glutamine and D-glutamate metabolism, Cyanoamino acid metabolism (Figure 4B). Compared with IS, the differential metabolic pathways of intestinal contents in APS at 7 days are Valine, leucine and isoleucine biosynthesis, Valine, leucine and isoleucine degradation, D-Glutamine and D-glutamate metabolism, Aminoacyl-tRNA biosynthesis, Butanoate metabolism, Alanine, aspartate and glutamate metabolism. At 14 days, it is Starch and sucrose metabolism, Pentose and glucuronate interconversions, Galactose metabolism, D-Glutamine and D-glutamate metabolism (Figure 4C).

Discussion

There was found that the species of bacteria in the caecum of laying hens varies with age and does not become stable until 12–16 weeks (28). The changing trend of intestinal flora may be similar during the growth and development of broilers and laying hens. Throughout the experiment, broiler birds grew from day 1–4 weeks of age possibly with changes in gut microbiota species occurring in this period. The effects of immunosuppression on the gut microbiota may coincide with changes in microbiota species. A tendency for the microbiota of each group to approach each other over time was observed in the NMDS analysis, and changes in gut microbiota species with age may be a factor in our study. In addition, the diversity of microbiota abundance was restored with the gradual recovery of immune functions with the cessation of immunosuppression. Nevertheless, in this process, there were no significant differences in the alpha diversity index except for the Shannon index. This trend of variation revealed that the process of immunosuppression had no significant effects on the number of species in the microbiota, but produced a certain degree of variation in the abundance of species in the microbiota.

Ten different genera were identified by differential analysis of the gut microbiota. *Anaerofustis*, *Anaeroplasma*, *Anaerotruncus*, *Blautia*, *cc_115*, *Eggerthella*, *Enterococcus*, *Holdemania*, *Lachnospira*, and *Ruminococcus*, respectively. Among them, *Anaerofustis*, *Anaeroplasma*, *Anaerotruncus*, *Lachnospira*, and *Ruminococcus* belong to *Firmicutes*. They often produce short-chain fatty acids (SCFAs) such as butyric

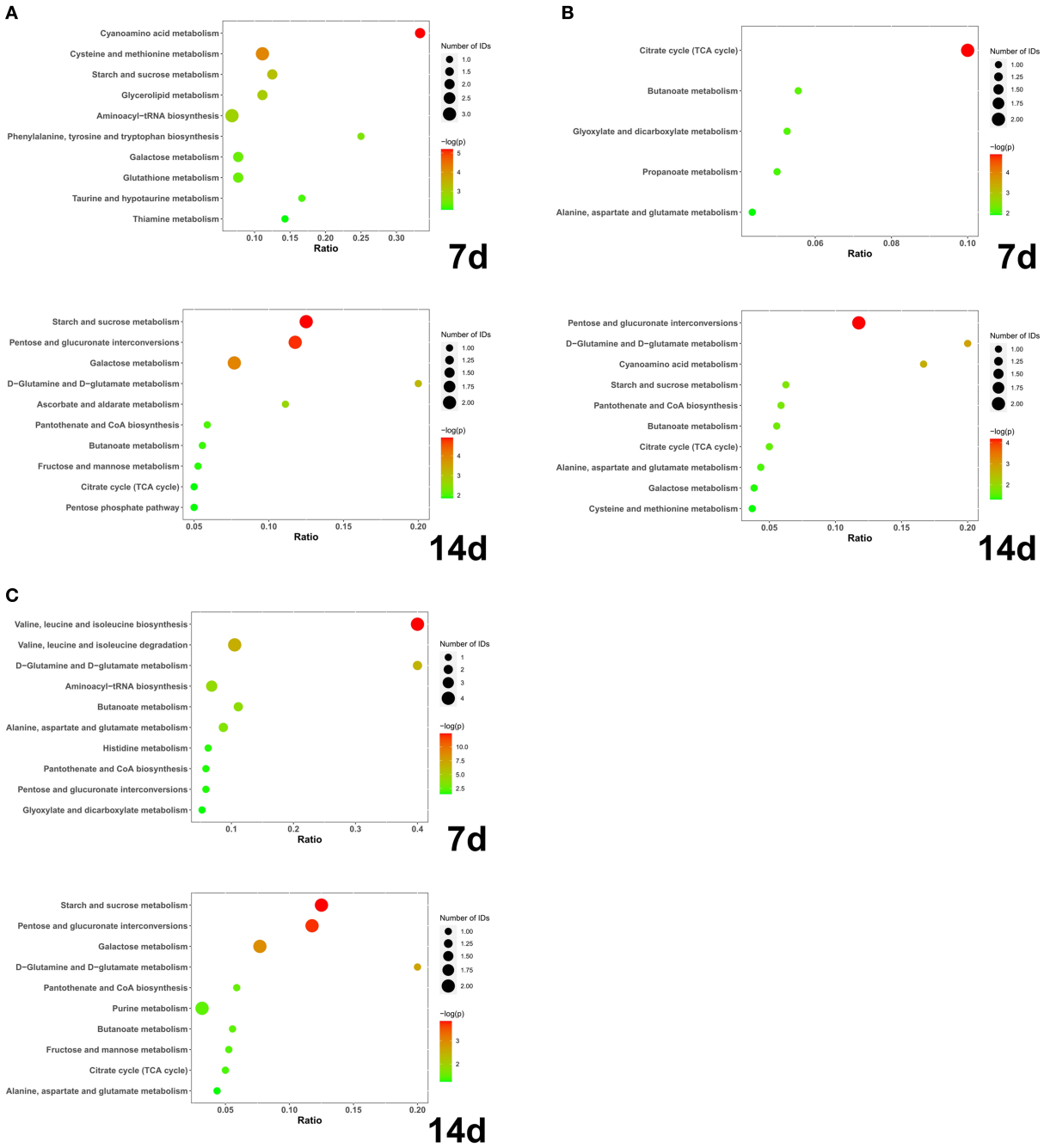


FIGURE 4
(A) IS-C; **(B)** EE-IS; **(C)** APS-IS. The abscissa axis Ratio represents the ratio of differential metabolites enriched to this metabolic pathway to total metabolites; the size of bubbles represents the number of differential metabolites enriched to this metabolic pathway; the larger the -log(*P*), the smaller the *P*-value and the more significant the difference ($N = 5$).

acid and propionic acid directly or indirectly (29–33). SCFAs has anti-inflammatory, anti-tumor, anti-bacterial and immunomodulatory functions. This type of metabolic derivative achieves an anti-inflammatory effects by inhibiting the proliferation of phagocytes and reducing the levels of inflammatory factors, thereby blocking the activation of the NF-κB pathways (30) which builds chemical and physical barriers in the gut and assists in intestinal immune balance.

The intestinal abundance of the above microbiota was significantly reduced in the immunosuppressed state, with the exception of *Ruminantococcus*. Under this condition, it may affect the metabolic production of SCFAs and increase

TABLE 2 Differential metabolites of intestinal contents in each group at 7 days (|Log[2]FC| ≥ 1).

	Metabolites	VIP	*p*-value	Log_2FC	HMDB	KEGG
IS-C	Maltose	1.46	1.46E-02	−1.98	HMDB0000163	C00208
	3-Cyanoalanine	1.74	3.66E-04	−1.95	METPA0300	C02512
	Glutaric acid	1.32	2.91E-02	−1.94	HMDB0000661	C00489
	3-Hydroxybenzoic acid	1.35	2.71E-02	−1.72	HMDB0002466	C00587
	Glutamine[-H_2O]	1.78	1.34E-04	−1.60	-	-
	4-Hydroxybenzoic acid	1.49	9.01E-03	−1.58	HMDB0000500	C00156
	4-Hydroxybutyric acid	1.33	3.27E-02	−1.13	HMDB0000710	C00989
	2-Deoxyinosine	1.36	1.93E-02	1.29	HMDB0000071	C05512
	Galactose	1.50	1.11E-02	1.32	HMDB0000143	C00984
	2-Deoxyguanosine	1.32	3.02E-02	1.35	HMDB0000085	C00330
	Glycerol-3-phosphate	1.22	3.43E-02	1.44	HMDB0000126	C00093
	Cystine	1.42	1.49E-02	1.48	HMDB0000192	C00491
	Orotic acid	1.49	1.50E-02	3.11	HMDB0000226	C00295
	Lactose	1.53	1.27E-02	3.22	HMDB0000186	C00243
EE-IS	Succinic acid	1.48	2.72E-02	−1.29	HMDB0000254	C00042
	Citric acid	1.60	2.00E-02	1.02	HMDB0000094	C00158
	Hexanoic acid	1.86	3.67E-03	1.37	HMDB0000535	C01585
	4-Methylvaleric acid	1.64	2.83E-02	1.63	HMDB0000689	-
	3-Hydroxyphenylacetic acid	1.94	9.78E-04	1.93	HMDB0000440	C05593
	Glutaric acid	1.98	1.39E-03	2.04	HMDB0000661	C00489
APS-IS	3,4-Dihydroxyphenylacetic acid	1.62	1.20E-02	−2.49	HMDB0001336	C01161
	Malic acid	1.42	4.97E-02	1.00	HMDB0000156	C00149
	4-Hydroxyphenylethanol	1.78	2.67E-03	1.01	HMDB0004284	C06044
	2-Hydroxyglutaric acid	1.40	4.71E-02	1.22	HMDB0000694	C03196
	4-Methylthio-2-ketobutyric acid	1.88	3.77E-04	1.50	HMDB0001553	C01180
	2-Ketoglutaric acid	1.68	6.60E-03	1.73	HMDB0000208	C00026
	2-Ketoisovaleric acid	1.74	3.75E-03	1.84	HMDB0000019	C00141
	3-Methyl-2-ketovaleric acid	1.81	1.54E-03	1.88	HMDB0000491	C03465
	2-Ketoisocaproic acid	1.87	5.55E-04	1.88	HMDB0000695	C00233

The underlined value means that the number has corresponding information in the database.

the risk to intestinal health. Instead, the two groups treated with EE and APS promoted the proliferation of colonies reduced in abundance by immunosuppression and increased their abundance, including *Ruminococcus*. In addition, an increased abundance of *Enterococcus* was found in both groups. *Enterococcus* was once considered to be a threatening pathogen. It has been found to be clinically resistant in human medicine, often causing bacteraemia and death (34). But recent research has found that *Enterococcus* has a protective role in the gut. Feeding *Enterococcus* to laying hens by mixing it into their diets could reduce damage to the intestinal mucosa during Salmonella infection. It enhances the immune functions of the organism by reducing oxidative stress and down-regulating serum malondialdehyde levels, while up-regulating IgG levels (35). The increase in the relative abundance of Enterococcus in the gut can protect the gut and have a positive effects on the immunity of the organism. The above evidence indicates that both EE and APS can regulate the immunity by increasing the relative abundance of beneficial bacteria in the gut, and relieve the organism's immune-suppressed state.

The relative abundance of *Eggerthella* was increased in the gut of immunosuppressed chickens. The relevance of *E. lenta* to autoimmune diseases in humans has been confirmed by research (36). It may induce the activation of Th17 cells through its microbial metabolism, contributing to an excessive inflammatory response and exacerbating symptoms in mice with colitis (22). Studies have shown that *E. lenta* can enhance host autoimmunity through the activation of helper T cells. The increased relative abundance of this genus may be associated with suppression of the overall immune functions of the host.

EE is a more targeted modulation of the organism's immune function than APS. EE significantly reduced the relative abundance of *Eggerthella*, *Holdemania* in the gut of immunosuppressed chickens but was not observed in APS

TABLE 3 Differential metabolites of intestinal contents in each group at 14 days (|Log[2]FC| ≥1).

	Metabolites	VIP	*p*-value	Log2FC	HMDB	KEGG
IS-C	2-Ketoglutaric acid	1.39	4.11E-02	−1.61	HMDB0000208	C00026
	Beta-glutamic acid	1.50	1.77E-02	−1.35	-	-
	4-Hydroxyphenylacetic acid	1.56	3.52E-02	1.00	HMDB0000020	C00642
	Fructofuranose	1.65	9.37E-03	1.12	-	-
	Gluconic acid	1.59	2.38E-02	1.49	HMDB0000625	C00257
	Glycyl-leucine	1.30	4.13E-02	1.63	HMDB0000759	C02155
EE-IS	2-Hydroxyglutaric acid	1.64	9.38E-03	−1.43	HMDB0000694	C03196
	2-Ketoglutaric acid	1.59	1.55E-02	−1.27	HMDB0000208	C00026
	4-Hydroxyphenylacetic acid	1.54	2.50E-02	−1.01	HMDB0000020	C00642
	3-Cyanoalanine	1.46	3.93E-02	−1.00	METPA0300	C02512
	Ethanolamine	1.52	2.50E-02	1.28	HMDB0000149	C00189
	5-Hydroxyindoleacetic acid	1.64	9.80E-03	1.43	HMDB0000763	C05635
	Coprostanol	1.47	3.46E-02	2.25	HMDB0000577	-
APS-IS	3-Hydroxybenzoic acid	1.48	1.43E-02	−2.77	HMDB0002466	C00587
	Fructose	1.74	1.86E-03	−1.59	HMDB0000660	C02336
	2,3-Dihydroxybutane	1.47	2.80E-02	−1.52	HMDB0003156	C00265
	Xylitol	1.47	1.34E-02	−1.25	HMDB0002917	C00379
	Glucose	1.57	1.50E-02	−1.22	HMDB0000122	C00031
	Fructofuranose	1.51	4.08E-02	−1.17	-	-
	Adenine	1.44	4.52E-02	1.13	HMDB0000034	C00147
	2-Deoxyadenosine	1.34	4.00E-02	1.15	HMDB0000101	C00559
	Stigmastanol	1.59	1.46E-02	1.40	HMDB0000494	-
	Octanoic acid	1.51	1.85E-02	1.68	HMDB0000482	C06423
	p-cresol	1.43	3.28E-02	1.68	HMDB0001858	C01468
	2-Ketoglutaric acid	1.55	1.91E-02	1.81	HMDB0000208	C00026
	Uric acid	1.44	2.05E-02	1.85	HMDB0000289	C00366

The underlined value means that the number has corresponding information in the database.

group. Additionally, in the EE group, the relative abundances of *Blautia* and *Ruminococcus* were found to be down-regulated. The relative abundance of *Blautia* in the gut is positively correlated with age (37, 38) and immune inflammatory response (39). It also correlates significantly with host physiological functions, such as obesity metabolism (40) and cancer. It has been reported that its biochemical metabolism can produce some carcinogens such as secondary bile acids, carbolic acid and deoxycholic acid (41). EE down-regulated its relative abundance in the gut, possibly revealing that EE plays a bidirectional regulatory role in regulating immune status in chickens.

Metabolomic analysis of intestinal contents revealed that EE and APS may influence butyrate metabolism, alanine, aspartate and glutamate metabolism, the interaction of pentose and glucuronide conversion, and D-glutamine and D-glutamate metabolism. Both may regulate the organism's gut immune functions through their effects on intestinal metabolic processes. This influence is reflected in the metabolic pathways of butyrate metabolism, alanine, aspartate and glutamate metabolism, with both butyrate and aspartate having a positive effect on intestinal immunity. For example, butyric acid, a member of the SCFAs, provides energy to the intestinal cells and maintains the integrity of the intestinal barrier. It also regulates the balance of the gut microbiota by acidifying the intestinal environment. Butyric acid has been shown to affect intestinal immune function by inhibiting the migration of immune cells and regulating cell proliferation and apoptosis (42–44). Together with glutamic acid, asparagine acts as precursors for the synthesis of various amino acids. They provide energy to the intestine, protect the intestinal mucosa, reduce mucosal damage caused by bacterial endotoxins (45), delay lymphocyte apoptosis and promote cell growth.

To reveal the effects of EE and APS on the gut of immunosuppressed chickens by correlating gut microbiota and content metabolomics. Not only is the gut involved in changes in the entire intestinal environment, but also the microbiota within it. The metabolites of the intestinal flora play an important role in the physiological processes of the organism, protecting intestinal health (46), providing energy, activating immune cells, participating in the intestinal immune process and regulating the balance of the intestinal flora. The succinate producing bacteria *Blautia* and *Ruminococcus* were discovered to be associated

with some differential metabolic pathways in the metabolomic analysis. Among these were alanine, aspartate and glutamate metabolism, butyrate metabolism, TCA cycle, glyoxylate and dicarboxylic acid metabolism and propionic acid metabolism. Succinate, an important signaling factor for immune activation, has a key role in the regulation of inflammation (47, 48) and a role in sustaining the stability of the intestinal lining in the gut (49). Elevated levels of succinate in the gut may promote intestinal inflammation (50). EE might have a reverse immunomodulatory effects by reducing the amount of succinic acid in the gut, affecting the energy metabolism of glucose metabolism, the metabolism of butyric and propionic acids and the metabolism of immune-related amino acids based on the results of the trial. But its more precise mechanism of action and related effects needs more experimental studies to verify the findings.

Conclusion

This trial used 16S-rRNA analysis of the gut microbiota and metabolomics to analyze the effects of EE and APS on immunosuppressed chickens. Overall, immunosuppression can affect the species diversity of the intestinal flora and reduce the relative abundance of some SCFAs-producing bacteria in the gut. Both EE and APS can play a role in mitigating the effects of immunosuppression on the intestinal flora, restoring the abundance of beneficial bacteria and anaerobic genera, and protecting the health of the intestinal environment. The comprehensive analysis of the metabolism of the microbiota revealed that EE might suppress the relative abundance of *Blautia* and *Ruminococcus*, reducing the succinic acid content in the intestine.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee of South China Agricultural University (License Number: 2017A087). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

SL, RL, JC, SZ, and YS were responsible for study conception and design. RH, DS, and AG revised the manuscript. SL, RL, JC, SZ, YS, and DS were involved in the drafting of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by General Program of Natural Science Foundation of Guangdong Province (2021A1515011010) and Key R&D Projects in Guangzhou (202206010189).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Balamurugan V, Kataria JM. Economically important non-oncogenic immunosuppressive viral diseases of chicken–current status. *Vet Res Commun.* (2006) 30:541–66. doi: 10.1007/s11259-006-3278-4

2. Gimeno IM, Schat KA. Virus-induced immunosuppression in chickens. *Avian Dis.* (2018) 62:272–85. doi: 10.1637/11841-041318-Review.1

3. Fussell LW. Poultry industry strategies for control of immunosuppressive diseases. *Poult Sci.* (1998) 77:1193–6. doi: 10.1093/ps/77.8.1193

4. Bossy A, Blaschek W, Classen B. Characterization and immunolocalization of arabinogalactan-proteins in roots of Echinacea purpurea. *Planta Med.* (2009) 75:1526–33. doi: 10.1055/s-0029-1185801

5. Gertsch J. Immunomodulatory lipids in plants: plant fatty acid amides and the human endocannabinoid system. *Planta Med.* (2008) 74:638–50. doi: 10.1055/s-2008-1034302

6. Goel V, Chang C, Slama JV, Barton R, Bauer R, Gahler R, et al. Alkylamides of Echinacea purpurea stimulate alveolar macrophage function in normal rats. *Int Immunopharmacol.* (2002) 2:381–7. doi: 10.1016/S1567-5769(01)00163-1

7. Hwang SA, Dasgupta A, Actor JK. Cytokine production by non-adherent mouse splenocyte cultures to Echinacea extracts. *Clin Chim Acta.* (2004) 343:161–6. doi: 10.1016/j.cccn.2004.01.011

8. Bauer R, Remiger P. TLC and HPLC analysis of alkamides in echinacea drugs 1,2. *Planta Med.* (1989) 55:367–71. doi: 10.1055/s-2006-962030

9. Kallon S, Li X, Ji J, Chen C, Xi Q, Chang S, et al. Astragalus polysaccharide enhances immunity and inhibits H9N2 avian influenza virus *in vitro* and *in vivo*. *J Anim Sci Biotechnol.* (2013) 4:22. doi: 10.1186/2049-1891-4-22

10. Zhang W, Ma W, Zhang J, Song X, Sun W, Fan Y. The immunoregulatory activities of astragalus polysaccharide liposome on macrophages and dendritic cells. *Int J Biol Macromol.* (2017) 105:852–61. doi: 10.1016/j.ijbiomac.2017.07.108

11. Liu L, Shen J, Zhao C, Wang X, Yao J, Gong Y, et al. Dietary astragalus polysaccharide alleviated immunological stress in broilers exposed to lipopolysaccharide. *Int J Biol Macromol.* (2015) 72:624–32. doi: 10.1016/j.ijbiomac.2014.08.057

12. Shan C, Sun B, Dalloul RA, Zhai Z, Sun P, Li M, et al. Effect of the oral administration of astragalus polysaccharides on jejunum mucosal immunity in chickens vaccinated against Newcastle disease. *Microb Pathog.* (2019) 135:103621. doi: 10.1016/j.micpath.2019.103621

13. Liu F, Yin J, Du M, Yan P, Xu J, Zhu X, et al. Heat-stress-induced damage to porcine small intestinal epithelium associated with downregulation of epithelial growth factor signaling. *J Anim Sci.* (2009) 87:1941–9. doi: 10.2527/jas.2008-1624

14. Hansen J, Gulati A, Sartor RB. The role of mucosal immunity and host genetics in defining intestinal commensal bacteria. *Curr Opin Gastroenterol.* (2010) 26:564–71. doi: 10.1097/MOG.0b013e32833f1195

15. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, et al. Gut microbiota functions: metabolism of nutrients and other food components. *Eur. J. Nutr.* (2018) 57:1–24. doi: 10.1007/s00394-017-1445-8

16. Song J, Jiao LF, Xiao K, Luan ZS, Hu CH, Shi B, et al. Cello-oligosaccharide ameliorates heat stress-induced impairment of intestinal microflora, morphology and barrier integrity in broilers. *Anim Feed Sci Technol.* (2013) 185:175–81. doi: 10.1016/j.anifeedsci.2013.08.001

17. Choi JH, Kim GB, Cha CJ. Spatial heterogeneity and stability of bacterial community in the gastrointestinal tracts of broiler chickens. *Poult Sci.* (2014) 93:1942–50. doi: 10.3382/ps.2014-03974

18. Allaire JM, Crowley SM, Law HT, Chang SY, Ko HJ, Vallance BA. The intestinal epithelium: central coordinator of mucosal immunity. *Trends Immunol.* (2018) 39:677–96. doi: 10.1016/j.it.2018.04.002

19. Mokkala K, Paulin N, Houttu N, Koivuniemi E, Pellonperä O, Khan S, et al. Metagenomics analysis of gut microbiota in response to diet intervention and gestational diabetes in overweight and obese women: a randomised, double-blind, placebo-controlled clinical trial. *Gut.* (2020) 70:gutjnl-2020–321643. doi: 10.1136/gutjnl-2020-321643

20. Michaudel C, Sokol H. The gut microbiota at the service of immunometabolism. *Cell Metab.* (2020) 32:514–23. doi: 10.1016/j.cmet.2020.09.004

21. Blaak EE, Canfora EE, Theis S, Frost G, Groen AK, Mithieux G, et al. Short chain fatty acids in human gut and metabolic health. *Benef Microbes.* (2020) 11:411–55. doi: 10.3920/BM2020.0057

22. Alexander M, Ang QY, Nayak RR, Bustion AE, Sandy M, Zhang B, et al. Human gut bacterial metabolism drives Th17 activation and colitis. *Cell Host Microbe.* (2022) 30:17–30.e9. doi: 10.1016/j.chom.2021.11.001

23. Kemp PF, Aller JY. Bacterial diversity in aquatic and other environments: what 16S rDNA libraries can tell us. *FEMS Microbiol Ecol.* (2004) 47:161–77. doi: 10.1016/S0168-6496(03)00257-5

24. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. *Science.* (2008) 320:1647–51. doi: 10.1126/science.1155725

25. Ramette A. Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol.* (2007) 62:142–60. doi: 10.1111/j.1574-6941.2007.00375.x

26. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* (2010) 7:335–6. doi: 10.1038/nmeth.f.303

27. Gao X, Pujos-Guillot E, Sebedio JL. Development of a quantitative metabolomic approach to study clinical human fecal water metabolome based on trimethylsilylation derivatization and GC/MS analysis. *Anal Chem.* (2010) 82:6447–56. doi: 10.1021/ac1006552

28. Liu Y, Yan T, Ren Z, Yang X. Age-associated changes in caecal microbiome and their apparent correlations with growth performances of layer pullets. *Anim Nutr.* (2021) 7:841–8. doi: 10.1016/j.aninu.2020.11.019

29. Tang W, Li DY, Hazen SL. Dietary metabolism, the gut microbiome, and heart failure. *Nat Rev Cardiol.* (2018) 16:137–54. doi: 10.1038/s41569-018-0108-7

30. Tan J, Mckenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. *Adv Immunol.* (2014) 121:91–119. doi: 10.1016/B978-0-12-800100-4.00003-9

31. Pieter VDA, Belzer C, Goossens M, Kleerebezem M, De Vos WM, Thas O, et al. Butyrate-producing Clostridium cluster XIVa species specifically colonize mucins in an *in vitro* gut model. *ISME J.* (2013) 7:949–61. doi: 10.1038/ismej.2012.158

32. Rey FE, Faith JJ, Bain J, Muehlbauer MJ, Stevens RD, Newgard CB, et al. Dissecting the *in vivo* metabolic potential of two human gut acetogens. *J Biol Chem.* (2010) 285:22082–90. doi: 10.1074/jbc.M110.117713

33. Peng L, Li ZR, Green RS, Holzman IR, Lin J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly *via* activation of AMP-activated protein kinase in Caco-2 cell monolayers. *J Nutr.* (2009) 139:1619–25. doi: 10.3945/jn.109.104638

34. Caballero-Granado FJ, Becerril B, Cuberos L, Bernabeu M, Cisneros JM, Pachon J. Attributable mortality rate and duration of hospital stay associated with enterococcal bacteremia. *Clin Infect Dis.* (2001) 32:587–94. doi: 10.1086/318717

35. Huang S, Rong X, Liu M, Liang Z, Geng Y, Wang X, et al. Intestinal mucosal immunity-mediated modulation of the gut microbiome by oral delivery of enterococcus faecium against salmonella enteritidis pathogenesis in a laying hen model. *Front Immunol.* (2022) 13:853954. doi: 10.3389/fimmu.2022.853954

36. Plichta DR, Somani J, Pichaud M, Wallace ZS, Fernandes AD, Perugino CA, et al. Congruent microbiome signatures in fibrosis-prone autoimmune diseases: IgG4-related disease and systemic sclerosis. *Genome Med.* (2021) 13:35. doi: 10.1186/s13073-021-00853-7

37. Xi Y, Shuling N, Kunyuan T, Qiuyang Z, Hewen D, Chencheng G, et al. Characteristics of the intestinal flora of specific pathogen free chickens with age. *Microb Pathogenesis.* (2019) 132:325–34. doi: 10.1016/j.micpath.2019.05.014

38. Vaiserman AM, Koliada AK, Marotta F. Gut microbiota: a player in aging and a target for anti-aging intervention. *Ageing Res Rev.* (2017) 35:36–45. doi: 10.1016/j.arr.2017.01.001

39. Kim CH, Park J, Kim M. Gut microbiota-derived short-chain fatty acids, T cells, and inflammation. *Immune Netw.* (2014) 14:277. doi: 10.4110/in.2014.14.6.277

40. Yang J, Bindels LB, Segura Munoz RR, Martínez I, Walter J, Ramer-Tait AE, et al. Disparate metabolic responses in mice fed a high-fat diet supplemented with maize-derived non-digestible feruloylated oligo- and polysaccharides are linked to changes in the gut microbiota. *PLoS ONE.* (2016) 11:e0146144. doi: 10.1371/journal.pone.0146144

41. Vaughn BP, Kaiser T, Staley C, Hamilton MJ, Reich J, Graiziger C, et al. A pilot study of fecal bile acid and microbiota profiles in inflammatory bowel disease and primary sclerosing cholangitis. *Clin Exp Gastroenterol.* (2019) 12:9–19. doi: 10.2147/CEG.S186097

42. Kushwaha V, Rai P, Varshney S, Gupta S, Khandelwal N, Kumar D, et al. Sodium butyrate reduces endoplasmic reticulum stress by modulating CHOP and empowers favorable anti-inflammatory adipose tissue immune-metabolism in HFD fed mice model of obesity. *Food Chem Mol Sci.* (2022) 4:100079. doi: 10.1016/j.fochms.2022.100079

43. Manrique Vergara D. Ácidos grasos de cadena corta (ácido butírico) y patologías intestinales. *Nutricion hospitalaria.* (2017) 34:58–61. doi: 10.20960/nh.1573

44. Berni CR, Di Costanzo M, Leone L. The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. *Clin Epigenetics.* (2012) 4:4. doi: 10.1186/1868-7083-4-4

45. Wang X, Liu Y, Li S, Pi D, Zhu H, Hou Y, et al. Asparagine attenuates intestinal injury, improves energy status and inhibits AMP-activated protein kinase signalling pathways in weaned piglets challenged with Escherichia coli lipopolysaccharide. *Br J Nutr.* (2015) 114:553–65. doi: 10.1017/S0007114515001877

46. Yamashiro Y. Gut microbiota in health and disease. *Ann Nutr Metab.* (2018) 71:242–6. doi: 10.1159/000481627

47. Littlewood-Evans A, Sarret S, Apfel V, Loesle P, Dawson J, Zhang J, et al. GPR91 senses extracellular succinate released from inflammatory macrophages and exacerbates rheumatoid arthritis. *J Exp Med.* (2016) 213:1655–62. doi: 10.1084/jem.20160061

48. Tannahill GM, Curtis AM, Adamik J, Palsson-Mcdermott EM, Mcgettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature.* (2013) 496:238–42. doi: 10.1038/nature11986

49. Fernandez-Veledo S, Vendrell J. Gut microbiota-derived succinate: friend or foe in human metabolic diseases? *Rev Endocr Metab Disord.* (2019) 20:439–47. doi: 10.1007/s11154-019-09513-z

50. Hagihara M, Ariyoshi T, Kuroki Y, Eguchi S, Higashi S, Mori T, et al. Clostridium butyricum enhances colonization resistance against Clostridioides difficile by metabolic and immune modulation. *Sci Rep.* (2021) 11:15007. doi: 10.1038/s41598-021-94572-z

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 06 September 2022
DOI 10.3389/fvets.2022.929112

OPEN ACCESS

EDITED BY
Dayou Shi,
South China Agricultural University, China

REVIEWED BY
Xiaolong Gu,
Yunnan Agricultural University, China
Haichong Wu,
Zhejiang University, China

*CORRESPONDENCE
Huabin Cao
chbin20020804@jxau.edu.cn

†These authors share first authorship

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 26 April 2022
ACCEPTED 04 July 2022
PUBLISHED 06 September 2022

CITATION
Wang X, Chen J, Yang F, Ali F, Mao Y, Hu A, Xu T, Yang Y, Wang F, Zhou G, Guo X and Cao H (2022) Two kinds of traditional Chinese medicine prescriptions reduce thymic inflammation levels and improve humoral immunity of finishing pigs. *Front. Vet. Sci.* 9:929112. doi: 10.3389/fvets.2022.929112

Two kinds of traditional Chinese medicine prescriptions reduce thymic inflammation levels and improve humoral immunity of finishing pigs

Xiaoyu Wang[1†], Jiajia Chen[2†], Fan Yang[1], Farah Ali[3], Yaqin Mao[4], Aiming Hu[5], Tianfang Xu[6], Yan Yang[6], Feibing Wang[7], Guangbin Zhou[8], Xiaowang Guo[9] and Huabin Cao[1*]

[1]Jiangxi Provincial Key Laboratory for Animal Health, College of Animal Science and Technology, Institute of Animal Population Health, Jiangxi Agricultural University, Nanchang, China, [2]Department of Animal Science and Technology, Jiangxi Biotech Vocational College, Nanchang, China, [3]Department of Theriogenology, Faculty of Veterinary & Animal Sciences, The Islamia University of Bahawalpur, Pakistan, Bahawalpur, Pakistan, [4]China Institute of Veterinary Drug Control, MOA Center for Veterinary Drug Evaluation, Beijing, China, [5]Jian City Livestock and Veterinary Bureau, Jiangxi, China, [6]Jiangxi Agricultural Technology Extension Center, Nanchang, China, [7]Agricultural Technology Extension Center, Jinxi County Agriculture and Rural Bureau, Fuzhou, China, [8]Animal Epidemic Prevention and Quarantine Unit, Fengcheng Agricultural and Rural Bureau, Fengcheng, China, [9]Yichun Agriculture and Rural Affairs Bureau, Yichun, China

In animal husbandry, traditional Chinese medicine (TCM) as a reasonable alternative to antibiotics has attracted more and more concerns to reduce microbial resistance. This study was aimed to investigate the effects of dietary supplementation with TCM prescriptions on serum parameters and thymus inflammation responses in finishing pigs. Thirty finishing pigs were randomly divided into three groups, which included the Con group (basal diet), the TCM1 group (basal diet supplemented with *Xiao Jian Zhong* prescriptions), and the TCM2 group (basal diet supplemented with *Jingsananli*-sepsis). The results showed that the contents of C3 and C4 in the serum were significantly increased in both the TCM1 and TCM2 groups compared to the Con group on day 30. Similarly, the levels of IgA, IgG, and IgM were increased in the TCM2 group, and only the level of IgM in TCM1 was increased on day 30. Meanwhile, the levels of classical swine fever virus (CSFV) and respiratory syndrome virus (PRRSV) antibodies had a notable increase in the TCM1 and TCM2 groups. Both TCM1 and TCM2 inhibited the levels of TLR4/MyD88/NF-κB signaling pathway-related mRNA (TLR4, MyD88, NF-κB, IL6, IL8, and TNF-α) and protein (p-IκBα and p-P65) expression levels in the thymus. In conclusion, dietary supplementation with TCM could reduce thymic inflammation levels and improve humoral immunity of finishing pigs.

KEYWORDS

traditional Chinese medicine, finishing pigs, immune function, inflammation, thymus

Introduction

As we all know, piglet breeding is the most critical period in the process of pig breeding. To safeguard the health of finishing pigs during the nursery period, the use of antimicrobials either in feed or in water has been an essential tool. Antibiotics are used in swine feed as a growth promoter, to improve feed efficiency, and to reduce susceptibility to bacterial infections (1, 2). Nevertheless, abuse of antibiotic feed additives not only leads to excessive antibiotic residues in animals but also endangers human food safety and health. The China Ministry of Agriculture (MOA) gradually reduced the types and doses of antibiotics allowed in feeds in 2017 with the intent to ban the usage of antibiotics in feed additives by 2020 (3). Thus, it is necessary to develop new alternatives instead of using antibiotics to control diseases.

Traditional Chinese medicine (TCM) has lately received greater attention from researchers because of its natural structure and biological activity that can enhance or restore the immune system (4, 5). A recent study has shown that some phytochemicals were beneficial to health by promoting immune function and reducing inflammation responses (6). According to the theory of TCM, prescriptions are usually composed of several herbs or minerals, one of which represents the principal component and the other serves as an auxiliary component to assist or promote the transmission of the principal component. In most cases, the mixture of a variety of TCM will have a synergistic therapeutic effect (7).

The TLR4/MyD88/NF-κB pathway is a classical toll-like receptor activation pathway that plays an essential role in anti-inflammatory and immune regulations. A large number of studies have reported that inhibition of the TLR4/MyD88/NF-κB pathway could effectively inhibit the activation of NF-κB and reduce the secretion of pro-inflammatory factors such as TNF-a and IL-6, thereby inhibiting the occurrence of inflammation (8, 9). A previous study demonstrated that cinnamaldehyde has anti-inflammatory effects that can prevent inflammation-related health problems associated with over-activation of the TLR4 signaling pathway (10). Meanwhile, Luo et al. (11) found that pachymaran enhanced the immune function of mice by regulating genes associated with T and B cell functions. Additionally, it is reported that saikosaponin-a strongly inhibited pro-inflammatory mediators (12). Thus, dietary supplementation with TCM may be a feasible strategy to replace or reduce the use of antibiotics by enhancing immune responses, consequently promoting growth performance in finishing pigs (13). In conclusion, natural plants with pharmacological activities are recommended as dietary supplements or therapeutic agents to effectively care for the organism.

There is little research on the effects of TCM prescriptions on the immunity of finishing pigs. Therefore, this study was conducted to assess the effects of TCM1 and TCM2 on serum parameters and thymus inflammation responses in finishing pigs, and aimed to provide valuable insights for improving the immune performance of finishing pigs.

Materials and methods

Animals and treatments

This trial was performed in the Researching and Teaching Base of Jiangxi Agricultural University and treated for 60 days. Thirty finishing pigs (white × landrace × Duroc) weighing 21.43 ± 2.86 kg were allowed *ad libitum* access to feed and water throughout the experimental period. Experimental animals of all three treatment groups were fed with the same basal diet, which was formulated to meet the nutrient requirements of finishing pigs (14). The composition and nutrient levels of basal diet are shown in Table 1. The finishing pigs were randomly allotted to three treatment groups ($n = 10$): The Con group (basal diet), the TCM1 group (basal diet + 10 g/kg *Xiao Jian Zhong*, XJZ) and the TCM2 group (basal diet + 3 g/kg *Jingsananli*-sepsis, JSS). The doses of TCM1 and TCM2 were evaluated according to our preliminary study and traditional Chinese pharmacopeia (2005). All raw materials of TCM1 were bought from Chinese Traditional Medicine Chang Sheng (Jiangxi, China), and TCM2 was provided by Jiangxi Chuang Dao Animal Health Products

TABLE 1 Composition and nutrient levels of basal diets (air-dry basis)[a].

Ingredients	Content (%)	Analyzed composition, g/kg	Content (%)
Maize	55.80	DM	89.21
Soybean meal	16.3	DE (MJ/kg)	14.36
Fermented soybean meal	7.0	CP	19.63
Wheat middling	4.5	Lysine	1.32
Fish meal	2.5	Methionine	0.43
Dried porcine solubles	2.5	Methionine+Cystine	0.77
Whey powder	6.25	Threonine	0.81
Soy oil	1.65	Calcium	0.96
Lysine	0.25	Total phosphorus	0.60
Methionine	0.1		
Limestone	1.05		
$CaHPH_4$	0.80		
NaCl	0.30		
premix	1.00		

[a]The premix provides the following per kilogram diet:Vitamin A 8 000 IU, Vitamin D 2 500 IU, Vitamin E 15 mg, nicotinic acid 20 mg, D-pantothenie 10 mg, riboflavin 4 mg, biotin 0.06 mg, folic acid 0.2 mg, thiamine 2mg, choline chloride 500 mg, Cu 165 mg, Fe 110 mg, Mn 80 mg, Zn 330 mg, Se 0.20 mg.

Co., Ltd. (Jiangxi, China). The composition and main active constituents of TCM1 and TCM2 are presented in Table 2. All experimental protocols were approved by the Committee for the Care and Use of Experimental Animals, Jiangxi Agricultural University, Jiangxi, China.

Sample collection

The experimental period lasted 60 days, and blood samples were collected on days 0, 30, and 60 and centrifuged (3,000 × g, 10 min) to obtain the serum which was then stored at −20°C for follow-up studies. All the finishing pigs were killed by euthanasia with an intravenous injection of sodium pentobarbital (40 mg/kg body weight), and the thymus of the finishing pigs was collected immediately after euthanasia, rapidly frozen in liquid nitrogen, and stored at −80°C for various analyses on day 60.

Serum parameter analysis

IgA, IgM, IgG, C3, and C4 were assayed following the manufacturer's instructions and using porcine-specific immunoturbidimetry kits (Nanjing Jiancheng Bioengineering Institute, China).

Blocking enzyme-linked immunosorbent assay (ELISA)

The serum samples were assayed following the manufacturer's instructions and using the IDEXX classical swine fever virus (CSFV) antibody test kit and IDEXX porcine reproductive and respiratory syndrome virus (PRRSV) antibody test kit (IDEXX Laboratories, Netherlands).

Real-time quantitative polymerase chain reaction (qRT-PCR)

The mRNA transcription levels of TLR4/MyD88/NF-κB signal pathway-related genes (TLR4, MyD88, NF-κB, IL-6, IL-8, and TNF-α) in the thymus were determined by qRT-PCR assay according to the manufacturer's instructions. Briefly, total RNA was isolated using the TransZol Up Reagent (TransGen Biotech, Beijing, China). Then, cDNA was synthesized using a TransScript® One-Step gDNA Removal and cDNA Synthesis

TABLE 2 Composition and main active constituents of TCM1 and TCM2 (air dry basis)[a].

Latin name	Main active constituent	Used part	Content (%)
TCM1			
Cassia Twig	*Cinnamaldehyde*	Dried twig	13
Glycyrrhiza uralensis	*Glycyrrhizin*	Dried root	4
Ziziphus zizyphus	*Jujuba polysaccharide*	Dried fructification	4
Cynanchum otophyllum	*Paeoniflorin*	Dried root	13
Zingiber officinale Roscoe	*Ginger oleoresin*	Dried root	6
Rhizoma Atractylodes	*Atractylodine*	Dried root	14
Atractylodes macrocephala	*Biatractylolide*	Dried root	10.5
Poria cocos	*Pachymaran*	Dried sclerotium	10.5
Coptis chinensis Franch.	*Berberine*	Dried root	4
Maltose	Maltose	-	21
Total			100
TCM2			
Nepeta cataria L.	*Nepeta Cataria Oil*	Dried stem	16.5
Radix Saposhnikoviae	*Chromone glycoside*	Dried root	16.5
Notopterygium incisum	*Notopterol*	Dried root and stem	16.5
Radix Angelicae	*Heraclenin*	Dried root	16.5
Radix bupleuri	*Saikosaponin*	Dried root	10
Radix Peucedani	*peucedanin*	Dried root	10
Poria cocos	*Pachymaran*	Dried sclerotium	10
Glycyrrhiza uralensis	*Glycyrrhizin*	Dried root	4
Total			100

[a]Main active constituents of TCM come from Chinese pharmacopeia (2005).

SuperMix reagent kit (TransGen Biotech, Beijing, China) according to the kit's instructions and stored at −20°C for SYBR Green qRT-PCR. Gene-specific primers of all the genes were designed using the Primer Premier software (PREMIER Biosoft International, CA, United States). The GAPDH gene was used as an internal reference, and the primer sequences are shown in Table 3. The qRT-PCR profiles were as follows: 95°C for 10 min, 40 cycles at 95°C for 15 s, 60°C for 60 s, and extension at 95°C for 15 s. All the reactions were carried out using an ABI 7900HT machine (Applied Biosystems, United States).

TABLE 3 Primers used in this study.

Target	Gene bank number	Primers sequences (5'-3')
TNF-α	NM_214022.1	F: CCAATGGGCAGAHTGGGTATG
		R: TGAAGAGGACCTGGGAGTAG
IL-6	NM_001252429.1	F: TGGCTACTGCCTTCCCTACC
		R: CAGAGATTTTGCCGAGGATG
IL-8	NM_213867.1	F: TTCGATGCCAGTGCATAAATA
		R: CTGTACAACCTTCTGCACCCA
GAPDH	NM_001206359	F: ACTCACTCTTCCACTTTTGATGCT
		R: TGTTGCTGTAGCCAAATTCA
MyD88	NM_001099923.1	F: TGGTAGTGGTTGTCTCTGATGA
		R: TGGAGAGAGGCTGAGTGCAA
NF-κB	NM_001048232.1	F: CTCGCACAAGGAGACATGAA
		R: ACTCAGCCGGAAGGCATTAT
TLR4	NM_001113039.1	F: GCCATCGCTGCTAACATCATC
		R: CTCATACTCAAAGATACACCATCGG

Western blot

The total protein from about 0.1g thymus of each piglet was extracted using a RIPA lysis buffer (APPLYGEN, Beijing, China) supplemented with 1 mM phenylmethanesulfonyl fluoride and phosphatase inhibitor, and the concentrations of total proteins were measured using a BCA (bicinchoninic acid) protein assay kit (Solarbio, China). Protein supernatant was separated by 10% SDS-PAGE and transferred into PVDF membranes. After blocking with 5% skimmed milk powder, the membranes were incubated with appropriate primary antibodies overnight at 4°C, followed by incubation with corresponding secondary antibodies for 1 h at room temperature. The membranes were washed thrice for 10 min each, incubated with a SuperSignal chemiluminescent substrate (Pierce), and imaged with ChemiDoc XRS+ Imaging System (Bio-Rad). Blots were semi-quantified using the ImageJ software. Primary antibodies for NF-κB p65 (Bioss, 1:1,000) and p-NF-κB p65, p-IκB-α (Bioss, 1:800) and phospho-IκB-α were used in this study.

Statistical analysis

A statistical analysis was performed using the SPSS 17.0 software (SPSS Inc., Chicago, United States). All the results are expressed in the format of mean ± standard deviation (SD). Comparisons between two or multiple groups were made by the Student's *t*-test or one-way ANOVA. A *P*-value of <0.05 was significant.

Results

Effects of TCM1 and TCM2 on immunoglobulins in serum

The effects of TCM1 and TCM2 on serum immunoglobulins are shown in Figure 1. Compared to the Con group, the TCM2 group showed a higher concentration of IgA ($P < 0.01$) on day 30 and a higher concentration of IgG ($P < 0.05$) on day 60. Furthermore, the TCM1 group showed higher serum levels of IgM ($P < 0.05$) on day 30, and the TCM2 group showed a higher concentration of IgM ($P < 0.01$) on day 30.

Effects of TCM1 and TCM2 on complement in serum

The effects of TCM1 and TCM2 on serum complement are shown in Figure 2. Compared to the Con group, the TCM1 and TCM2 groups showed the highest concentration of C3 ($P < 0.01$) on day 30. No dramatic difference was observed in the serum concentrations of C3 among the three groups on day 60. The TCM2 group showed the highest concentration of C4 ($P < 0.01$) on day 30, and the TCM1 group showed a higher concentration of C4 ($P < 0.05$) on day 30. Furthermore, the TCM1 group showed highest serum levels of C4 ($P < 0.01$) on day 60.

CSFV and PRRSV antibodies in finishing pigs

CSFV and PRRSV antibodies in the finishing pigs are evaluated in Figure 3. Compared to the Con group, the TCM1 and TCM2 groups showed the highest concentration of CSFV and PRRSV antibodies ($P < 0.01$) on day 30.

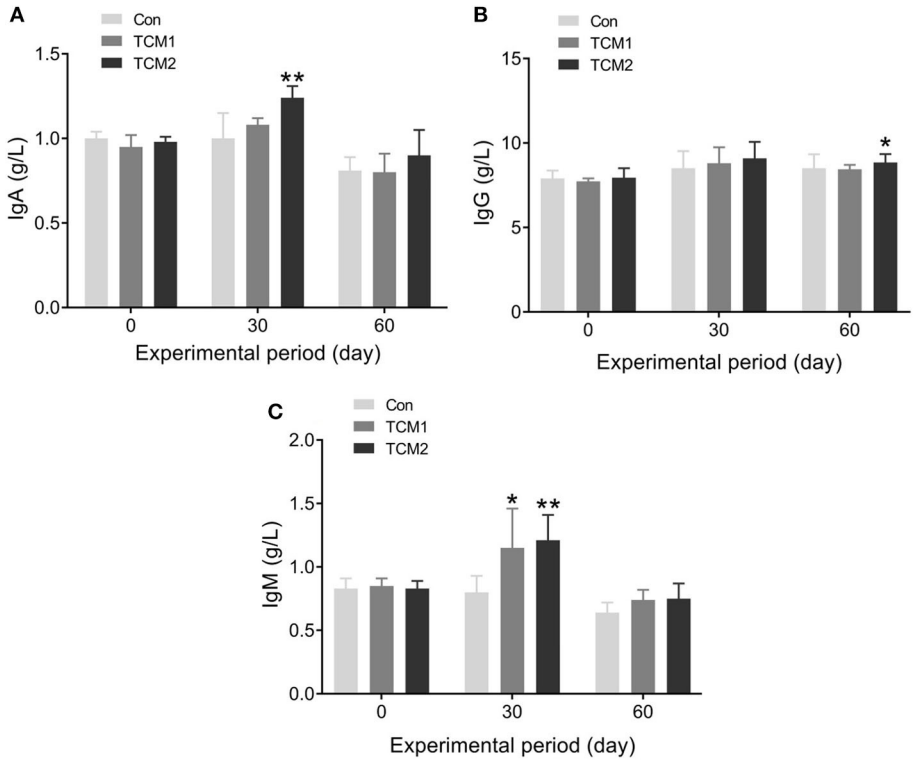


FIGURE 1
Effect of TCM1 and TCM2 on serums IgA, IgG, and IgM in finishing swine. "*" indicates significant difference compared with the control group (*$P < 0.05$, **$P < 0.01$). Below is the same. **(A)** IgA level. **(B)** IgG level. **(C)** IgM level.

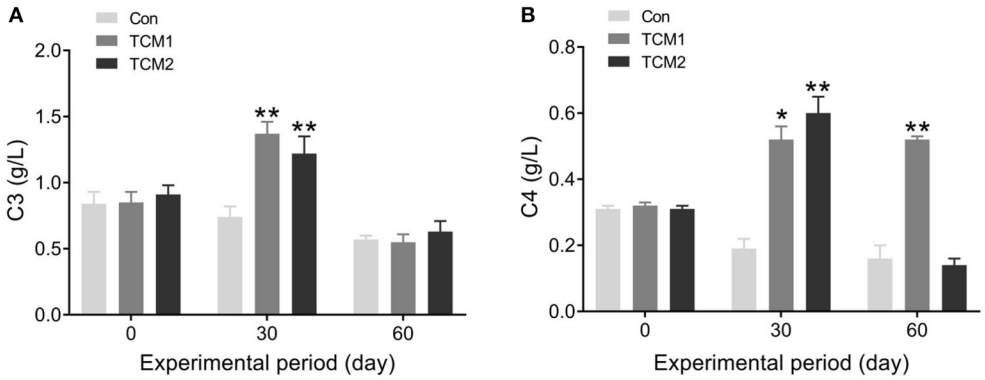


FIGURE 2
Effect of TCM1 and TCM2 on the levels of serums C3 and C4 in finishing swine. **(A)** The serum C3 level. **(B)** The serum C4 level. "*" indicates significant difference compared with control group (*$P < 0.05$ and **$P < 0.01$).

Effects of TCM1 and TCM2 on TLR4/MyD88/NF-κB pathway-related mRNA and protein levels

The effects of TCM1 and TCM2 treatments on mRNA expression levels of the TLR4/MyD88/NF-κB signaling pathway in thymus tissues of the finishing pigs are shown in Figures 4A,B. Compared to the Con group, the mRNA expression levels of MyD88 were decreased in the TCM1 and TCM2 ($P < 0.01$) treatments; NF–κB levels in all the treatments group were decreased and significant in TCM1 ($P < 0.05$) and TCM2 ($P < 0.01$). Similarly, the levels of TLR4 were downregulated in

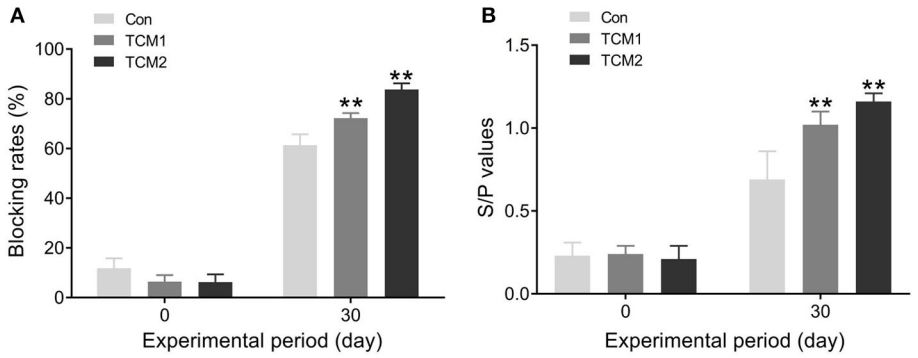


FIGURE 3
Effect of TCM1 and TCM2 on CSFV and PRRSV antibodies in finishing swine. **(A)** The concentration of CSFV antibody. **(B)** The concentration of PRRSV antibody. "*" indicates a significant difference compared with control group (*$P < 0.05$ and **$P < 0.01$).

the TCM1 ($P < 0.05$) and TCM2 ($P < 0.01$) treatments. The protein levels of P65, p-P65, IκBα, and p-IκBα were notably increased ($P < 0.01$ or $P < 0.001$) in the TCM1 and TCM2 groups (Figures 5A–C). Additionally, the p-P65/P65 ratio and the p-IκBα/IκBα ratio in both TCM1 and TCM2 groups was down-regulated ($P < 0.01$ or $P < 0.001$) in comparison with the control group.

Effects of TCM1 and TCM2 on inflammatory cytokine mRNA levels

The effects of TCM supplementation on thymus tissue mRNA expression levels of IL-6, IL-8 and TNF-α are shown in Figures 4C,D. Compared to the Con group, the mRNA expression levels of IL-8 and TNF-α declined. IL8 levels in all the treatment groups were decreased and significant in TCM1 and TCM2 ($P < 0.05$). Similarly, the levels of TNF-α were downregulated in the TCM1 ($P < 0.01$) and TCM2 ($P < 0.05$) treatments.

Discussion

In recent decades, medicinal plants have attracted much attention because of their significant bioactivities such as increasing growth performance, antioxidant activity, antiviral activity, and immunomodulatory activity, which make them suitable as antibiotic replacements (15). TCM as an alternative to antibiotics is receiving more and more attention. Numerous studies have been demonstrated that TCM poses lots of function to livestock and poultry breeding, such as anti-inflammatory, oxidation resistance, resistance to allergy, protect the cardiovascular and antitumor (16, 17). Animal immune response to infection is closely related to immunoglobulin levels. Humoral immunity could help the body prevent infections and diseases, which are mediated by the IgA, IgM, and IgG antibodies (18, 19). It has been found that polysaccharides of traditional Chinese medicine alleviated the decrease of serum IgG concentration in broilers treated with cyclophosphamide (20). Similarly, polysaccharides of traditional Chinese medicine significantly increased the values of immune organ indices and serum IgM and IgG in mice and rats (21, 22). The polysaccharides of traditional Chinese medicine injection increased the content of IgG, IgM, and IgA in weaned piglets (15). We found that adding TCM1 and TCM2 as feed additives in the finishing pigs increased IgA, IgG, and IgM levels compared to the Con group. The complement system restricts viral infections through both classical and alternative pathways (23, 24). They contain numerous small proteins that enhance the ability of antibodies and phagocytic cells to clear microbes and damaged cells in the blood (25, 26). In addition, they also can facilitate inflammation and attack cells infected with pathogens. C3, the body's most abundant complement, is the body's innate immune system central link. It can interact with at least 25 soluble or membrane-bound proteins to activate the complement system through classical pathways, bypass pathways, and lectin pathways (14). In our study, the C3 and C4 in the TCM1 and TCM2 groups were significantly higher than those in the Con group on day 30. The results indicated that TCM1 and TCM2 could enhance immune function in finishing pigs.

Vaccination is often conducted in the pig industry to prevent CSF and PRRS, but there are many factors that can affect its effectiveness. It was described that astragalus polysaccharide and oxymatrine can synergistically improve the immune efficacy of Newcastle disease vaccine in chicken (27). Additionally, Chinese herbal medicine additives can improve the level of antibody against classic swine fever (28). We found that adding TCM1 and TCM2 could increase the levels of antibody against CSFV and PRRSV compared to the Con group. These results indicated that TCM1 and TCM2 could enhance the immune response of finishing pigs by increasing the levels of CSFV and PRRSV antibodies.

In the past few years, a study has shown that many kinds of polysaccharides of TCM have immunomodulatory effects through the TLR4/MyD88/NF-κB signaling pathway

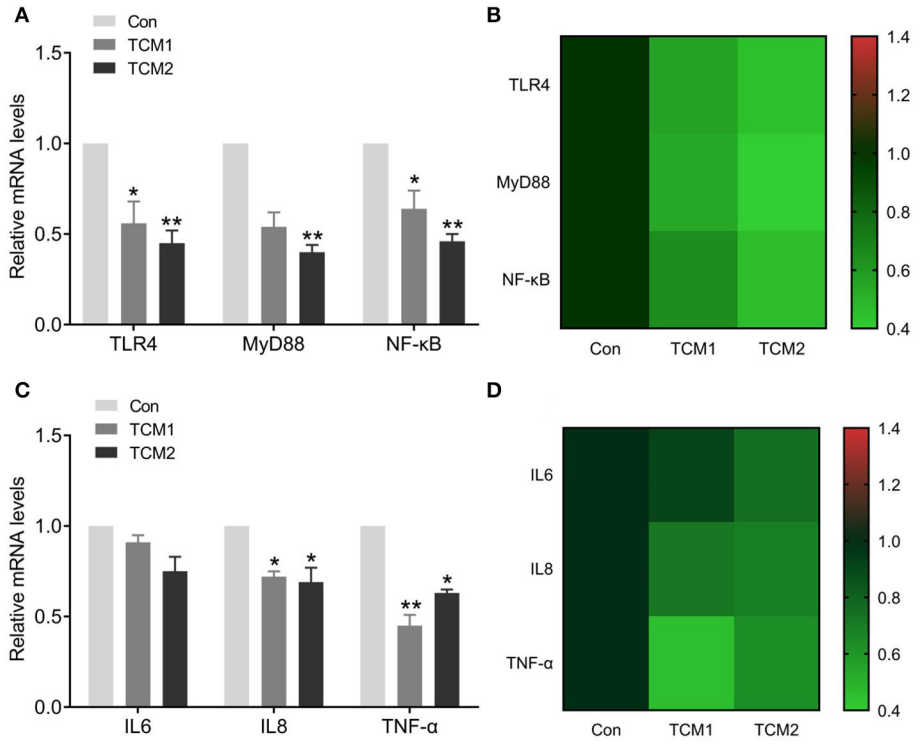


FIGURE 4
Effects of TCM supplement on mRNA levels of the TLR4/MyD88/NF-κB signaling pathway and inflammatory cytokine genes. **(A)** Effects of TCM supplement on mRNA levels of TLR4/MyD88/NF-κB pathway-related genes. **(B)** Heat map shows the mRNA levels of TLR4/MyD88/NF-κB signaling pathway-related genes. **(C)** Effects of TCM supplement on mRNA levels of inflammatory cytokine genes. **(D)** Heat map shows the mRNA levels of inflammatory cytokines genes. "*" indicates a significant difference compared with control group ($*P < 0.05$ and $**P < 0.01$).

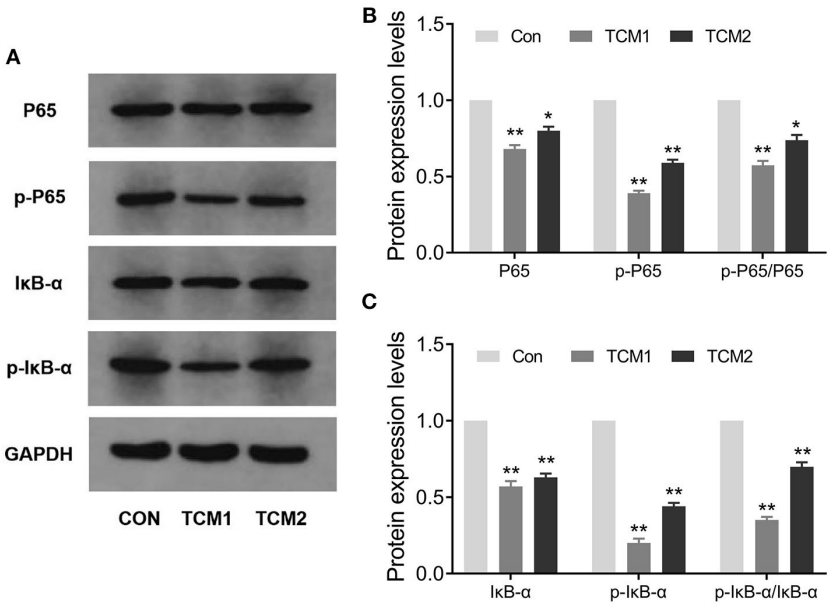


FIGURE 5
Effects of TCM supplement on key proteins levels of the NF-κB signaling pathway. **(A,B)** Immunoblot analysis of P65 and p-P65 proteins. **(C)** Immunoblot analysis of IκBα and p-IκBα proteins. "*" indicates a significant difference compared with control group ($*P < 0.05$ and $**P < 0.01$).

(29). TNF-α has the characteristics of a multifunctional pro-inflammatory cytokine with an important role in the pathogenesis of inflammatory diseases in this pathway (30). TNF-α activated phosphorylate IκB and induced its degradation, in parallel with leading to the liberation of NF-κB, and evoking the expression of a variety of genes, which participate in inflammatory responses (31). Our results showed that TCM1 and TCM2 could reduce the expression levels of genes (TLR-4, MyD88, NF-κB, TNF-α, IL6, and IL8) and proteins (IκBα, p-IκBα, p65, and p-p65) to different degrees. Furthermore, this activation was significantly inhibited by TCM1 and TCM2, suggesting that TCM1 and TCM2 exerted important effects resulting in reduced inflammation. Our results showed that TCM1 and TCM2 could act on the NF-κB signaling pathway and reduce the body's inflammatory response, thereby improving the immune performance.

Conclusion

In summary, the results showed that TCM1 and TCM2 had a significant inhibition of inflammation and low toxicity. These positive effects indicate that TCM1 and TCM2 can be dietary additives for animals to enhance humoral immunity.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by the Committee for the Care and Use of Experimental Animals, Jiangxi Agricultural University, Jiangxi, China. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

XW, JC, and FY contributed to conception and design of the study. YM, AH, and TX organized the database. FW, GZ, and YY performed the statistical analysis. HC wrote the first draft of the manuscript. XG, AH, and TX wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

The authors declare that this study received funding from Spirit Jinyu Biological Pharmaceutical Co. Ltd. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Zhou X, Zhang Y, Wu X, Wan D, Yin Y. Effects of dietary serine supplementation on intestinal integrity, inflammation and oxidative status in early-weaned piglets. *Cell Physiol Biochem*. (2018) 48:993–1002. doi: 10.1159/000491967

2. Wang L, Zhu F, Yang H, Li J, Li Y, Ding X, et al. Effects of dietary supplementation with epidermal growth factor on nutrient digestibility, intestinal development and expression of nutrient transporters in early-weaned piglets. *J Anim Physiol Anim Nutr*. (2019) 103:618–25. doi: 10.1111/jpn.13059

3. Qin L, Ji W, Wang J, Li B, Hu J, Wu X, et al. Effects of dietary supplementation with yeast glycoprotein on growth performance, intestinal mucosal morphology, immune response and colonic microbiota in weaned piglets. *Food Funct*. (2019) 10:2359–71. doi: 10.1039/C8FO02327A

4. Kamatou GPP, Makunga NP, Ramogola WPN, Viljoen AM. South African Salvia species: a review of biological activities and phytochemistry. *J Ethnopharmacol*. (2008) 119:664–72. doi: 10.1016/j.jep.2008.06.030

5. Zhao LM, Jia YL, Ma M, Duan YQ, Liu LH. Prevention effects of Schisandra polysaccharide on radiation-induced immune system dysfunction. *Int J Biol Macromol*. (2015) 76:63–9. doi: 10.1016/j.ijbiomac.2015.02.020

6. Shin HJ, Hwang KA, Choi KC. Antitumor effect of various phytochemicals on diverse types of thyroid cancers. *Nutrients*. (2019) 11:125. doi: 10.3390/nu11010125

7. Wang L, Zhou GB, Liu P, Song JH, Liang Y, Yan XJ, et al. Dissection of mechanisms of Chinese medicinal formula realgar-Indigo naturalis as an effective treatment for promyelocytic leukemia. *Proc Natl Acad Sci U S A*. (2008) 105:4826–31. doi: 10.1073/pnas.0712365105

8. Su Q, Li L, Sun Y, Yang H, Ye Z, Zhao J, et al. Effects of the TLR4/Myd88/NF-κB signaling pathway on NLRP3 inflammasome in coronary microembolization-induced myocardial injury. *Cell Physiol Biochem*. (2018) 47:1497–508. doi: 10.1159/000490866

9. Wang L, Li N, Lin D, Zang Y. Curcumin protects against hepatic ischemia/reperfusion induced injury through inhibiting TLR4/NF-κB pathway. *Oncotarget*. (2017) 8:65414–20. doi: 10.18632/oncotarget.18676

10. Lee SC, Wang SY, Li CC, Liu CT. Anti-inflammatory effect of cinnamaldehyde and linalool from the leaf essential oil of Cinnamomum osmophloeum Kanehira in endotoxin-induced mice. *J Food Drug Anal*. (2018) 26:211–20. doi: 10.1016/j.jfda.2017.03.006

11. Luo X, Huang S, Luo S, Liao H, Wang Y, Deng X, et al. Identification of genes underlying the enhancement of immunity by a formula of lentinan, pachymaran and tremelia polysaccharides in immunosuppressive mice. *Sci Rep*. (2018) 8:10082. doi: 10.1038/s41598-018-28414-w

12. Ali J, Khan AU, Shah FA, Ali H, Islam SU, Kim YS, et al. Mucoprotective effects of Saikosaponin-A in 5-fluorouracil-induced intestinal mucositis in mice model. *Life Sci*. (2019) 239:116888. doi: 10.1016/j.lfs.2019.116888

13. Li LL, Yin FG, Zhang B, Peng HZ, Li FN, Zhu NS, et al. Dietary supplementation with Atractylodes Macrophala Koidz polysaccharides ameliorate metabolic status and improve immune function in early-weaned pigs. *Livest Sci*. (2011) 142:33–41. doi: 10.1016/j.livsci.2011.06.013

14. National Research Council. *Nutrient Requirements of Swine*. Washington, DC: National Academy Press (2012).

15. Xie JH, Jin ML, Morris GA, Zha XQ, Xie M. Advances on bioactive polysaccharides from medicinal plants. *Crit Rev Food Sci Nutr*. (2016) 56(Suppl 1):S60. doi: 10.1080/10408398.2015.1069255

16. Kong XF, Wu GY, Liao YP, Hou ZP, Liu HJ, Yin FG, et al. Effects of Chinese herbal ultra-fine powder as a dietary additive on growth performance, serum metabolites and intestinal health in early-weaned piglets. *Livestock Sci*. (2007) 108:272–5. doi: 10.1016/j.livsci.2007.01.079

17. Kommera SK, Mateo RD, Neher FJ, Kim SW. Phytobiotics and organic acids as potential alternatives to the use of antibiotics in nursery pig diets. *Asian-Au J Animal Sci*. (2006) 19:1784–9. doi: 10.5713/ajas.2006.1784

18. Salmon H, Berri M, Gerdts V, Meurens F. Humoral and cellular factors of maternal immunity in swine. *Develop Compar Immunol*. (2009) 33:384–93. doi: 10.1016/j.dci.2008.07.007

19. Rahe MC, Murtaugh MP. Effector mechanisms of humoral immunity to porcine reproductive and respiratory syndrome virus. *Vet Immunol Immunopathol*. (2017) 186:15–8. doi: 10.1016/j.vetimm.2017.02.002

20. Li J, Li R, Li N, Zheng F, Dai Y, Ge Y, et al. Mechanism of antidiabetic and synergistic effects of ginseng polysaccharide and ginsenoside Rb1 on diabetic rat model. *J Pharm Biomed Anal*. (2018) 158:451–60. doi: 10.1016/j.jpba.2018.06.024

21. Rui L, Chen W, Wang W, Tian W, Zhang X. Extraction, characterization of Astragalus polysaccharides and its immune modulating activities in rats with gastric cancer. *Carbohyd Polym*. (2009) 78:738–42. doi: 10.1016/j.carbpol.2009.06.005

22. Meng F, Xu P, Wang X, Huang Y, Wu L, Chen Y, et al. Investigation on the immunomodulatory activities of Sarcodon imbricatus extracts in a cyclophosphamide (CTX)-induced immunosuppressanted mouse model. *Saudi Pharm J*. (2017) 25:460–3. doi: 10.1016/j.jsps.2017.04.006

23. Gialeli C, Gungor B, Blom AM. Novel potential inhibitors of complement system and their roles in complement regulation and beyond. *Mol Immunol*. (2018) 102:73–83. doi: 10.1016/j.molimm.2018.05.023

24. Moreno-Navarrete JM, Fernandez-Real JM. The complement system is dysfunctional in metabolic disease: Evidences in plasma and adipose tissue from obese and insulin resistant subjects. *Semin Cell Dev Biol*. (2019) 85:164–72. doi: 10.1016/j.semcdb.2017.10.025

25. Liao Z, Wan Q, Yuan G, Su J. The systematic identification and mRNA expression profiles post viral or bacterial challenge of complement system in grass carp Ctenopharyngodon idella. *Fish Shellfish Immunol*. (2019) 86:107–15. doi: 10.1016/j.fsi.2018.11.032

26. Zhao P, Wu J, Lu F, Peng X, Liu C, Zhou N, et al. The imbalance in the complement system and its possible physiological mechanisms in patients with lung cancer. *Bmc Cancer*. (2019) 19:201. doi: 10.1186/s12885-019-5422-x

27. Chen Y, Wang D, Hu Y, Guo Z, Wang J, Zhao X, et al. Astragalus polysaccharide and oxymatrine can synergistically improve the immune efficacy of Newcastle disease vaccine in chicken. *Int J Biol Macromol*. (2010) 46:428. doi: 10.1016/j.ijbiomac.2010.02.004

28. Fu-Zhuang LU, Wang ZG, Yuan FU, Shi TY, Hua WD, Zhang XJ, et al. Effects of Chinese herbal medicine additives on productive performances and levels of antibody against classical swine fever (CSF) for swine. *Acta Agriculturae Zhejiangensis*. (2011) 23:56.

29. Shan Y, Jiang B, Yu J, Wang J, Wang X, Li H, et al. Protective effect of Schisandra Chinesis polysaccharides against the immunological liver injury in mice based on Nrf2/ARE and TLR4/NF-kappaB signaling Pathway. *J Med Food*. (2019). doi: 10.1089/jmf.2018.4377

30. Du L, Li J, Zhang X, Wang L, Zhang W, Yang M, et al. Pomegranate peel polyphenols inhibits inflammation in LPS-induced RAW264, 7. macrophages via the suppression of TLR4/NF-kappaB pathway activation. *Food Nutr Res*. (2019) 63:3392. doi: 10.29219/fnr.v63.3392

31. Sun T, Dong W, Jiang G, Yang J, Liu J, Zhao L, et al. Cordyceps militaris improves chronic kidney disease by affecting TLR4/NF-kappaB Redox signaling pathway. *Oxid Med Cell Longev*. (2019) 2019:7850863. doi: 10.1155/2019/7850863

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 16 September 2022
DOI 10.3389/fvets.2022.993018

OPEN ACCESS

EDITED BY
Fazul Nabi,
Lasbela University of Agriculture, Water and Marine Sciences, Pakistan

REVIEWED BY
Cong Zhang,
Henan Agricultural University, China
Hui Zhang,
South China Agricultural University, China

*CORRESPONDENCE
Huabin Cao
chbin20020804@jxau.edu.cn

†These authors share first authorship

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 13 July 2022
ACCEPTED 26 August 2022
PUBLISHED 16 September 2022

CITATION
Chen J, Hu N, Mao Y, Hu A, Jiang W, Huang A, Wang Y, Meng P, Hu M, Yang X, Cao Y, Yang F and Cao H (2022) Traditional Chinese medicine prescriptions (XJZ, JSS) ameliorate spleen inflammatory response and antioxidant capacity by synergistically regulating NF-κB and Nrf2 signaling pathways in piglets.
Front. Vet. Sci. 9:993018.
doi: 10.3389/fvets.2022.993018

Traditional Chinese medicine prescriptions (XJZ, JSS) ameliorate spleen inflammatory response and antioxidant capacity by synergistically regulating NF-κB and Nrf2 signaling pathways in piglets

Jian Chen[1†], Nianqing Hu[1†], Yaqing Mao[2], Aiming Hu[3], Wenjuan Jiang[4], Aimin Huang[1], Yun Wang[5], Puyan Meng[6], Mingwen Hu[1], Xiaobin Yang[7], Yuandong Cao[8], Fan Yang[1] and Huabin Cao[1*]

[1]Jiangxi Provincial Key Laboratory for Animal Health, Institute of Animal Population Health, College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang, China, [2]MOA Center for Veterinary Drug Evaluation, China Institute of Veterinary Drug Control, Beijing, China, [3]Jian Animal Husbandry and Veterinary Bureau, Jian, China, [4]Animal Husbandry and Aquatic Products Technology Application Extension Office, Jiangxi Agricultural Technology Extension Center, Nanchang, China, [5]Jiangxi Vocational College of Technology, Nanchang, China, [6]Jiangxi Academy of Forestry, Nanchang, China, [7]Jiangxi Zhongchengren Pharmaceutical Co., Ltd., Nanchang, China, [8]Jiangxi Jiabo Biological Engineering Co., Ltd., Jiujiang, China

Weaning transition generally impairs the immune system, inducing immune disturbance, which may be associated with post-weaning diarrhea and high mortality in piglets. The spleen is a pivotal lymphatic organ that plays a key role in the establishment of the immune system. Traditional Chinese medicine (TCM) prescriptions, XiaoJianZhong (XJZ) and Jiansananli-sepsis (JSS), are widely used prescriptions for treating spleen damage and diarrhea. Here, we hypothesized that XJZ and JSS maintain the spleen physiological function by ameliorating antioxidant capacity and inflammatory response in weaned piglets. In this study, 18 weaned piglets were assigned to the Control, XJZ and JSS groups. By hematoxylin and eosin staining, hematological analysis, flow cytometric analysis, qRT-PCR and western blot, the effects of both TCM prescriptions on the spleen antioxidant defense system and inflammatory pathway were explored. Results showed that both TCM treatment significantly ameliorated the weaning-induced morphological damage in piglets, as evidenced by clearer and more perfect spleen histology, as well as higher relative area of white pulp. Meanwhile, both XJZ and JSS exerted better blood parameters, as supported by the changes of monocyte level and lymphocyte subpopulations CD4+/CD8+ ratio. Furthermore, the levels of inflammatory markers, IL1β, IL6, IL8, and TNF-α in the spleen were markedly decreased after supplemented with both TCM prescriptions. Importantly, the inhibition of nuclear factor-kappaB (NF-κB) and its downstream effector genes (IL6, IL8, and TNF-α) in both XJZ and JSS treatment groups further confirmed alleviation of

inflammatory responses in the spleen. In addition, both XJZ and JSS enhanced the antioxidant capacity of the spleen by activating the nuclear factor erythroid 2-related factor 2 (Nrf2)-activated antioxidant defense system. Notably, the results of PCA and network correlation analysis indicated that XJZ and JSS treatment altered the expression profiles of inflammatory and antioxidant-related factors in the spleen of weaned-piglets, which may involve the synergy of NF-κB and Nrf2 signaling pathways. In summary, our study showed that TCM prescriptions, XJZ and JSS could ameliorate inflammatory response and antioxidant capacity in the spleen by synergistically regulating NF-κB and Nrf2 signaling pathways in piglets.

KEYWORDS

weaning stress, spleen function, traditional Chinese medicine, antioxidant capacity, anti-inflammatory

Introduction

Weaning stress could impair immune system development and intestinal barrier function in piglets, resulting in growth inhibition, secondary infection, diarrhea and even death (1). Meanwhile, due to its weak immune and digestive system in weaned piglets, once the potential diseases are induced by stress, it will not only cause economic losses, but also damage animal welfare (2). Diarrhea and stress-induced secondary bacterial or viral infections are leading causes of mortality in weaned piglets. Hence, improving the immunity of piglets may be a feasible method to against weaning stress (3, 4). In the past decades, antibiotic, as a feed additive, has been employed in increasing daily gain and preventing disease of piglets or other economical animals (5, 6). Likewise, it also plays an important role in bacterial diarrhea control (6). However, the abuse of antibiotics has led to the multi-antibiotic and resistance antibiotic residue (7, 8). Many countries or regions, such as the United States, China, and European Union, have clearly stipulated that the use of antibiotics as feed additives is prohibited. Crucially, the antibiotics cannot fundamentally ameliorate the immune state of piglets, but affect the colonization and proliferation of normal or beneficial gut microflora (9). Therefore, it is imperative to find an alternative medicine with low toxicity, few side effects, and high efficiency.

Traditional Chinese medicine (TCM), derived from natural plants, has been shown to exert immune-potentiating efficacy (10). It has almost no side effects, which effectively solves the adverseness caused by antibiotics application. Importantly, multiple active compounds in TCM prescription could hit various targets and exert synergistic therapeutic efficacy (11). As classic TCM prescriptions, XiaoJianZhong (XJZ) and Jiansananli-sepsis (JSS) prescriptions have been used in China and Asian countries for hundreds of years, and they have been proven to boost immunity and treat diarrhea. However, the mechanism is still not fully understood. Previous study showed that both XJZ and JSS could increase the growth performance and control diarrhea induced by weaning in piglets (9, 12, 13). Here, we speculated that the alleviation of weaning-induced growth inhibition and diarrhea by XJZ and JSS is related to the improved spleen development in piglets. It is well-known that the spleen, as largest secondary immune organ, is responsible for initiating immune response to blood-borne antigens in the body, and filtering non-self-substances and damaged cells from the blood (14). Meanwhile, the spleen is a storage organ for T lymphocytes (Lym), which provides protection against pathogens and tissue damage (15). Maintenance of normal spleen function is extremely important for growth and development of young animal as it is involved in the maturation of digestive and immune processes (16). However, the spleen is sensitive to stress, inducing inflammatory responses and oxidative damage. It is well-known that nuclear factor-kappaB (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2) are transcription factors that regulate genes responsible for inflammatory and anti-oxidant response, respectively. NF-κB signaling and its subsequent pro-inflammatory cytokine expression, oxidative stress, apoptotic and anti-apoptotic genes play a critical role in stress-induced spleen injury. Recent studies have shown that weaning-induced inflammatory response and oxidative stress are the main causes of spleen damage in piglets (17, 18). Therefore, the main purpose of this study was to investigate the effects of TCM prescription, XJZ and JSS on spleen morphology, inflammatory response and antioxidant defense system in weaned piglets to test our above hypothesis.

Our results found that both XJZ and JSS could ameliorate blood parameters, relieve spleen morphological damage, elevate the peripheral blood Lym subpopulations CD4+/CD8+ ratio, alleviate inflammation and improve antioxidant capacity in the spleen of weaned-piglets by synergistically regulating NF-κB and Nrf2 signaling pathways.

Materials and methods

Animals, experimental design and rations

The experiment was performed at JiangXi Agricultural University's Researching and Teaching Base lasted 60 days. All experimental protocols were approved by the Committee for the Care and Use of Experimental Animals, Jiangxi Agricultural University, Jiangxi, China. Eighteen weaned-piglets (17.37 ± 1.32 kg, Large White × Landrace × Duroc) at 40 days of age were randomly divided into three groups ($n = 6$) according to weight and sex. The three experimental groups were fed with the same basic diet, and it was designed to meet the nutritional level of the piglets ((19), Supplementary Table 1). Experimental groups included: (I) Control group (basal diet), (II) XJZ group (basal diet plus 10 g/kg XJZ prescription), or (III) JSS group (basal diet plus 3 g/kg JSS prescription). The doses for TCM groups (XJZ and JSS) were determined based on our preliminary tests and previous study (9). All raw materials for XJZ and JSS prescriptions were provided by The Spirit Jinyu Biological Pharmaceutical Co., Ltd (Huhhot, Inner Mongolia, China). All dried Chinese herbs were smashed through a 2.5 mm screen sieve. Composition and main active constituents of XJZ and JSS are presented in Table 1.

TABLE 1 Composition and main active constituents of XJZ and JSS (air-dried basis)[a].

Scientific name	Main active ingredients	Proportion (%)
XJZ prescription		
Cassia twig	Cinnamaldehyde	13
Glycyrrhiza uralensis	Glycyrrhizin	4
Ziziphus zizyphus	*Jujuba* polysaccharide	4
Cynanchum otophyllum	Paeoniflorin	13
Zingiber officinale Roscoe	Ginger oleoresin	6
Rhizoma atractylodes	Atractylodine	14
Atractylodes macrocephala	Biatractylolide	10.5
Poria cocos	Pachymaran	10.5
Coptis chinensis Franch	Berberine	4
Maltose	Maltose	21
Total		100
JSS prescription		
Nepeta cataria L.	*Nepeta cataria* oil	16.5
Radix saposhnikoviae	Chromone glycoside	16.5
Notopterygium incisum	Notopterol	16.5
Radix angelicae pubescentis	Heraclenin	16.5
Radix bupleuri	Saikosaponin	10
Radix peucedani	Peucedanin	10
Poria cocos	Pachymaran	10
Glycyrrhiza uralensis	Glycyrrhizin	4
Total		100

[a]Main active constituents of TCM come from Chinese pharmacopeia (2005).

Sample collection

Blood samples were collected from the jugular vein using EDTA tubes for hematological analysis and flow cytometry. Piglets were euthanized by intravenous injection of sodium pentobarbital 40 mg/kg·BW. The abdominal cavity was then opened, and the spleen tissue was immediately harvested. The spleen index was calculated following the formula: spleen index = weight of spleen (g)/body weight (kg). Small pieces of spleen tissue were fixed in 4% formaldehyde solution until hematoxylin and eosin (H&E) staining. All remnant spleen tissues were kept frozen at −80°C until needed.

H&E staining

The spleen tissues were taken from 4% formaldehyde solution and embedded in paraffin. The paraffin sections were then deparaffinized in xylene and rehydrated in a high-ethanol-to-water gradient (from 70 to 100%). Following that, sections were stained with hematoxylin and differentiated with 1% HCl in 70% alcohol. The sections were stained with eosin after being rinsed with tap water, then dehydrated and differentiated as described above. Finally, the sections were washed with xylene and mounted with neutral resin. To observe the histopathological changes of the spleen, the sections were examined under a light microscope. The spleen damage score was performed using a blinded fashion method according to previous studies (20, 21). The scoring rules are as follows: normal structure (0 point), granule denaturation (1 point); vacuolar degeneration (2 points), and apoptotic or necrosis (3 points).

Hematological analysis

Hematology was performed in Ethylene Diamine Tetraacetic Acid (EDTA) full blood by automatic whole blood count (BC-5000 Vet, Mindray, China). The levels of white blood cells (WBC), Lym, monocytes (Mon), neutrophil (Neu), eosnophils (Eos) and basophil (Bas), red blood cell (RBC), hemoglobin (HGB), hematokrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), and platelets (PLT) were analyzed.

Flow cytometric analysis

Peripheral blood single cell suspensions of lymphocytes and flow cytometric were performed as described in previous studies

(22, 23). For T-cell phenotyping, blood samples were double-stained with monoclonal antibodies for CD4 (PE-Cy™7 Mouse Anti-Pig CD4, BD, USA) and CD8 (APC Mouse Anti-Pig CD8, BD, USA) or the corresponding isotype controls. The ratio CD4+/CD8+ was calculated based on the relative percentage of CD4+ and CD8+.

ELISA kit assay

The levels of inflammatory markers in the spleen were measured using the Interleukin (IL)-1β (Beijing Chenglin Biological Technology Co., LTD., China, AD0125Po), Porcine IL-6 (Beijing Chenglin Biological Technology Co., LTD., China, AD0120Po), Porcine IL-8 (Beijing Chenglin Biological Technology Co., LTD., China, AD0056Po), and tumor necrosis factor alpha (TNF-α, Beijing Chenglin Biological Technology Co., LTD., China, AD0070Po) ELISA assay kits according to the manufacturer's protocol, respectively.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the mRNA expression of inflammation and antioxidant-related genes. Briefly, the total RNA was isolated using the TransZol Reagent (TransGen Biotech, Beijing, China). The complementary DNA (cDNA) was synthesized using a *TransScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix reagent kit (TransGen Biotech, Beijing, China) according to the kit's instructions. Then,

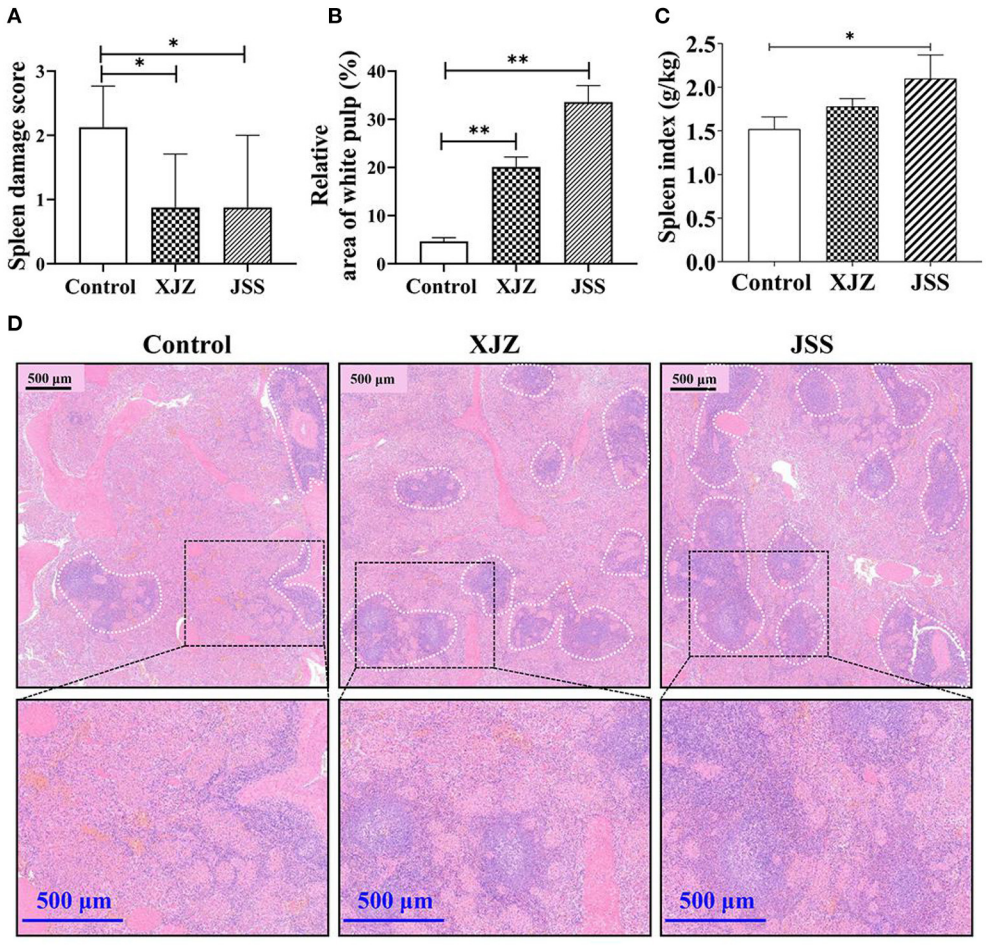


FIGURE 1
Effect of XJZ and JSS prescriptions on spleen health in weaned piglets. *$P < 0.05$ and **$P < 0.01$ vs. the control group. Data are expressed as means ± SD. **(A)** Spleen damage score. Blind scoring based on HE-stained sections. **(B)** Relative area of white pulp in the spleen. **(C)** Spleen index. Ratio of spleen weight to animal weight. **(D)** H&E staining imaging of spleen tissues in weaned-piglets. The white dotted line marks the white pulp area of the spleen.

it was stored at −20°C for SYBR Green qRT-PCR. All primer sequences were designed by OLIGO7 and presented in Supplementary Table 2. Relative gene expression levels were assessed using the $2^{-\Delta\Delta CT}$ method and normalized to the housekeeping genes GAPDH and β-actin.

Western blot

Total protein of spleen was extracted with RIPA Lysis buffer. The details of Western Blot were consistent with our previous (9). The blots were semi-quantified using Image J software. The primary antibodies for NF-κB p65 (Cell Signaling, 6956, 1:1,000), phospho-NF-κB p65 (p-NF-κB p65, Cell Signaling, 3033, 1:1,000), inhibitor of NF-κBα (IκB-α, Cell Signaling, 4814, 1:1,000), phospho-IκB-α (p-IκB-α, Cell Signaling, 9246, 1:1,000), Nrf2 (Proteintech Group, 16396-1-AP, 1:1,000), Heme oxygenase-1 (HO-1, Proteintech Group, 27282-1-AP, 1:1,000) and superoxide dismutase 2 (SOD2, Abclonal, A19576, 1:1,500), and the HRP-conjugated secondary antibody (Bioss, bs-40295G-HRP, 1:3,000) were used in this study.

Statistical analysis

All data in this study were presented as the mean ± standard deviation (SD). One-way analysis of variance was used for the statistical analysis, and then Tukey's multiple comparison test was performed with SPSS software (SPSS Inc., Chicago, IL, USA). Principal component analysis (PCA) and network analysis and correlation analysis were performed using WEKEMO Bioincloud online software (https://www.bioincloud.tech/). The statistical difference was considered significant at $P < 0.05$ and highly significant at $P < 0.01$, respectively.

Results

XJZ and JSS prescriptions ameliorated spleen morphological damage in piglets

To confirm the potential ameliorative effects of XJZ and JSS on spleen microstructure, H&E staining was performed (Figure 1). As shown in Figure 1D, in both XJZ and JSS groups, the splenocytes were tightly and adequately arranged with distinct nuclei and boundary between the red and white medulla, whereas its boundary in the control group were blurred. Notably (Figure 1A), the spleen damage score was significantly reduced in both TCM groups ($P < 0.05$). Surprisingly, the relative area of white pulp was markedly elevated after XJZ and JSS treatment ($P < 0.01$). Consistent with this result, there a marked increase of spleen index was observed after JSS treatment ($P < 0.05$). These results indicated that TCM prescriptions, XJZ and JSS, could ameliorate the histological structure of spleen and alleviated its morphological damage induced by weaning in piglets.

XJZ and JSS prescriptions increased monocytes numbers in peripheral blood

To evaluate the effect of TCM on blood parameters, a blood routine test was performed on the peripheral blood of weaned piglets. As presented in Table 2, all blood parameters in the Control, XJZ and JSS groups were within the physiological range. Compared to the Control group, supplementation with XJZ and JSS was no significant ($P > 0.05$) difference on WBC, Lym, Neu, Eos, Bas, RBC, HGB, HCT, MCV, MCH, MCHC, and PLT. The levels of Mon in the control group were the lowest, which was significantly lower than those in the XJZ and JSS group ($P < 0.05$). These results suggested that the supplementation with XJZ and JSS is safe for weaned piglets at the doses used in this study and they have the potential to improve immunity.

TABLE 2 Effects of XJZ and JSS prescriptions supplement on hematological variables of piglets.

Items	Groups		
	Control	XJZ	JSS
WBC, 10^9/L	16.01 ± 2.38	15.89 ± 2.53	17.57 ± 2.10
Lym, 10^9/L	9.92 ± 1.65	9.57 ± 1.26	10.42 ± 1.38
Lym (%)	58.64 ± 10.11	61.99 ± 7.96	61.18 ± 5.33
Mon, 10^9/L	1.06 ± 0.48	1.25 ± 0.50	1.18 ± 0.18
Mon (%)	5.6 ± 1.13	6.99 ± 1.36*	6.73 ± 1.07*
Neu, 10^9/L	5.91 ± 1.83	5.98 ± 1.43	6.02 ± 1.57
Neu (%)	33.34 ± 5.95	36.54 ± 9.03	32.43 ± 7.15
Eos, 10^9/L	0.48 ± 0.16	0.48 ± 0.21	0.58 ± 0.17
Eos (%)	2.71 ± 0.78	2.67 ± 1.42	3.38 ± 0.89
Bas, 10^9/L	0.14 ± 0.03	0.18 ± 0.05	0.15 ± 0.03
Bas (%)	0.80 ± 0.10	0.95 ± 0.09	0.87 ± 0.22
RBC, 10^{12}/L	5.79 ± 0.35	5.87 ± 0.43	5.94 ± 0.43
HGB, g/L	108.00 ± 8.44	101.30 ± 6.58	106.90 ± 9.35
HCT (%)	31.86 ± 2.50	30.44 ± 1.71	31.73 ± 2.69
MCV (fl)	55.03 ± 2.32	54.01 ± 2.95	53.39 ± 2.30
MCH (pg)	18.67 ± 0.84	17.40 ± 0.93	17.83 ± 1.02
MCHC (g/L)	339.33 ± 6.81	332.30 ± 6.43	337.10 ± 5.04
PLT, 10^{12}/L	351.50 ± 39.44	393.87 ± 75.69	326.00 ± 87.46

WBC, white blood cells; Lym, lymphocyte; Mon, monocytes; Neu, neutrophil; Eos, eosnophils; Bas, baosphil; RBC, red blood cell; HGB, hemoglobin; HCT, hematokrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; PLT, platelets. Results are expressed as mean ± SD, one-way ANOVA, n = 6. *P < 0.05 vs. the control group.

XJZ and JSS prescriptions improved the Lym subpopulations CD4+/CD8+ ratio

To explore the effect of TCM on immune performance, we analyzed the levels of Lym subpopulations CD4+ and CD8+. As shown in Figures 2A,B, there was no apparently difference in the relative proportions of CD4+ and CD8+ subpopulations ($P > 0.05$). Surprisingly, both XJZ and JSS significantly increased the ratio of CD4+/CD8+ ($P < 0.05$). These results showed that both TCM treatments could improve immune performance by increasing the ratio of CD4+/CD8+.

XJZ and JSS prescriptions reduced the levels of inflammatory marker in the spleen

Activation of inflammatory response is a hallmark of stress-induced spleen damage in piglets. As shown in Table 3, both TCM prescriptions obviously reduced the inflammatory markers level of IL1β ($P < 0.01$), IL6 ($P < 0.01$), IL8 ($P < 0.01$ or $P < 0.05$), and TNF-α ($P < 0.01$). Hence, there is no doubt that XJZ and JSS prescriptions have anti-inflammatory potential.

XJZ and JSS prescriptions inhibited NF-κB signaling pathway

NF-κB is a key regulator of inflammation and it is involved in both innate and adaptive immune responses. Our results showed that both TCM treatments conspicuously suppressed the mRNA expression of pattern recognition receptor (Toll-like Receptor 4, TLR4, $P < 0.01$, Figure 3A) and adapter protein (myeloid differentiation primary response 88, Myd88, $P < 0.01$ or $P < 0.05$, Figure 3B). Likewise, the NF-κB transcription level also significantly decreased as expected after XJZ ($P < 0.05$) and JSS ($P < 0.01$) treatment (Figure 3C). Moreover, the mRNA levels of NF-κB downstream pro-inflammatory factors, IL-6 ($P < 0.05$ or $P < 0.01$) and IL-8 ($P < 0.01$) were also prominently decreased (Figures 3D–F). At the protein level, there was no significant change in the total protein level of NF-κB in the XJZ and JSS groups (Figure 4A). As an inhibitor of NF-κB, the levels of IκB-α were markedly increased ($P < 0.05$) after XJZ treatment (Figure 4B). Importantly, the protein levels of p-NF-κB (Figure 4C) and p-IκB-α (Figure 4D) were notably decreased in the spleen after XJZ and JSS supplementation ($P < 0.01$). Furthermore, both TCM prescriptions, XJZ and JSS, remarkably reduced the ratio of p-NF-κB/NF-κB and p-IκB-α/IκB-α ($P < 0.01$, Figure 4E). These results revealed that XJZ and JSS could relieve inflammatory response of spleen in weaned piglets *via* TLR4-Myd88-NF-κB dependent pathway.

XJZ and JSS prescriptions activated antioxidant system of spleen in weaned-piglets

As a therapeutic target for many diseases and damage, Nrf2 is a switch on the endogenous antioxidant defense system. As shown in Figures 5A–D, the protein levels of Nrf2 ($P < 0.01$) and antioxidases (HO-1, $P < 0.05$ or $P < 0.01$ and SOD 2, $P < 0.01$) were significantly upregulated in both TCM groups. Likewise, both XJZ and JSS also markedly increased the gene expression of Nrf2 ($P < 0.05$, Figure 5E) HO-1 ($P < 0.05$ or $P < 0.01$, Figure 5F) NQO1 ($P < 0.05$, Figure 5G) and SOD-1 ($P < 0.05$ or $P < 0.01$, Figure 5H). These results indicated that XJZ and JSS prescriptions could promote the spleen antioxidant capacity in weaned piglets by stimulating Nrf2 antioxidant defense response.

XJZ and JSS prescriptions alleviated spleen damage *via* crosstalk of NF-κB and Nrf2

To determine the role of NF-κB and Nrf2 signaling pathways in TCM prescription ameliorating spleen damage, the PCA, network and correlation analysis were applied. As shown in Figure 6A, there was no overlap among these groups, indicating that the expression profiles of inflammation-related genes and antioxidant-related factors were significantly changed after XJZ and JSS treatment. Network analysis (Figure 6B) showed that the damage score and SOD2 were located at the center, suggesting that they are associated with the expression of multiple factors. It is worth emphasizing that there was a markedly ($P < 0.05$ or $P < 0.01$) positive correlation between the damage score and the levels of p-NF-κB/NF-κB, p-IκB-α/IκB-α, IL-1β, and IL-6. Moreover, an obvious negative correlation was observed between the damage score and the expression of HO-1, and SOD2 ($P < 0.05$ or $P < 0.01$). Of note, correlation analysis revealed that there was a crosstalk between inflammatory signaling pathway and antioxidant defense system, (Figure 6C) as reflected by a significant ($P < 0.05$ or $P < 0.01$) correlation between antioxidant molecules (Nrf2, HO-1, SOD2) and NF-κB signaling pathway (TLR4, Myd88, p-NF-κB, p-IκB-α, p-NF-κB/NF-κB, p-IκB-α/IκB-α, IL-1β, IL-6, IL-8, and TNF-α). These results suggested that XJZ and JSS intervention may alleviate spleen injury in weaned piglets *via* the crosstalk of NF-κB-mediated inflammatory signaling pathway and Nrf2-mediated antioxidant defense system.

Discussion

For thousands of years, TCM prescription has contributed to human health in China and Asian countries. The compatibility

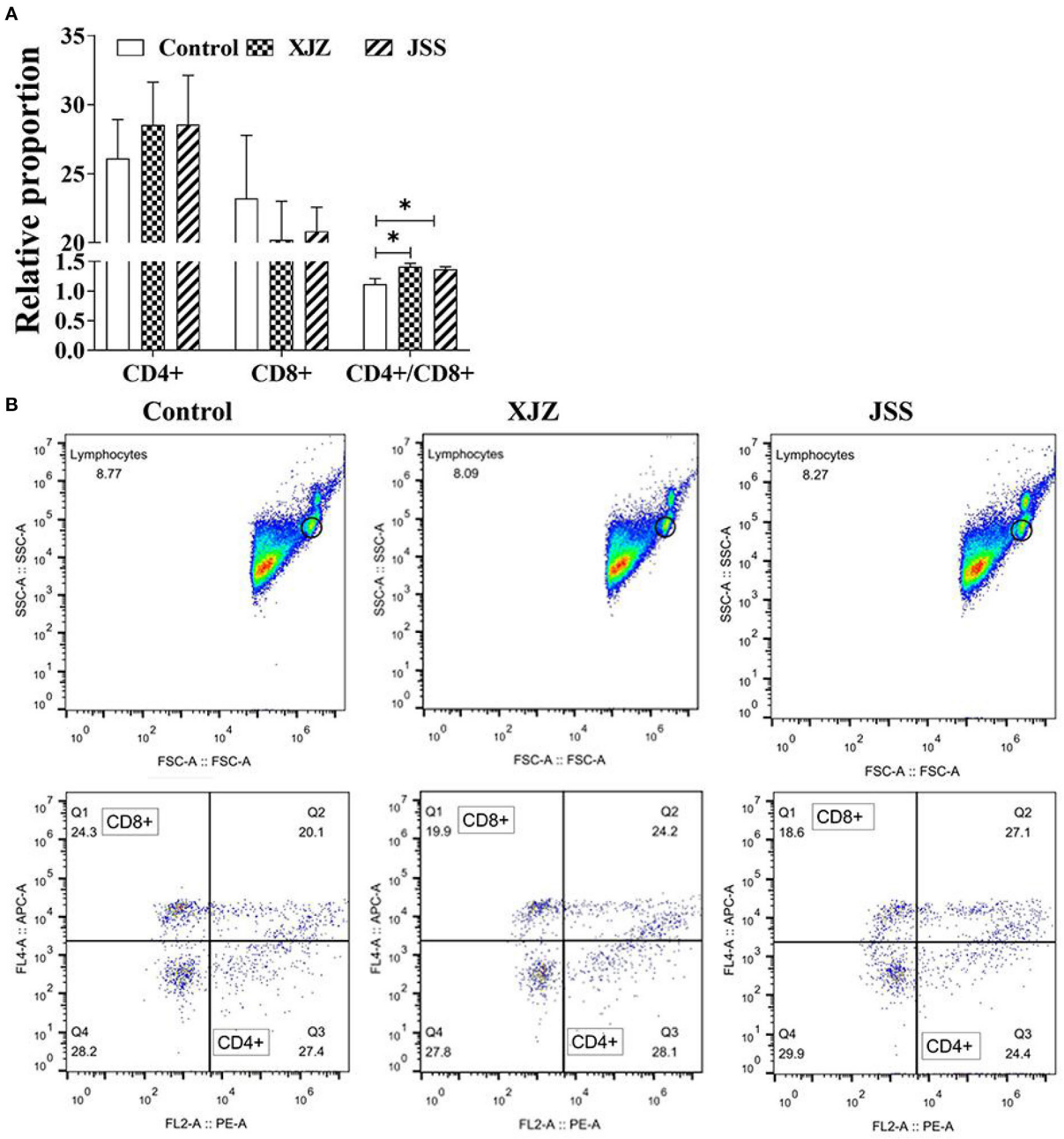


FIGURE 2
Effects of XJZ and JSS prescriptions on proportion of CD4+ and CD8+ subpopulations in peripheral blood of piglets. *$P < 0.05$ vs. the control group. Data are expressed as means ± SD. **(A)** Statistical analysis of Lymphocyte Subpopulations CD4+ and CD8+. **(B)** Flow cytometry analysis of CD4+ and CD8+.

of prescription drugs is strictly in accordance with the theory of TCM. It is well-known that most of TCM are derived from natural plants or minerals, and the pharmaceutical activities and therapeutic effects of these natural ingredients have been proven and witnessed in long-term clinical applications (24). XJZ and JSS prescriptions have been used in China for hundreds of years, mainly for the treatment of organ function injury and body fatigue. The main ingredients of the XJZ prescription include *Cassia Twig* (13%), *Cynanchum otophyllum* (13%), *Rhizoma Atractylodes* (14%), *Atractylodes macrocephala* (10.5%), *Poria cocos* (10.5%), Maltose (21%). Among these materials, the main effect of maltose is to improve

the palatability of the prescription, and *Cassia Twig* (25), *Cynanchum otophyllum* (26), *Rhizoma Atractylodes* (27) have been shown to have anti-inflammatory and antiviral properties, and extracts of *Atractylodes macrocephala* (28), *Poria cocos* (29) exert antioxidant properties. In JSS prescription, the main components, *Nepeta cataria L.*, *Radix Saposhnikoviae*, *Notopterygium incisum*, *and Radix Angelicae pubescentis* have an extensive range of biological activities, including antimicrobial, antioxidant and anti-inflammatory, as well as anti-ulcer and insecticidal properties (13, 30, 31). Due to the complex pathogenic factors and various diseases caused by weaning stress, the clinical effect of monotherapy may not meet expectations (1). In addition, Western medicine is increasingly inclined to use multiple drugs to synergistically treat some multi-organ damage diseases and immunosuppressive diseases, such as AIDS (32). Weaning stress induces oxidative damage (33), diarrhea (9), multi-organ inflammation (34), and reduced immune function in piglets. Therefore, the use of TCM treatment has unparalleled advantages.

The spleen is an immune organ innervated by sympathetic nerves, which together with the adrenomedullary system control splenic immune functions (35). In addition, as an important lymph organ, the spleen is the center of cellular immunity and humoral immunity, which can exert anti-inflammatory and antiviral effects by regulating lymphoid tissues and immune-related cytokines (36). Meanwhile, the structure and function of the spleen is comprehensive and is involved in digestion, absorption, energy conversion, and immune regulation. Based on the above concept, for weaned-piglets, with immature digestive and immune systems, maintaining or improving the spleen structure and function is critical. Study indicated that the immune state of organisms can be reflected by the spleen index (37, 38). In our study, JSS notably elevated the spleen index of piglets, meanwhile, both XJZ and JSS groups ameliorated the microstructure of spleen, and alleviated the morphological damage induced by weaning stress. Notably, the relative area of white pulp was increased after supplementation of XJZ and JSS. The white pulp and the red pulp are two distinct parts of the spleen's anatomy. Lymphoid tissue, mostly lymphocytes (T cells and B cells) and macrophages, make up the white pulp. Thus, the promotion in the white pulp and spleen index suggested an increase in the immune function of the spleen. In line with Lee et al. (22) reported that exposure of spleen cells to a turmeric

TABLE 3 Effect of XJZ and JSS prescriptions on spleen inflammatory markers of weaned piglets.

Item	Con	XJZ	JSS
IL-1β, ng/L	88.32 ± 4.98	74.33 ± 5.54**	76.05 ± 4.83**
IL-6, ng/L	45.74 ± 3.69	36.88 ± 2.85**	36.89 ± 2.63**
IL-8, ng/L	71.10 ± 4.29	58.55 ± 3.45**	65.34 ± 3.51*
TNF-α, ng/L	79.25 ± 5.82	65.44 ± 5.16**	66.42 ± 4.88**

IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrosis factor-alpha. Results are expressed as mean ± SD, one-way ANOVA, n = 6. *P < 0.05 and **P < 0.01 vs. the control group.

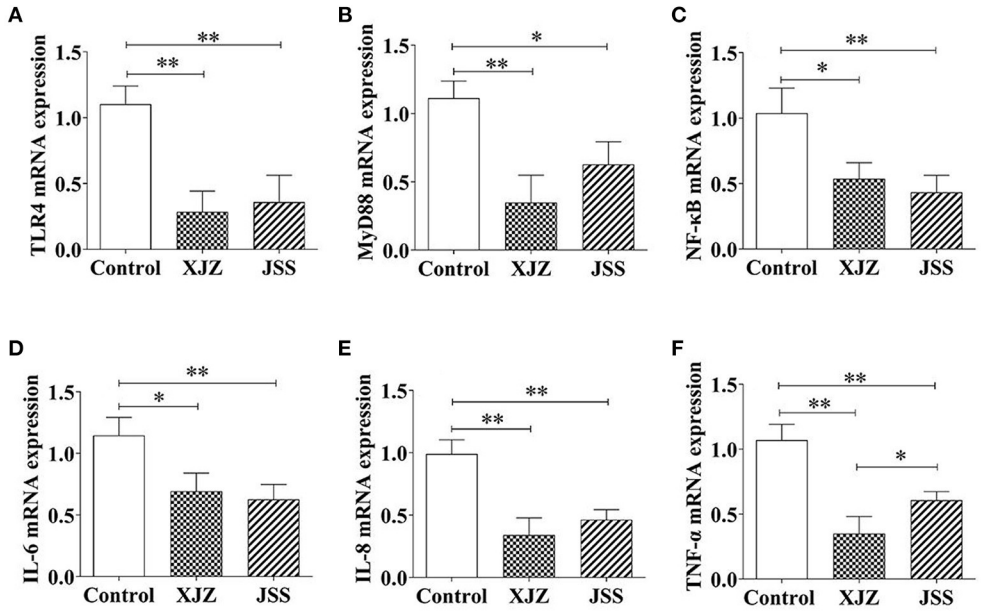


FIGURE 3
Effects of XJZ and JSS prescriptions on mRNA levels of NF-κB signaling pathway in the spleen of weaned-piglets. *$P < 0.05$ and **$P < 0.01$ vs. the control group. Data are expressed as means ± SD. **(A)** TLR4, **(B)** MyD88, **(C)** NF-κB, **(D)** IL-6, **(E)** IL-8, and **(F)** TNF-α.

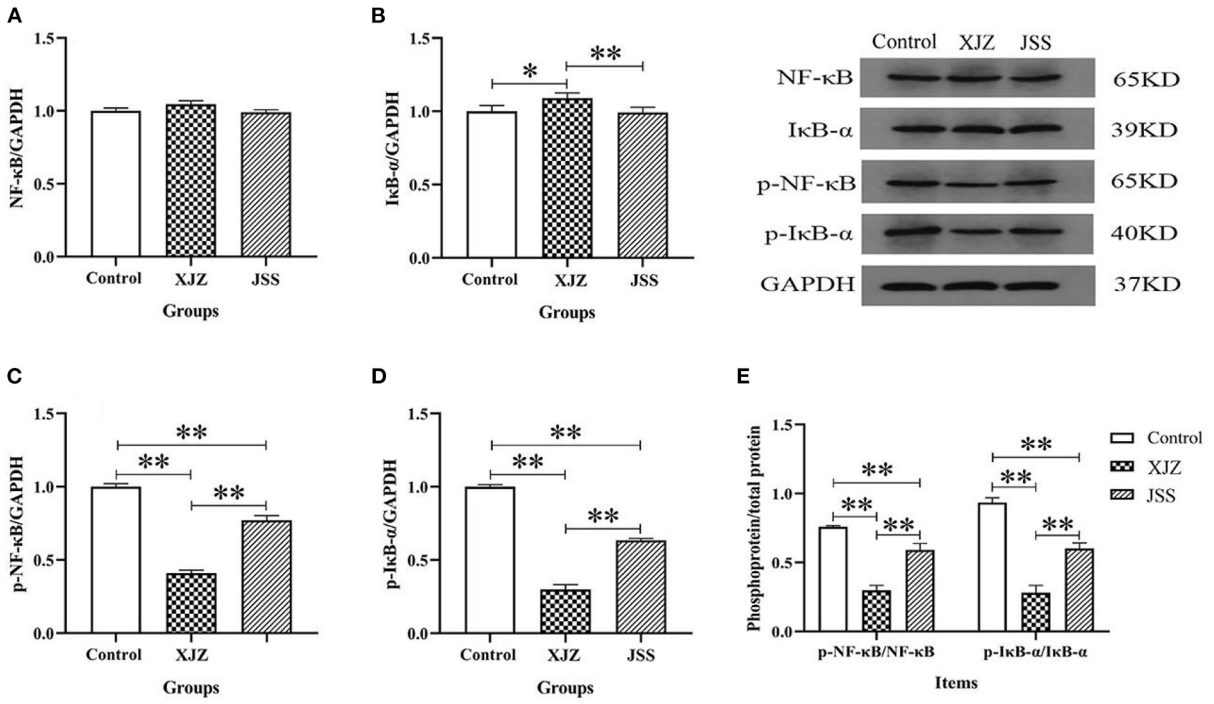


FIGURE 4
Effect of both XJZ and JSS prescriptions on protein level of NF-κB signaling pathway in the spleen of weaned-piglets. *$P < 0.05$ and **$P < 0.01$ vs. the control group. Data are expressed as means ± SD. **(A)** NF-κB/GAPDH, **(B)** IκB-α/GAPDH, **(C)** p-NF-κB/GAPDH, **(D)** p-IκB-α/GAPDH, and **(E)** Phosphoprotein/total protein.

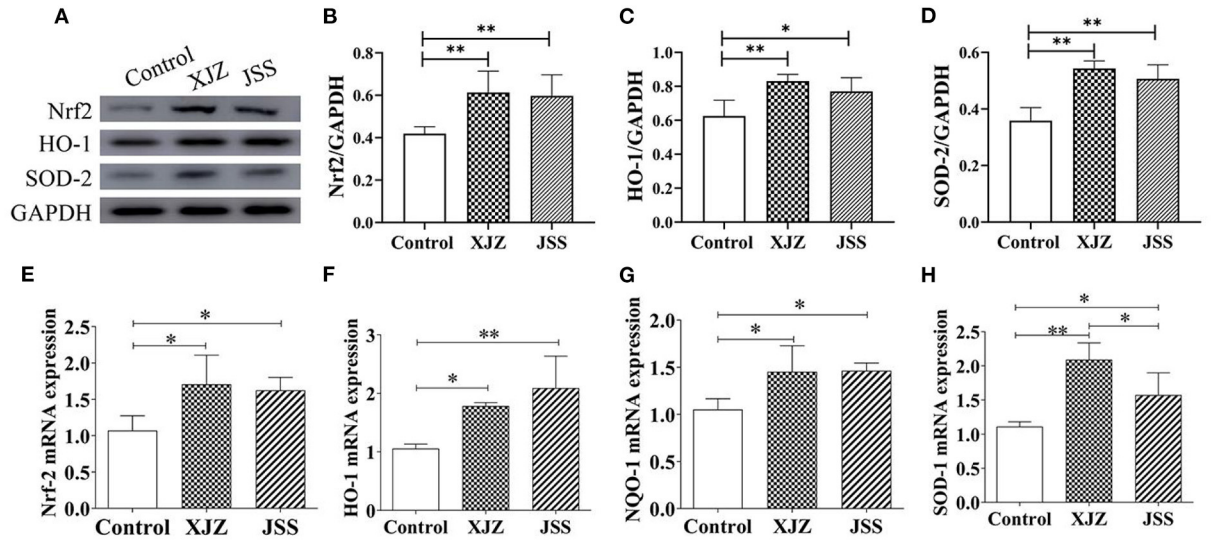


FIGURE 5
Effects of XJZ and JSS prescriptions on antioxidant defense system of spleen in weaned piglets. *$P < 0.05$ and **$P < 0.01$ vs. the control group. Data are expressed as means ± SD. **(A)** Bands of Nrf2, HO-1, SOD-2. **(B)** Nrf2/GAPDH. **(C)** HO-1/GAPDH. **(D)** SOD-2/GAPDH. **(E)** Nrf2 mRNA expression. **(F)** HO-1 mRNA expression. **(G)** NQO-1 mRNA expression. **(H)** SOD-1 mRNA expression.

extract (zingiberaceae, similar to *zingiber officinale* roscoe) *in vitro* elevated the proliferation of lymphocyte. Speculatively, the promotion of lymphocyte proliferation by certain active ingredients can be linked to the increase in the region of white pulp in the spleen. Importantly, the results of peripheral blood Mon proportion and CD4+/CD8+ ratio of T-lymphocytes

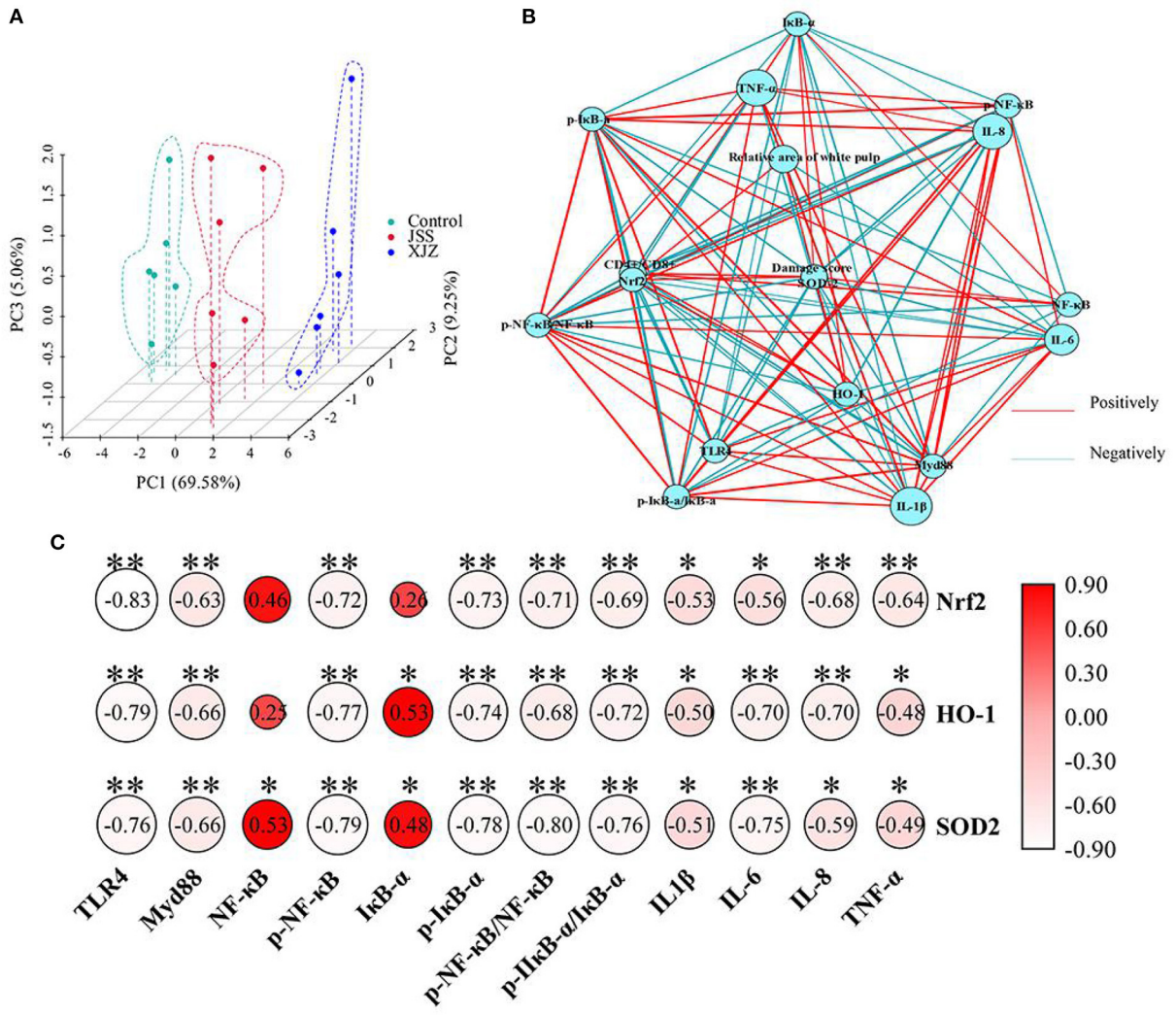


FIGURE 6
Effects of XJZ and JSS prescriptions on the interaction of inflammation-related and antioxidant-related molecules in the spleen of weaned piglets. **(A)** Principal component analysis (PCA) of inflammation-related and antioxidant-related molecules expression. **(B)** Network analysis of spleen damage score, inflammation/antioxidant-related molecules. The threshold for the absolute value of the correlation coefficient is 0.4. **(C)** Correlation analysis between NF-κB-mediated inflammatory pathway and Nrf2-mediated antioxidant defense system. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the control group.

also supported the enhancement of immune function. As the largest white blood cell, Mon plays a vital role in immune response, phagocytosis, and clearing injured senescent cells or its debris (39). Furthermore, CD4+ and CD8+ T cells are the central factors of immune regulation and immune response. The CD4+/CD8+ ratio was significantly increased after treatment with XJZ and JSS prescriptions, suggesting that the blood immunity of piglets in both XJZ and JSS groups were improved. In other words, the susceptibility to the disease was reduced. The ameliorative effect of TCM prescription on the blood immunity of piglets may be related to the enhancement of immune response activity by some of their active ingredients. For example, in chickens, the expression of a family of innate immune response genes were up-regulated when supplemented with cinnamaldehyde in the diet (40). Moreover, our previous study found that both XJZ and JSS prescriptions could improve the growth rate and reduce the rate of diarrhea in piglets (9). Therefore, these results suggested that XJZ and JSS could promote the growth performance, control diarrhea induced by weaning stress, and improve the blood immune-related factors level, which may be related to maintaining the spleen function of weaned piglets.

Inflammatory and oxidative damage induced by weaning or other stress are the key factors contributing to the decline

of spleen physiological function in piglets (15). NF-κB is a nuclear transcription factor that is activated by pattern recognition receptors and mediates the splenic inflammatory response. TLR4 is the first pattern recognition receptors discovered, which can activate Myd88 and NF-κB, thereby promoting the release pro-inflammatory cytokines and gene transcription (29). An earlier study showed that the TCM extract reduced proinflammatory cytokines, reduced oxidative stress, and boosted antioxidant protection system in a mouse model of sepsis, preventing spleen and liver injury (41). Similarly, Rao et al. demonstrated that, in LPS-stimulated RAW264.7 cells, JSS prescription exerted precise anti-inflammatory effects that are regulated by suppression of the NF-κB signaling pathway (13). In our study, both TCM groups apparently reduced pro-infammatory factors level of IL-1β, IL-6, IL-8, and TNF-α in the spleen, consequently verifying the anti-inflammatory effect of both XJZ and JSS. Moreover, excessive release of inflammatory factors causes cytokine imbalance and immune dysfunction, further injuring the spleen function. The activation of the NF-κB inflammatory signaling pathway is linked to the production of these inflammatory cytokines. Consistent with Xu et al. (42) findings, both XJZ and JSS treatments reduced TLR4, MyD88, and NF-κB mRNA levels, together with the phosphorylated protein of p-NF-κB and p-IκB-α. These results suggested that XJZ and JSS prescriptions targeted the NF-κB inflammatory signaling pathway and ameliorated weaning-induced splenic inflammatory injury. In addition, the inhibition of inflammatory response may be related to the activation of spleen antioxidant defense system by TCM prescription.

Oxidative damage occurs when biomolecules such as lipids, DNA, and proteins are oxidized, resulting in reduced functionality of basic physiological processes. Additionally, oxidative stress is often associated with inflammation. As ulcerative colitis (UC) strikes, for example, a vast of Lym and macrophages are activated; these cells then migrate to the damaged mucous membrane, where they produce an excessive amount of oxygen free radicals, damaging or even killing the inflammation region (43). Oxidative stress activates molecular pathways that trigger inflammation and can directly contribute to spleen damage. This type of tissue injury is considered

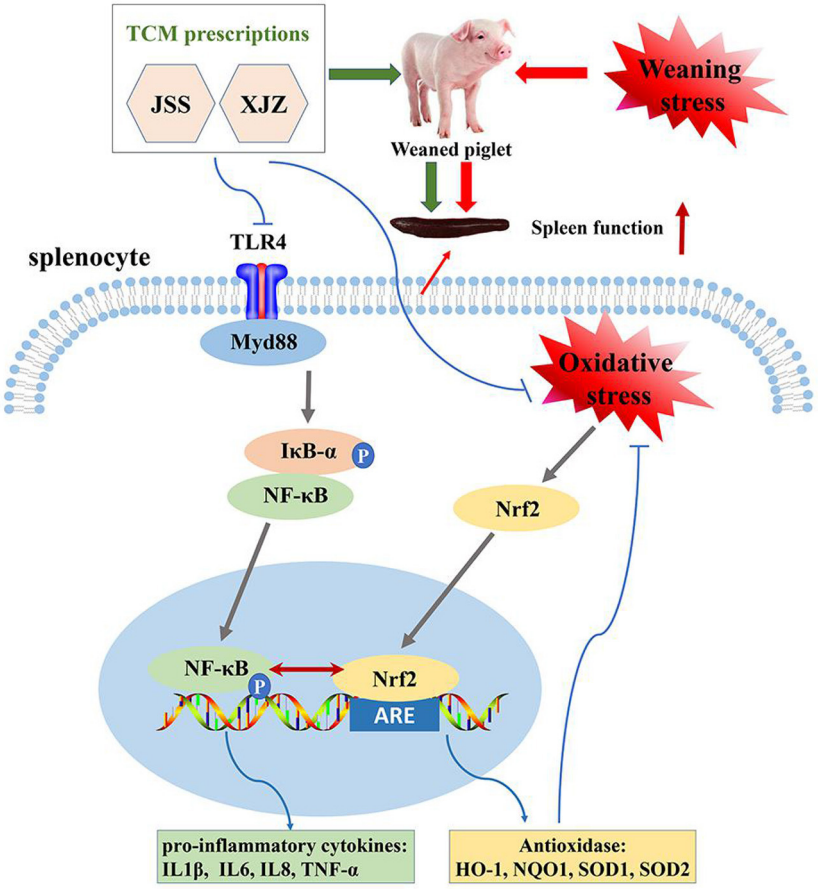


FIGURE 7
Schematic diagram illustrating the proposed mechanism of XJZ and JSS ameliorates spleen inflammatory response and antioxidant capacity by synergistically regulating NF-κB and Nrf2 signaling pathways in weaned-piglets.

one of the most significant mechanisms leading to organ dysfunction. As previous study showed, weaning and other stress induced oxidative damage to multiple organs in piglets, such as the hepatic and intestine (33). The Nrf2 signaling pathway, as one of the most essential defense systems, is a vital path to stimulate the expression of antioxidants and phase II enzymes. Meanwhile, many natural compounds exert their activity through it (44). Nrf2 is a transcription factor that usually found in the cytoplasm as a dormant protein tethered to Keap1. By binding to ARE, Nrf2 activates the expression of phase II enzymes, such as HO-1 and NQO1, once it translocates from the cytoplasm to the nucleus in response to oidative signals. Liu et al. (45) found that soyasaponin, a natural active ingredient derived from plant, provided its defense effect of anti-oxidative stress *via* Nrf2 signaling pathway. Likewise, Li et al. (46) reported that the active ingredients in TCM, such as flavonoids, triterpenoid saponins, quinones, terpenoids, and phthalide class, can improve the body's antioxidant capacity by activating the Nrf2 signaling pathway. Moreover, these active ingredients, such as triterpenoid saponins and terpenoids, were included in both XJZ and JSS prescriptions. In our study, we observed that both TCM prescription treatments prominent increased antioxidant-related gene levels of Nrf-2, HO-1, NQO-1, and SOD-1, which suggested that XJZ and JSS prescriptions could ameliorate the antioxidant capacity and alleviate the oxidative damage of spleen by activating the Nrf2 signaling pathway. Meanwhile, the higher Nrf2, HO-1 and SOD2 protein levels of TCM treatments also supported it. Therefore, based on the above results, both XJZ and JSS prescriptions have potential protective effects on oxidative injury in the spleen of piglets by activating Nrf2-mediated endogenous antioxidant system. Concomitantly, according to the results of PCA and network analysis, we found the spleen damage scores was at the center of the network, suggested that it correlates with the expression of multiple molecules. Surprisingly, there was a significant correlation between NF-κB-mediated inflammatory signaling axis and Nrf2-mediated antioxidant defense system, which suggested that both XJZ and JSS intervention may alleviate spleen damage by crosstalk of NF-κB and Nrf2 signaling pathways.

In conclusion, our study found that both XJZ and JSS prescriptions ameliorated spleen antioxidant capacity and inflammatory response by synergistically regulating NF-κB and Nrf2 signaling pathways in weaned-piglets (Figure 7). Our study provides a new and effective strategy to antagonism immune weakening.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of JiangXi Agricultural University.

Author contributions

JC, NH, and HC designed the study. YM, AHu, WJ, AHua, YW, and PM performed the experiments. JC and NH analyzed the data. JC and HC drafted the article. NH, MH, and FY revised the article and rewrote the discussion. XY did the final review. YC checked the language of the article. HC administrated and supervised the study. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by major research and development projects of Jiangxi province (20194ABC28008).

Conflict of interest

Author XY was employed by Jiangxi Zhongchengren Pharmaceutical Co., Ltd. Author YC was employed by Jiangxi Jiabo Biological Engineering Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.993018/full#supplementary-material

References

1. Campbell JM, Crenshaw JD, Polo J. The biological stress of early weaned piglets. *J Anim Sci Biotechnol.* (2013) 4:19. doi: 10.1186/2049-1891-4-19

2. Merlot E, Quesnel H, Prunier A. Prenatal stress, immunity and neonatal health in farm animal species. *Animal.* (2013) 7:2016–25. doi: 10.1017/S175173111300147X

3. Kick AR, Tompkins MB, Flowers WL, Whisnant CS, Almond GW. Effects of stress associated with weaning on the adaptive immune system in pigs. *J Anim Sci.* (2012) 90:649–56. doi: 10.2527/jas.2010-3470

4. Chen J, Xu YR, Kang JX, Zhao BC, Dai XY, Qiu BH, et al. Effects of alkaline mineral complex water supplementation on growth performance, inflammatory response and intestinal barrier function in weaned piglets. *J Anim Sci.* (2022) skac251. doi: 10.1093/jas/skac251 [Epub ahead of print].

5. Valini GAC, Duarte MS, Calderano AA, Teixeira LM, Rodrigues GA, Fernandes KM, et al. Dietary nucleotide supplementation as an alternative to in-feed antibiotics in weaned piglets. *Animal.* (2021) 15:100021. doi: 10.1016/j.animal.2020.100021

6. Hu J, Ma L, Nie Y, Chen J, Zheng W, Wang X, et al. A microbiota-derived bacteriocin targets the host to confer diarrhea resistance in early-weaned piglets. *Cell Host Microbe.* (2018) 24:817–32.e8. doi: 10.1016/j.chom.2018.11.006

7. McEwen SA, Collignon PJ. Antimicrobial resistance: a one health perspective. *Microbiol Spectr.* (2018) 6:ARBA-0009-2017. doi: 10.1128/microbiolspec.ARBA-0009-2017

8. Zhu Q, Sun P, Zhang B, Kong L, Xiao C, Song Z. Progress on gut health maintenance and antibiotic alternatives in broiler chicken production. *Front Nutr.* (2021) 8:692839. doi: 10.3389/fnut.2021.692839

9. Chen J, Mao Y, Xing C, Hu R, Xu Z, Cao H, et al. Traditional Chinese medicine prescriptions decrease diarrhea rate by relieving colonic inflammation and ameliorating caecum microbiota in piglets. *Evid Based Complement Alternat Med.* (2020) 2020:3647525. doi: 10.1155/2020/3647525

10. Wang Y, Zhang Q, Chen Y, Liang CL, Liu H, Qiu F, et al. Antitumor effects of immunity-enhancing traditional Chinese medicine. *Biomed Pharmacother.* (2020) 121:109570. doi: 10.1016/j.biopha.2019.109570

11. Chen Y, Dong J, Yang D, Qian Q, Wang P, Yang X, et al. Synergistic network pharmacology for traditional Chinese medicine Liangxue Tongyu formula in acute intracerebral hemorrhagic stroke. *Neural Plast.* (2021) 2021:8874296. doi: 10.1155/2021/8874296

12. Du XL. [Essence of professor SHI Xin-de's experience in application of persicae ramulus-associated prescriptions]. *Zhongguo Zhong Yao Za Zhi.* (2014) 39:2569–72. Available online at: https://pubmed.ncbi.nlm.nih.gov/25276984/

13. Rao Z, Cao H, Shi B, Liu X, Luo J, Zeng N. Inhibitory effect of Jing-Fang powder n-butanol extract and its isolated fraction D on lipopolysaccharide-induced inflammation in RAW2647 cells. *J Pharmacol Exp Ther.* (2019) 370:62–71. doi: 10.1124/jpet.118.255893

14. Cesta MF. Normal structure, function, and histology of the spleen. *Toxicol Pathol.* (2006) 34:455–65. doi: 10.1080/01926230600867743

15. Ding D, Mou D, Zhao L, Jiang X, Che L, Fang Z, et al. Maternal organic selenium supplementation alleviates LPS induced inflammation, autophagy and ER stress in the thymus and spleen of offspring piglets by improving the expression of selenoproteins. *Food Funct.* (2021) 12:11214–28. doi: 10.1039/D1FO01653A

16. Wu X, Cao W, Jia G, Zhao H, Chen X, Wu C, et al. New insights into the role of spermine in enhancing the antioxidant capacity of rat spleen and liver under oxidative stress. *Anim Nutr.* (2017) 3:85–90. doi: 10.1016/j.aninu.2016.11.005

17. Fathy SM, Abdelkader IY. Effect of resveratrol on the inflammatory status and oxidative stress in thymus gland and spleen of sulfoxaflor-treated rats. *Environ Toxicol.* (2021) 36:1326–37. doi: 10.1002/tox.23129

18. Zhang R, Guo R, Liu Q, Li G, Sun B, Huang X. Selenium deficiency *via* the TLR4/TRIF/NF-kappaB signaling pathway leading to inflammatory injury in chicken spleen. *Biol Trace Elem Res.* (2021) 199:693–702. doi: 10.1007/s12011-020-02173-0

19. NRC. *Nutrient Requirements of Swine. 11th rev. ed.* Washington, DC: National Academy Press (2012).

20. Murthy SN, Cooper HS, Shim H, Shah RS, Ibrahim SA, Sedergran DJ. Treatment of dextran sulfate sodium-induced murine colitis by intracolonic cyclosporin. *Dig Dis Sci.* (1993) 38:1722–34. doi: 10.1007/BF01303184

21. Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM. Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. *Mol Cell Biol.* (1995) 15:5258–67. doi: 10.1128/MCB.15.10.5258

22. Lee SH, Lillehoj HS, Chun HK, Tuo W, Park HJ, Cho SM, et al. *In vitro* treatment of chicken peripheral blood lymphocytes, macrophages, and tumor cells with extracts of Korean medicinal plants. *Nutr Res.* (2007) 27:362–6. doi: 10.1016/j.nutres.2007.04.001

23. Drong C, Meyer U, von Soosten D, Frahm J, Rehage J, Schirrmeier H, et al. Effects of monensin and essential oils on immunological, hematological and biochemical parameters of cows during the transition period. *J Anim Physiol Anim Nutr.* (2017) 101:791–806. doi: 10.1111/jpn.12494

24. Kamatou GP, Makunga NP, Ramogola WP, Viljoen AM. South African salvia species: a review of biological activities and phytochemistry. *J Ethnopharmacol.* (2008) 119:664–72. doi: 10.1016/j.jep.2008.06.030

25. Peng L, Lei Z, Rao Z, Yang R, Zheng L, Fan Y, et al. Cardioprotective activity of ethyl acetate extract of cinnamomi ramulus against myocardial ischemia/reperfusion injury in rats *via* inhibiting NLRP3 inflammasome activation and pyroptosis. *Phytomedicine.* (2021) 93:153798. doi: 10.1016/j.phymed.2021.153798

26. Dong J, Peng X, Lu S, Zhou L, Qiu M. Hepatoprotective steroids from roots of *Cynanchum otophyllum*. *Fitoterapia.* (2019) 136:104171. doi: 10.1016/j.fitote.2019.104171

27. Qu L, Liu C, Ke C, Zhan X, Li L, Xu H, et al. *Atractylodes lancea* rhizoma attenuates DSS-induced colitis by regulating intestinal flora and metabolites. *Am J Chin Med.* (2022) 50:525–52. doi: 10.1142/S0192415X22500203

28. Zhu B, Zhang QL, Hua JW, Cheng WL, Qin LP. The traditional uses, phytochemistry, and pharmacology of *Atractylodes macrocephala* Koidz: a review. *J Ethnopharmacol.* (2018) 226:143–67. doi: 10.1016/j.jep.2018.08.023

29. Tian H, Liu Z, Pu Y, Bao Y. Immunomodulatory effects exerted by *Poria cocos* polysaccharides *via* TLR4/TRAF6/NF-kappaB signaling *in vitro* and *in vivo*. *Biomed Pharmacother.* (2019) 112:108709. doi: 10.1016/j.biopha.2019.108709

30. Rao Z, Zeng J, Li X, Peng L, Wang B, Luan F, et al. JFNE-A isolated from Jing-Fang n-butanol extract attenuates lipopolysaccharide-induced acute lung injury by inhibiting oxidative stress and the NF-kappaB signaling pathway *via* promotion of autophagy. *Phytomedicine.* (2022) 96:153891. doi: 10.1016/j.phymed.2021.153891

31. Yuan A, Zeng J, Zhou H, Liu Q, Rao Z, Gao M, et al. Anti-type I allergic effects of Jing-Fang powder extracts *via* PI3K/Akt pathway *in vitro* and *in vivo*. *Mol Immunol.* (2021) 135:408–20. doi: 10.1016/j.molimm.2021.01.015

32. Symington B, Mapanga RF, Norton GR, Essop MF. Resveratrol co-treatment attenuates the effects of HIV protease inhibitors on rat body weight and enhances cardiac mitochondrial respiration. *PLoS ONE.* (2017) 12:e0170344. doi: 10.1371/journal.pone.0170344

33. Luo Z, Zhu W, Guo Q, Luo W, Zhang J, Xu W, et al. Weaning induced hepatic oxidative stress, apoptosis, and aminotransferases through MAPK signaling pathways in piglets. *Oxid Med Cell Longev.* (2016) 2016:4768541. doi: 10.1155/2016/4768541

34. Ma X, Zhang Y, Xu T, Qian M, Yang Z, Zhan X, et al. Early-life intervention using exogenous fecal microbiota alleviates gut injury and reduce inflammation caused by weaning stress in piglets. *Front Microbiol.* (2021) 12:671683. doi: 10.3389/fmicb.2021.671683

35. Laukova M, Vargovic P, Rokytova I, Manz G, Kvetnansky R. Repeated stress exaggerates lipopolysaccharide-induced inflammatory response in the rat spleen. *Cell Mol Neurobiol.* (2018) 38:195–208. doi: 10.1007/s10571-017-0546-5

36. Ren J, Liu C, Zhao D, Fu J. The role of heat shock protein 70 in oxidant stress and inflammatory injury in quail spleen induced by cold stress. *Environ Sci Pollut Res Int.* (2018) 25:21011–23. doi: 10.1007/s11356-018-2142-8

37. Yang B, Xiao B, Sun T. Antitumor and immunomodulatory activity of *Astragalus membranaceus* polysaccharides in H22 tumor-bearing mice. *Int J Biol Macromol.* (2013) 62:287–90. doi: 10.1016/j.ijbiomac.2013.09.016

38. Wu F, Ding XY, Li XH, Gong MJ, An JQ, Huang SL. Correlation between elevated inflammatory cytokines of spleen and spleen index in acute spinal cord injury. *J Neuroimmunol.* (2020) 344:577264. doi: 10.1016/j.jneuroim.2020.577264

39. Wan J, Yuan L, Jing H, Zheng Q, Xiao H. Defective apoptotic cell clearance activates innate immune response to protect *Caenorhabditis elegans* against pathogenic bacteria. *Virulence.* (2021) 12:75–83. doi: 10.1080/21505594.2020.1857982

40. Kim DK, Lillehoj HS, Lee SH, Jang SI, Bravo D. High-throughput gene expression analysis of intestinal intraepithelial lymphocytes after oral feeding of carvacrol, cinnamaldehyde, or capsicum oleoresin. *Poult Sci.* (2010) 89:68–81. doi: 10.3382/ps.2009-00275

41. Dkhil MA, Al-Quraishy S, Moneim AEA. Ziziphus spina-christi leaf extract pretreatment inhibits liver and spleen injury in a mouse model of sepsis *via* anti-oxidant and anti-inflammatory effects. *Inflammopharmacology.* (2018) 26:779–91. doi: 10.1007/s10787-017-0439-8

42. Xu X, Zhang L, Liu Z, Pan Y, Chen D, Yang Z, et al. Therapeutic efficacy of the traditional Chinese medicine baishaoqiwu on TNBS-induced colitis is associated with down-regulation of the TLR4/MyD88/NF-kappaB signaling pathway. *In Vivo.* (2016) 30:181–6. Available online at: https://iv.iiarjournals.org/content/30/3/181.long

43. Marquez L, Perez-Nievas BG, Garate I, Garcia-Bueno B, Madrigal JL, Menchen L, et al. Anti-inflammatory effects of *Mangifera indica* L extract in a model of colitis. *World J Gastroenterol.* (2010) 16:4922–31. doi: 10.3748/wjg.v16.i39.4922

44. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol.* (2010) 12:213–23. doi: 10.1038/ncb2021

45. Liu XY, Chen KK, Zhu LJ, Liu H, Ma T, Xu QY, et al. Soyasaponin Ab protects against oxidative stress in HepG2 cells *via* Nrf2/HO-1/NQO1 signaling pathways. *J Funct Foods.* (2018) 45:110–7. doi: 10.1016/j.jff.2018.03.037

46. Li B, Nasser MI, Masood M, Adlat S, Huang Y, Yang B, et al. Efficiency of traditional Chinese medicine targeting the Nrf2/HO-1 signaling pathway. *Biomed Pharmacother.* (2020) 126:110074. doi: 10.1016/j.biopha.2020.110074

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 23 September 2022
DOI 10.3389/fvets.2022.937745

OPEN ACCESS

EDITED BY
Dayou Shi,
South China Agricultural University, China

REVIEWED BY
Ahmed Elsayed Noreldin,
Damanhour University, Egypt
Mohammed A. E. Naiel,
Zagazig University, Egypt

*CORRESPONDENCE
Fan Yang
yfan@jxau.edu.cn

†These authors share first authorship

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 06 May 2022
ACCEPTED 15 July 2022
PUBLISHED 23 September 2022

CITATION
Wang X, Wang Y, Mao Y, Hu A, Xu T, Yang Y, Wang F, Zhou G, Guo X, Cao H and Yang F (2022) The beneficial effects of traditional Chinese medicine on antioxidative status and inflammatory cytokines expression in the liver of piglets. *Front. Vet. Sci.* 9:937745. doi: 10.3389/fvets.2022.937745

The beneficial effects of traditional Chinese medicine on antioxidative status and inflammatory cytokines expression in the liver of piglets

Xiaoyu Wang[1†], Yun Wang[2†], Yaqin Mao[3], Aiming Hu[4], Tianfang Xu[5], Yan Yang[5], Feibing Wang[6], Guangbin Zhou[7], Xiaowang Guo[8], Huabin Cao[1] and Fan Yang[1*]

[1]Jiangxi Provincial Key Laboratory for Animal Health, Institute of Animal Population Health, College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang, China, [2]Department of Animal Science and Technology, Jiangxi Biotech Vocational College, Nanchang, China, [3]China Institute of Veterinary Drug Control, MOA Center for Veterinary Drug Evaluation, Beijing, China, [4]Jian City Livestock and Veterinary Bureau, Ji'an, China, [5]Jiangxi Agricultural Technology Extension Center, Nanchang, China, [6]Agricultural Technology Extension Center, Jinxi County Agriculture and Rural Bureau, Fuzhou, China, [7]Animal Epidemic Prevention and Quarantine Unit, Fengcheng Agricultural and Rural Bureau, Fengcheng, China, [8]Yichun Agriculture and Rural Affairs Bureau, Yichun, China

Oxidative stress and inflammation seriously affected the growth and development of piglets. Traditional Chinese medicine (TCM) prescriptions has been used to prevent various diseases of piglets, including anti-inflammatory and antioxidant. Here, we identified the effects of Xiao-Jian-Zhong-Tang (XJZT) and Jingsananli-sepsis (JJS) on the oxidative stress and inflammatory in the liver of piglets. The piglets were fed with the basal diet (Control group), basal diet affixed with 10 g/kg XJZT (TCM I group), and basal diet affixed with 3 g/kg JJS (TCM II group), respectively. The serum was gathered on days 30 and 60 and the liver samples were also collected on day 60. Results showed that the TCM I and TCM II markedly increased the activities of the glutathione peroxidase (GSH-Px) and total antioxidant capacity (T-AOC), and reduced the levels of malonaldehyde (MDA), TNF-α, IL-6, and IL-8 in serum. In addition, compared to the control group, Nrf2, SOD-1, NQO-1, and HO-1 mRNA expression levels and the protein levels of Nrf2 and HO-1 were significantly increased while NF-κB, TNF-α, IL-6, and IL-8 mRNA expression levels and the phosphorylation levels of NF-κB and IκB-α were decreased in TCM I and TCM II groups. Collectively, these findings suggested that TCM I and TCM II could enhance anti-oxidative and anti-inflammatory capabilities in the liver of piglets via the Nrf2/NF-κB pathway, providing a basis for the functional exploration of TCM prescriptions.

KEYWORDS

traditional Chinese medicine, piglet, antioxidant capability, inflammation, liver

Introduction

The climbing requirement for animal products in recent decades has led to the development of intensive animal production systems which has been demonstrated to produce stress responses in animals (1). Most human practices toward animals could lead to stress responses like climate change, social, environmental, and immunological stress, thereby causing oxidative stress (2–4). In consideration of global climate change, heat stress as a kind of oxidative stress brings about hundreds of millions of dollars of economic losses in the swine industry annually (5). Therefore, combatting oxidative stress is absolutely crucial for the swine industry. Generally, there is an imbalance between the production of reactive oxygen species (ROS) and the biological ability to clear reactive intermediates (6). When out of control, the imbalance of ROS dynamic is hazardous to cellular macromolecules, posing toxic effects in the function and survival of numerous organs (7). Elevated levels of oxidative stress can impair milk production and reproductive performance of sows, which can affect not only the life of sows, but also the health of piglets (8). On the other hand, the occurrence of inflammation could be caused by increased ROS (9). Inflammation is an aspect of the immune response to injury and disease, whose process is closely related to the occurrence of oxidative stress (10). Thus, the two processes are crucial targets of developing therapeutics against numerous diseases.

TABLE 1 Composition and nutrient levels of basal diet (air-dry basis).

Ingredients	Content (%)	Analyzed composition, g/kg	Content (%)
Maize	55.80	DM	89.21
Soybean meal	16.30	DE[2] (MJ/kg)	14.36
Fermented soybean meal	7.00	Crude Protein (CP, %)	19.63
Wheat middling	4.50	Lysine	1.32
Fish meal	2.50	Methionine	0.43
Dried porcine solubles	2.50	Methionine + Cystine	0.77
Whey powder	6.25	Threonine	0.81
Soy oil	1.65	Calcium	0.96
Lysine	0.25	Total phosphorus	0.60
Methionine	0.10	Total	100.00
Limestone	1.05		
$CaHPH_4$	0.80		
NaCl	0.30		
Vitamin-mineral premix[1]	1.00		
Total	100.00		

[1]The premix provides following per kilogram diet: Vitamin A 8 000 IU, Vitamin D 2 500 IU, Vitamin E 15 mg, nicotinic acid 20 mg, D-pantothenie 10 mg, riboflavin 4 mg, biotin 0.06 mg, folic acid 0.2 mg, thiamine 2 mg, choline chloride 500 mg, copper 165 mg, iron 110 mg, manganese 80 mg, zinc 330 mg, selenium 0.20 mg.
[2]Digestible energy is calculated value according to ingredients energy, the others are measured values.

Currently, traditional Chinese medicine (TCM) composed of natural plant derivative has been practiced to improve farm-animal health and prevent various diseases (11, 12). Compound preparations can potentially exert multiple effects in a distinct mechanism that is expected to reach more comprehensive effect by targeting multi pathways and multi targets (13). Xiao-Jian-Zhong-Tang (XJZT) consists of 10 medicinal herbs (Cassia Twig, Glycyrrhiza uralensis, Ziziphus zizyphus, Cynanchum otophyllum, Zingiber officinale Roscoe, Rhizoma Atractylodes, Atractylodes macrocephala, Poria cocos, Coptis chinensis Franch and Maltose) and is currently used to treat chronic liver diseases (14). Jingsananli-sepsis (JSS) composed of 8 medicinal herbs (Nepeta cataria L, Radix Saposhnikoviae, Notopterygium incisum, Radix Angelicae pubescentis, Radix bupleuri, Radix Peucedani, Poria cocos, and Glycyrrhiza uralensis), which have been used in the treatment of grippe, and fever with a long history (15). These may be related to their prescriptions composition consists with various types of antimicrobial,

TABLE 2 Composition and main active constituents of TCM I and TCM II (air dry basis)[1].

Latin name	Main active constituent	Used part	Content (%)
TCM I			
Cassia Twig	Cinnamaldehyde	Dried twig	13.0
Glycyrrhiza uralensis	Glycyrrhizin	Dried root	4.0
Ziziphus zizyphus	Jujuba polysaccharide	Dried fructification	4.0
Cynanchum otophyllum	Paeoniflorin	Dried root	13.0
Zingiber officinale Roscoe	Ginger oleoresin	Dried root	6.0
Rhizoma atractylodes	Atractylodine	Dried root	14.0
Atractylodes macrocephala	Biatractylolide	Dried root	10.5
Poria cocos	Pachymaran	Dried sclerotium	10.5
Coptis chinensis Franch.	Berberine	Dried root	4.0
Maltose	Maltose	-	21.0
Total			100.0
TCM II			
Nepeta cataria L.	Nepeta Cataria Oil	Dried stem	16.5
Radix Saposhnikoviae	Chromone glycoside	Dried root	16.5
Notopterygium incisum	Notopterol	Dried root and stem	16.5
Radix Angelicae pubescentis	Heraclenin	Dried root	16.5
Radix bupleuri	Saikosaponin	Dried root	10.0
Radix Peucedani	Peucedanin	Dried root	10.0
Poria cocos	Pachymaran	Dried sclerotium	10.0
Glycyrrhiza uralensis	Glycyrrhizin	Dried root	4.0
Total			100.0

[1]Main active constituents of TCM come from Chinese pharmacopeia (2005).

immunoregulatory, anti-oxidative and anti-inflammatory active substances (16, 17). Many functional components in these two of TCM, including flavonoids, volatile oils, polysaccharides and organic acids have been demonstrated that were closely related to the immunity enhancement. The flavonoids have antioxidant activity of reduction of free radical formation and free radical scavenging (18). Volatile oil, a major active compound of many herbs, is known to inhibit oxidative stress, and inflammation (19). Additionally, polysaccharides and organic acids are also widely used in inhibits oxidative stress, bacterium and virus (20, 21).

Nuclear factor E2 related factor 2 (Nrf2) acts as a main regulatory factor in preserving cellular defense against oxidative stress. Evidence have been provided that it is responsible for the protection of liver injury and inflammation caused by oxidative stress in the way of regulating antioxidant proteins expression levels (14, 22). On the other hand, many *in vivo* and *in vitro* experiments have demonstrated that oxidative stress could activate NF-κB pathway. Additionally, the activation of NF-κB is thought to be a response to oxidative stress. This signaling pathway is a master regulator of inflammation and may the target of the anti-inflammatory effect of TCM. TCM are usually used to counteract diseases. Whereas studies on the effects of TCM with respect to their anti-inflammatory and anti-oxidative response are rather scanty. Hence, this study explored the potential effects of TCM prescriptions (10 g/kg XJZT and 3g/kg JSS) as feed additives on antioxidative status and inflammatory reaction in piglets, to shed light on the functional role of XJZT and JSS prescriptions.

Materials and methods

Animal treatments

All experimental procedures were performed by Jiangxi Agricultural University Animal Care and Use Committee. The experiment lasted for 60 days, and a total of thirty crossbred (Durox × Landrace × Yorkshire) piglets (weight 21.43 ± 2.86 kg) were randomly allotted to three dietary treatments groups on average according to initial body weight. The dietary treatments were basal diet (control group), 10 g/kg Xiao-Jian-Zhong-Tang (XJZT) combination with basal diet (TCM I group) and 3 g/kg Jingsananli-sepsis combination with basal diet (TCM II group). The composition and nutrient levels of basal diet are shown in Table 1. All raw materials for TCM I were bought from Changsheng pharmacy (Jiangxi, China) and TCM II was provided by The Spirit Jinyu Biological Pharmaceutical Co., Ltd. (Huhhot, Inner Mongolia, China). All dried herbs are crushed through a 2.5 mm screen. Composition and main active constituents of TCM I and TCM II are presented in Table 2.

TABLE 3 Primers used in this study.

Gene	GeneBank Number	Primers Sequences (5′-3′)	AAnnealing temp (°C)	Amplification size (bp)
Nrf2	XM_005671982.1	F: CCCATTCACAAAAGACAAACATTC	58	72
		R: GCTTTTGCCCTTAGCTCATCTC		
SOD-1	NM_001190422.1	F: GAGACCTGGGCAATGTGACT	57	139
		R: CTGCCCAAGTCATCTGGTT		
NQO1	NM_001159613.1	F: CCAGCAGCCCGGCCAATCTG	66	160
		R: AGGTCCGACACGGCGACCTC		
HO-1	NM_001004027	F: CGCTCCCGAATGAACAC	55	112
		R: GCTCCTGCACCTCCTC		
NF-κB	NM_001048232.1	F: CTCGCACAAGGAGACATGAA	58	147
		R: ACTCAGCCGGAAGGCATTAT		
TNF-α	NM_214022.1	F: CCAATGGGCAGAGTGGGTATG	56	117
		R: TGAAGAGGACCTGGGAGTAG		
IL-6	NM_001252429.1	F: TGGCTACTGCCTTCCCTACC	58	132
		R: CAGAGATTTTGCCGAGGATG		
IL-8	NM_213867.1	F: TTCGATGCCAGTGCATAAATA	57	176
		R: CTGTACAACCTTCTGCACCCA		
IL-10	NM_214041	F: CGGCGCTGTCATCAATTTCTG	58	89
		R: CCCCTCTCTTGGAGCTTGCTA		
GAPDH	NM_001206359	F: ACTCACTCTTCCACTTTTGATGCT	57	100
		R: TGTTGCTGTAGCCAAATTCA		

F, Forward primer; R, Reverse Primer.

Sample collection

Blood was obtained from the jugular vein in the collection tube on days 30 and 60. Serum was separated and stored at −20°C to detect the antioxidant indices and inflammatory cytokines. On the day 60 of the experiment, all piglets in each group were euthanized with sodium pentobarbital (40 mg/kg body weight). Livers were dissected immediately from all piglets. The blood of the liver was washed by precooled normal saline (0.9% NaCl, Beyotime, China), and the surface liquid was removed with filter paper, and then stored at −80°C for analysis after rapidly frozen in liquid nitrogen.

Determination of antioxidant indices and inflammatory cytokines in serum

The levels of T-AOC, SOD, GSH-PX, and MDA in serum were measured strictly based on the instructions of the kits (Nanjing Jiancheng Bioengineering Institute, China; T-AOC, A015-2-1; SOD, A001-3-1; GSH-PX, A005-1-2; MDA, A003-1-2). The levels of TNF-α, IL-6, IL-8, and IL-10 in serum were measured by enzyme linked immunosorbent assay kits (R&D, USA; TNF-α, MTA00B; IL-6, D6050; IL-8, D8000C; IL-10, M1000B) according to the instructions. The optical density of each well was read at 450 nm with an Absorbance Microplate Reader (SpectraMax, China).

Quantitative real-time PCR analysis

RT-qPCR assay was consistent with the method previously (23, 24). The primer sequences of Nrf2, SOD-1, NQO-1, HO-1, NF-κB, TNF-α, IL-6, IL-8, IL-10, and GAPDH are shown in Table 3.

Western blot analysis

Western blotting assay was performed in accordance with the procedure in a previous study (Dai et al., 2021). The primary antibodies were Nrf2 (Proteintech, 1:1000), HO-1

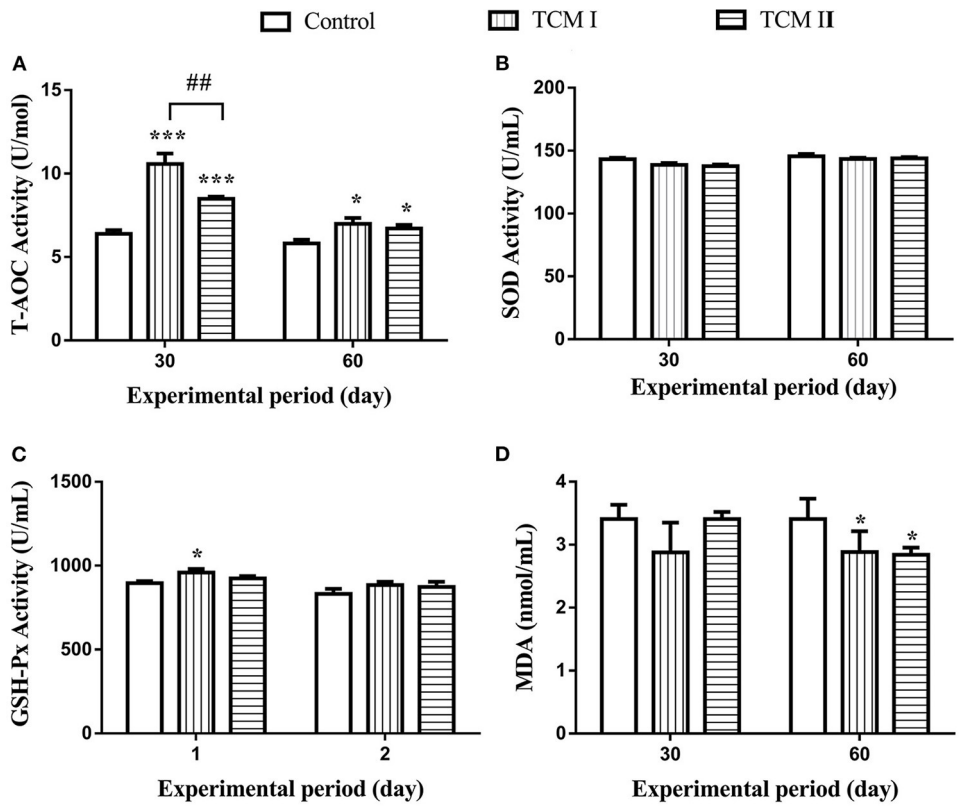


FIGURE 1
Determination of **(A)** T-AOC, **(B)** SOD, **(C)** GSH-Px, and **(D)** MDA levels in serum on days 30 and 60. The data are presented as the mean ± SD of at least three independent experiments ($n \geq 3$). "*" indicates a significant difference compared with control group (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). "#" indicates a significant difference between the indicated groups (#$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$). The same scheme also applies to the remaining figures.

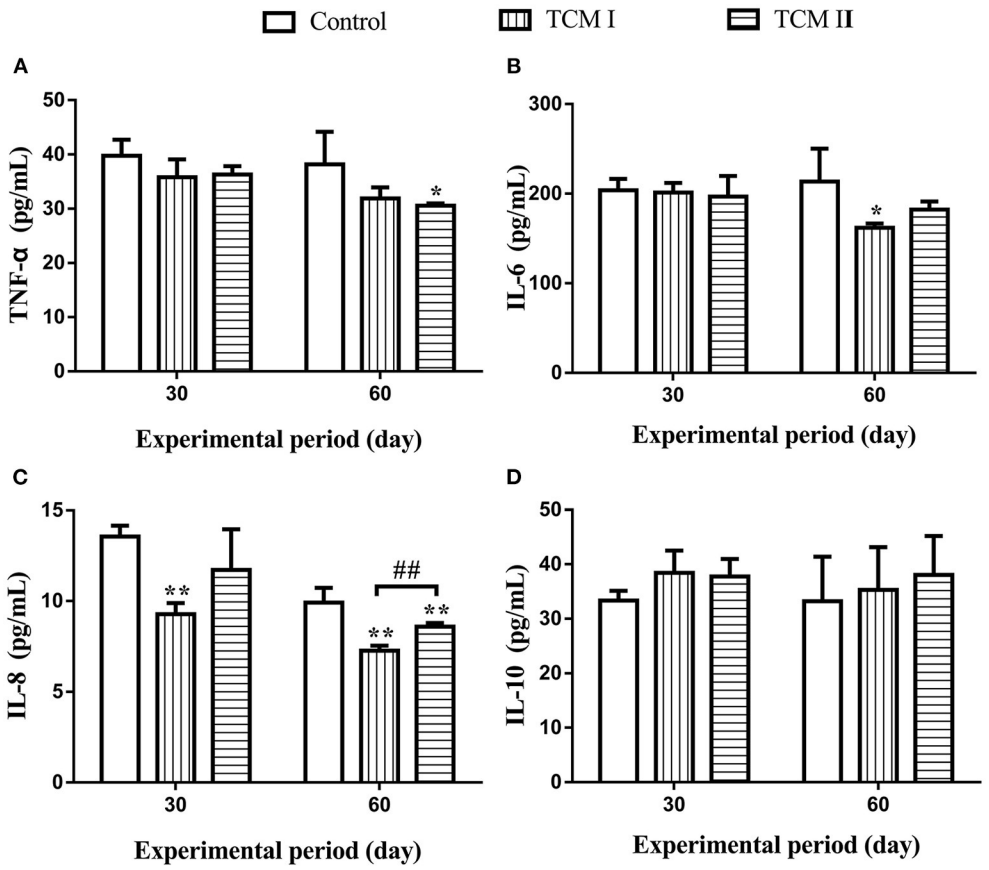


FIGURE 2
Determination of **(A)** TNF-α, **(B)** IL-6, **(C)** IL-8, and **(D)** IL-10 levels in serum on days 30 and 60.

(Wanleibio, 1:1000), NF-κB P65 (Bioss, 1:1000), phospho-NF-κB (Bioss, 1:1000), IκB-α (Wanleibio, 1:1000), phospho-IκB-α (Bioss, 1:1000), and GAPDH (1:8000; Bioss, China).

Statistical analysis

Quantitative variables are expressed as the mean ± standard deviation (SD). All statistical analyses were calculated by one-way analysis of variance and the least significant difference test. P values < 0.05 was considered significant.

Results

Effects of TCM I and TCM II on antioxidant capabilities in serum

As shown in Figures 1A,B, T-AOC level in TCM I and TCM II groups was obviously higher than the control group ($P < 0.05$ or $P < 0.001$) on days 30 and 60. In addition, GSH-Px level was notably increased ($P < 0.05$) in TCM I group compared to the control group (Figure 1C). Furthermore, the T-AOC activity in TCM I and TCM II groups was markedly upregulated ($P < 0.05$) compared to the control group on day 60. However, MDA content was significantly decreased ($P < 0.05$) in TCM I and TCM II groups compared to the control group on day 60 (Figure 1D).

Effects of TCM I and TCM II on inflammatory cytokines in serum

As shown in Figures 2A–C, TNF-α, IL-6 and IL-8 levels in the TCM I and TCM II groups were significantly lower than the control group ($P < 0.05$ or $P < 0.01$) on days 30 and 60. Moreover, IL-8 level was markedly decreased ($P < 0.01$) in the TCM I group compared to the TCM II group on day 60. Additionally, IL-10 level was increased in TCM I and TCM II groups in comparison with the control group (Figure 2D).

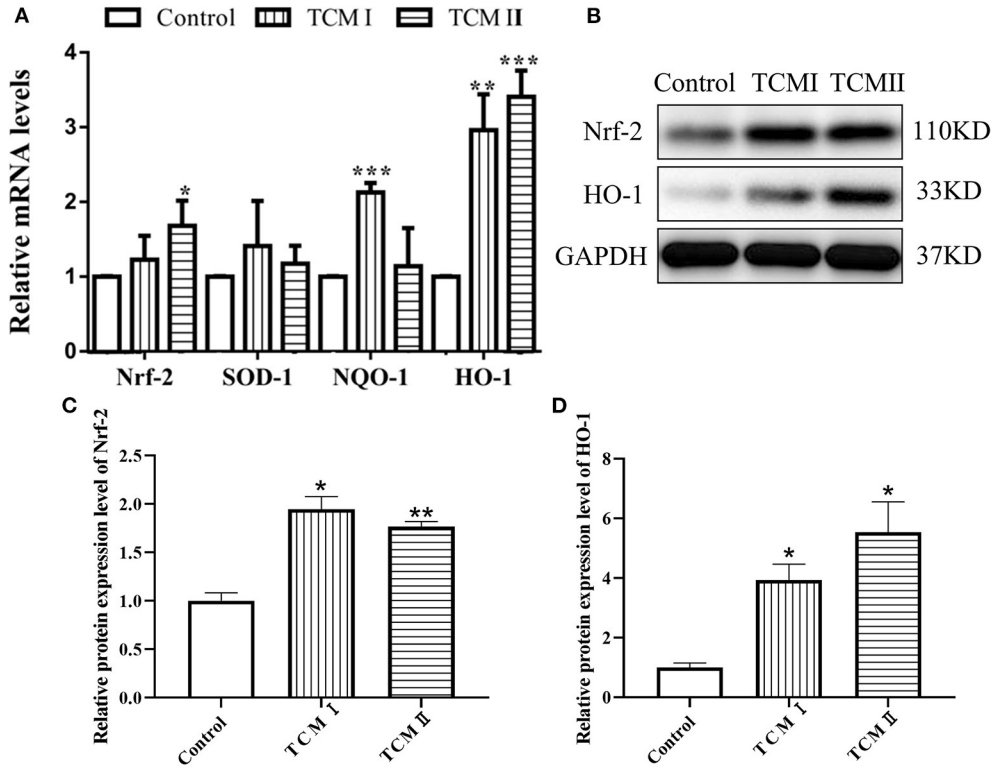


FIGURE 3
Effects of TCM I and TCM II on the mRNA levels of antioxidant-related genes and protein levels in liver. **(A)** mRNA levels of Nrf-2, SOD-1, NQO-1, and HO-1. **(B)** Protein levels of Nrf-2 and HO-1. **(C)** Graph showing the protein level of Nrf-2. **(D)** Graph showing the protein levels of HO-1.

Effects of TCM I and TCM II on mRNA levels of antioxidant-related genes and protein levels in liver

As described in Figure 3A, HO-1 and NQO-1 mRNA levels of liver in TCM and TCM II groups were higher than that in control group ($P < 0.05$ or $P < 0.01$). Otherwise, the mRNA level of SOD-1 was increased in TCM I and TCM II groups compared to the control group, whereas the differences were not significant. The protein levels of Nrf2 and HO-1 are presented in Figures 3B–D. Both Nrf2 and HO-1 protein levels in TCM I and TCM II groups were dramatically increased in comparison with the control group ($P < 0.05$ or $P < 0.01$).

Effects of TCM I and TCM II on mRNA levels of inflammatory cytokines and protein levels in liver

As shown in Figure 4A, NF-κB, IL-8, and TNF-α mRNA levels in TCM I group were significantly downregulated compared to control group ($P < 0.05$ or $P < 0.01$), while IL-10 mRNA level was significantly increased ($P < 0.01$). Additionally, Figure 4A shows that NF-κB and IL-6 mRNA levels in the TCM II group also dramatically declined in comparison with control group ($P < 0.01$ or $P < 0.001$). However, compared to the control group, IL-10 mRNA level was markedly up-regulated in TCM II group ($P < 0.001$). The protein expressions of IκB-α and NF-κB had no significant differences in TCM I and TCM II groups ($P > 0.05$) in comparison with the control group (Figures 4B,C). As shown in Figures 4E,F the protein expressions of the p-IκB-α and p-NF-κB in TCM I and TCM II groups were dramatically downregulated in comparison with the control group ($P < 0.01$ or $P < 0.001$).

Discussion

In intensive farming systems, piglets face numerous challenges like change in the nutritional source, feed contamination with mycotoxins, pathogenic micro-organisms, and some chemical agents, leading to excessive ROS accumulation that cause the occurrence of oxidative stress and inflammation (3, 25, 26). Accordingly, it is necessary to find functional products that can effectively protect piglets from

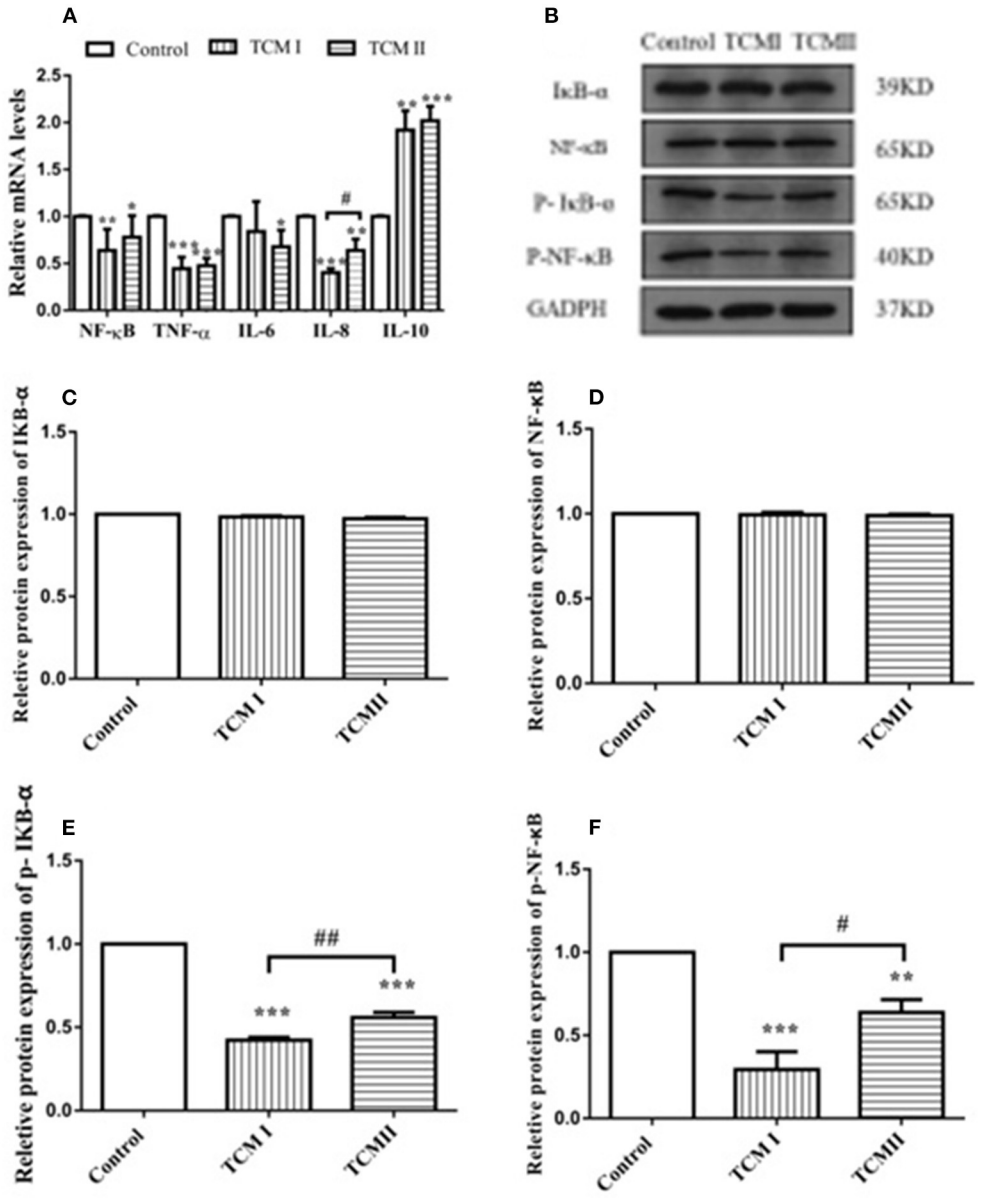


FIGURE 4
Effects of TCM I and TCM II on the mRNA levels of inflammatory cytokines and protein levels in liver. **(A)** mRNA levels of NF-κB, TNF-α, IL-6, IL-8, and IL-10. **(B)** Protein levels of IκB-α, NF-κB p65, p-IκB-α, and p-NF-κB. **(C)** Graph showing the protein level of IκB-α. **(D)** Graph showing the protein level of NF-κB p65. **(E)** Graph showing the protein level of p-IκB-α. **(F)** Graph showing the protein level of p-NF-κB.

oxidative stress and inflammation. Chinese herbal medicine is a unique medical resource in China. In most condition, many Chinese herbal medicines share the advantages of low toxicity, small side effects, low drug resistance and no residual, etc. (27, 28). Their application in livestock and poultry production not only enhanced the immune function of the body and improve disease resistance, but also promote the growth and development of animals. Due to the complicated process of oxidative stress and inflammation, the therapeutic effect of a single herb may not be ideal. XJZT and JSS are well-known traditional herbal medicines which have been used to alleviate oxidative stress and inflammation and improve immunity for a long time, containing with multiple antimicrobial, immunoregulatory, anti-oxidative and anti-inflammatory active substances (29–31). The flavonoids as a powerful antioxidant can scavenge free radicals from multiple targets, and they are safer and more effective than other antioxidants (19, 32). Additionally, polysaccharides and organic acids have abundant

biological activities, so they can also widely used in inhibits oxidative stress and inflammation (20, 21). Therefore, this study investigated the effects of dietary supplementation of XJZT or JJS on the anti-oxidative capacity and inflammatory response in the liver of piglets.

Oxidative stress refers to a state of imbalance between oxidation and antioxidation in which numerous oxidized intermediates are produced. The impulse induction of protective antioxidant enzymes can exert profitable effects on the ability of the body to maintain homeostasis. T-AOC represents the enzymatic and non-enzymatic antioxidant defense systems. Superoxide anion radicals (O^{2-}) can be scavenged by T-SOD and peroxides and hydroxyl radicals produced in the process of cell metabolism can be eliminated by GSH-Px, thereby protecting the body from oxidative damage (33, 34). Additionally, the degree of lipid peroxidation could be reflected by MDA content, so its level is proportional to the degree of cell damage (35). Previous reports have shown that subcutaneous lipopolysaccharide injection and supplement ampelopsin in diet for pigs improved the anti-oxidative capacity in plasma (36, 37). In this study, we found that supplementation with XJZT and JJS increased GSH-Px and T-AOC levels but decreased MDA content in plasma, indicating that XJZT and JJS supplementations in piglets could save tissues from lipid peroxidation. This benefit might be due to the reduction of the antioxidative system burden by dietary total flavonoids, polysaccharides, and phenolicsand. Additionally, changes in the levels of these antioxidant indexes (SOD, GSH-Px, and MDA) have affinities with Nrf2 expression level. Moreover, Nrf2 binds to antioxidant response elements such as HO-1 and NQO1 to prevent oxidative damage (38). Our results illustrated that XJZT and JJS could significantly increase Nrf2, SOD-1, HO-1, and NQO-1 mRNA levels and Nrf2 and HO-1 protein levels. These results demonstrated that XJZT and JJS against oxidative damage by activating the Nrf2 signal pathway in liver.

Additionally, there is a strong relationship between oxidative stress and inflammation. As a redox-sensitive transcription factor, NF-κB expression level can be promoted by oxidative stress, whose level can mediate the transcription of numerous inflammatory genes (39). TNF-α occupies an important position in the inflammatory responses which is responsible for plenty of cytokines and chemokines production (40, 41). IL-10 acts as an anti-inflammatory cytokine with important immunoregulatory functions in the way of restraining the inflammatory cytokines expression levels like TNF-α, IL-6 and IL-1 (40). In our study, the XJZT and JJS decreased the concentrations of TNF-α, IL-6, and IL-8 and increased IL-10 level in the plasma. NF-κB is of great importance for controlling the expression levels of inflammatory response related proteins. Under normal conditions, there is covalently binding between NF-κB and IκB in the cytoplasm. After receiving immune stimulation signals such as TNF-α and lipopolysaccharides, IκB is phosphorylated and degradated, followed by NF-κB translocation to the nucleus, promoting transcription of inflammatory genes like IL-1β, and IL-6. In this study, XJZT and JJS were significantly decreased the mRNA expression levels of NF-κB, IL-6, IL-8, and TNF-α, but remarkably increased the IL-10 mRNA expression. Furthermore, the results showed that XJZT and JJS could decrease the protein levels of IκB-α and NF-κB, and remarkably decrease the p-IκB-α and p-NF-κB protein levels in liver. These results illustrated that the XJZT and JJS inhibited the production of pro-inflammatory mediators in livers through down-regulating the NF-κB signal pathway.

Conclusion

Our data confirmed that 10 g/kg XJZT and 3 g/kg JJS prescriptions exhibited strong hepatoprotective effect on the liver of piglets, which have a strong relationship with their anti-oxidative and anti-inflammatory capabilities by activating the Nrf2/NF-κB pathway. Accordingly, 10 g/kg XJZT and 3 g/kg JJS prescriptions can act as potential materials for drug and functional food development to prevent oxidative stress and inflammatory of piglets.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Experimental Animal Care and Use Committee of Jiangxi Agricultural University.

Author contributions

XW, YW, and FY contributed to conception and design of the study. YM, AH, and TX organized the database. FW, GZ, and YY performed the statistical analysis. HC wrote the first draft of the manuscript. XG, AH, and TX wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This work was supported by Major Research and Development Projects of Jiangxi Province (20194ABC28008)

and Spirit Jinyu Biological Pharmaceutical Co., Ltd. (Huhhot, Inner Mongolia, China). The authors declare that this study received funding from Spirit Jinyu Biological Pharmaceutical Co. Ltd. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Martínez-Miró S, Tecles F, Ramón M, Escribano D, Hernández F, Madrid J, et al. Causes, consequences and biomarkers of stress in swine: an update. *BMC Vet Res.* (2016) 12:171. doi: 10.1186/s12917-016-0791-8

2. Barnett JL, Hemsworth PH, Cronin GM, Jongman EC, Hutson GDA. review of the welfare issues for sows and piglets in relation to housing. *Aust J Agric Res.* (2001) 52:1–28. doi: 10.1071/AR00057

3. Frankič T, Salobir J. In vivo antioxidant potential of Sweet chestnut (Castanea sativa Mill) wood extract in young growing pigs exposed to n-3 PUFA-induced oxidative stress. *J Sci Food Agric.* (2011) 91:1432–9. doi: 10.1002/jsfa.4328

4. Bacou E, Haurogne K, Mignot G, Allard M, De Beaurepaire L, Marchand J, et al. Acute social stress-induced immunomodulation in pigs high and low responders to ACTH. *Physiol Behav.* (2017) 169:1–8. doi: 10.1016/j.physbeh.2016.11.012

5. Cruzen SM, Baumgard LH, Gabler NK, Pearce SC, Lonergan SM. Temporal proteomic response to acute heat stress in the porcine muscle sarcoplasm. *J Anim Sci.* (2017) 95:3961–71. doi: 10.2527/jas.2017.1375

6. Pi J, Zhang Q, Fu J, Woods CG, Hou Y, Corkey BE, et al. ROS signaling, oxidative stress and Nrf2 in pancreatic beta-cell function. *Toxicol Appl Pharmacol.* (2010) 244:77–83. doi: 10.1016/j.taap.2009.05.025

7. Yin J, Wu MM, Xiao H, Ren WK, Duan JL, Yang G, et al. Development of an antioxidant system after early weaning in piglets. *J Anim Sci.* (2014) 92:612–9. doi: 10.2527/jas.2013-6986

8. Jang KB, Kim SW. Supplemental effects of dietary nucleotides on intestinal health and growth performance of newly weaned pigs. *J Anim Sci.* (2019) 97:4875–82. doi: 10.1093/jas/skz334

9. Tsai WH, Yang CC, Li PC, Chen WC, Chien CT. Therapeutic potential of traditional chinese medicine on inflammatory diseases. *J Tradit Complement Med.* (2013) 3:142–51. doi: 10.4103/2225-4110.114898

10. Shingnaisui K, Dey T, Manna P, Kalita J. Therapeutic potentials of Houttuynia cordata Thunb. against inflammation and oxidative stress: A review. *J Ethnopharmacol.* (2018) 220:35–43. doi: 10.1016/j.jep.2018.03.038

11. He DY, Dai SM. Anti-inflammatory and immunomodulatory effects of paeonia lactiflora pall. a traditional chinese herbal medicine. *Front Pharmacol.* (2011) 2:10. doi: 10.3389/fphar.2011.00010

12. Jiang C, Liu P, Zhang J, Bao W, Qiu S, Liang Y, et al. Clinical study of effects of jian ji ning, a chinese herbal medicine compound preparation, in treating patients with myasthenia gravis via the regulation of differential MicroRNAs expression in serum. *Evid Based Complement Alternat Med.* (2014) 2014:518942. doi: 10.1155/2014/518942

13. Zhang YT, Xi YL, Liu M, Ma HB. The study of flavonoids and polysaccharide content and antioxidant capacity *in vitro* of compound chinese herbal medicine in changbai mountain. *Adv Mater Res.* (2014) 181:1036–9. doi: 10.4028/www.scientific.net/AMR.926-930.1036

14. Kai L, Lei S. From "Preventive Treatment" theory to discuss xiaojianzhong soup in the treatment of liver disease. *Acta Chin Med.* (2014) 29:1743–4. doi: 10.16368/j.issn.1674-8999.2014.12.018

15. Yin X, Song F, Gong Y, Tu X, Wang Y, Cao S, et al. A systematic review of antibiotic utilization in China. *J Antimicrob Chemother.* (2013) 68:2445–52. doi: 10.1093/jac/dkt223

16. Alshathly MR. Efficacy of Ginger (Zingiber officinale) in ameliorating streptozotocin-induced diabetic liver injury in rats: histological and biochemical studies. *J Microsc Ultrastruct.* (2019) 7:91–101. doi: 10.4103/JMAU.JMAU_16_19

17. Shao YX, Gong Q, Qi XM, Wang K, Wu YG. Paeoniflorin ameliorates macrophage infiltration and activation by inhibiting the TLR4 signaling pathway in diabetic nephropathy. *Front Pharmacol.* (2019) 10:566. doi: 10.3389/fphar.2019.00566

18. Pietta PG. Flavonoids as antioxidants. *J Nat Prod.* (2000) 63:1035–42. doi: 10.1021/np9904509

19. Edris AE. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. *Phytother Res.* (2007) 21:308–23. doi: 10.1002/ptr.2072

20. Polycarpo GV, Andretta I, Kipper M, Cruz-Polycarpo VC, Dadalt JC, Rodrigues PHM, et al. Meta-analytic study of organic acids as an alternative performance-enhancing feed additive to antibiotics for broiler chickens. *Poult Sci.* (2017) 96:3645–53. doi: 10.3382/ps/pex178

21. Zhang L, Hu Y, Duan X, Tang T, Shen Y, Hu B, et al. Characterization and antioxidant activities of polysaccharides from thirteen boletus mushrooms. *Int J Biol Macromol.* (2018) 113:1–7. doi: 10.1016/j.ijbiomac.2018.02.084

22. Tian Y, Li Z, Shen B, Zhang Q, Feng H. Protective effects of morin on lipopolysaccharide/d-galactosamine-induced acute liver injury by inhibiting TLR4/NF-κB and activating Nrf2/HO-1 signaling pathways. *Int Immunopharmacol.* (2017) 45:148–55. doi: 10.1016/j.intimp.2017.02.010

23. Dai XY, Zhao Y, Ge J, Zhu SY, Li MZ, Talukder M, et al. Lycopene attenuates di(2-ethylhexyl) phthalate-induced mitophagy in spleen by regulating the sirtuin3-mediated pathway. *Food Funct.* (2021) 12:4582–90. doi: 10.1039/D0FO03277H

24. Dai XY, Li XW, Zhu SY, Li MZ, Zhao Y, Talukder M, et al. Lycopene Ameliorates Di(2-ethylhexyl) Phthalate-Induced Pyroptosis in Spleen via Suppression of Classic Caspase-1/NLRP3 Pathway. *J Agric Food Chem.* (2021) 69:1291–9. doi: 10.1021/acs.jafc.0c06534

25. Zheng P, Yu B, He J, Yu J, Mao X, Luo Y, et al. Arginine metabolism and its protective effects on intestinal health and functions in weaned piglets under oxidative stress induced by diquat. *Br J Nutr.* (2017) 117:1495–502. doi: 10.1017/S0007114517001519

26. Novais AK, Deschêne K, Martel-Kennes Y, Roy C, Laforest JP, Lessard M, et al. Weaning differentially affects mitochondrial function, oxidative stress, inflammation and apoptosis in normal and low birth weight piglets. *PLoS ONE.* (2021) 16:e0247188. doi: 10.1371/journal.pone.0247188

27. Er J, Xian Z, Qi S, Jin Z, Sheng L, Guang J, et al. The effect of compound Chinese herbal preparation on muscle development and meat quality of broiler. *Jiangsu J Agric Sci.* (2018) 34:374–84. doi: 10.3969/j.issn.1000-4440.2018.02.022

28. Hu H, Xu K, Wang K, Zhang F, Bai X. Dissecting the effect of berberine on the intestinal microbiome in the weaned piglets by metagenomic sequencing. *Front Microbiol.* (2022) 13:862882. doi: 10.3389/fmicb.2022.862882

29. Wang WK, Hsu TL, Huang ZY, Wang YY. Collective effect of a Chinese formula–a study of xiao-jian-zhong-tang. *Am J Chinese Med.* (1995) 23:299–304. doi: 10.1142/S0192415X95000353

30. Yong Z, Qian L, Bin W. Effect of xiaojianzhong decoction on lipid peroxidation and cyclic nucleotides in rat with deficiency and cold of spleen and stomach. *Chin J Exp Tradit Med Formulae.* (2011) 17:151–4. doi: 10.13422/j.cnki.syfjx.2011.23.065

31. Yan T, Rui L, Xiao W. Alleviating action of jingfang baidu san on acute alcoholism in mise. *Chung-kuo Shih Yen Fang Chi Hsueh Tsa Chih.* (2011) 17:221–3. doi: 10.13422/j.cnki.syfjx.2011.21.067

32. El-Demerdash FM, El-Sayed RA, Abdel-Daim MM. Rosmarinus officinalis essential oil modulates renal toxicity and oxidative stress induced by potassium dichromate in rats. *J Trace Elem Med Biol.* (2021) 67:126791. doi: 10.1016/j.jtemb.2021.126791

33. Zhang L, Zhang W, Zheng B, Tian N. Sinomenine attenuates traumatic spinal cord injury by suppressing oxidative stress and inflammation via Nrf2 pathway. *Neurochem Res.* (2019) 44:763–75. doi: 10.1007/s11064-018-02706-z

34. Guo Z, Chen X, Huang Z, Chen D, Yu B, Chen H, et al. Dietary dihydromyricetin supplementation enhances antioxidant capacity and improves lipid metabolism in finishing pigs. *Food Funct.* (2021) 12:6925–35. doi: 10.1039/D0FO03094E

35. Wu QJ, Wang ZB, Wang GY, Li YX, Qi YX. Effects of feed supplemented with fermented pine needles (Pinus ponderosa) on growth performance and antioxidant status in broilers. *Poult Sci.* (2015) 94:1138–44. doi: 10.3382/ps/pev013

36. Milbury PE, Kalt W. Xenobiotic metabolism and berry flavonoid transport across the blood–brain barrier. *J Agric Food Chem.* (2010) 58:3950–6. doi: 10.1021/jf903529m

37. Hou X, Zhang J, Ahmad H, Zhang H, Xu Z, Wang T, et al. Evaluation of antioxidant activities of ampelopsin and its protective effect in lipopolysaccharide-induced oxidative stress piglets. *PLoS ONE.* (2014) 9:e108314. doi: 10.1371/journal.pone.0108314

38. Wang R, Wang J, Song F, Li S, Yuan Y. Tanshinol ameliorates CCl4-induced liver fibrosis in rats through the regulation of Nrf2/HO-1 and NF-κB/IκBα signaling pathway. *Drug Des Devel Ther.* (2018) 12:1281–92. doi: 10.2147/DDDT.S159546

39. Kaltschmidt C, Greiner JFW, Kaltschmidt B. The transcription factor NF-κB in stem cells and development. *Cells.* (2021) 10:2042. doi: 10.3390/cells10082042

40. Kumar P, Sulakhiya K, Barua CC, Mundhe N. TNF-alpha, IL-6 and IL-10 expressions, responsible for disparity in action of curcumin against cisplatin-induced nephrotoxicity in rats. *Mol Cell Biochem.* (2017) 431:113–22. doi: 10.1007/s11010-017-2981-5

41. Hira K, Sajeli Begum A. Methods for evaluation of TNF-α inhibition effect. *Methods Mol Biol.* (2021) 2248:271–9. doi: 10.1007/978-1-0716-1130-2_21

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 13 October 2022
DOI 10.3389/fvets.2022.980381

OPEN ACCESS

EDITED BY
Dayou Shi,
South China Agricultural University, China

REVIEWED BY
Hongxu Du,
Southwest University, China
Xiaona Zhao,
Shandong Agricultural University, China

*CORRESPONDENCE
Zhiwen Xu
abtcxzw@126.com
Youjun Hu
61368851@163.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 28 June 2022
ACCEPTED 12 September 2022
PUBLISHED 13 October 2022

CITATION
Liu Z, Zhu L, Zhao X, Liu J, Cheng H, Zhang L, Tang H, Sun X, Hu Y and Xu Z (2022) Effects of oral of administration of monoglycide laurate on virus load and inflammation in PEDV infected porcine. *Front. Vet. Sci.* 9:980381. doi: 10.3389/fvets.2022.980381

Effects of oral of administration of monoglycide laurate on virus load and inflammation in PEDV infected porcine

Zheyan Liu[1†], Ling Zhu[1,2†], Xiaonan Zhao[3], Jian Liu[3], Huangzuo Cheng[3], Lina Zhang[4], Huaqiao Tang[1], Xiangang Sun[1], Youjun Hu[3]* and Zhiwen Xu[1,2]*

[1]College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, China, [2]Key Laboratory of Animal Diseases and Human Health of Sichuan Province, Chengdu, China, [3]Innovation Center of Guangdong Nuacid Biotechnology Co., Ltd., Qingyuan, China, [4]College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang, China

To investigate the effect of monoglyceryl laurate (GML) against PEDV *in vivo*, the clinical signs, pathological changes, tissue viral load and cytokine levels of piglets were compared in different GML treatment groups and PEDV infected group. The diets of experimental groups were supplemented with different doses of GML (5g for A1, 10g for A2, 20g for A3) on day 1, 2, and 3 after PEDV challenge, and the virus challenge group (group C) and blank group (group B) were set as control. The results showed that compared with group C, groups As could reduce the mortality rate of piglets, among which the protection rates of groups A2 and A3 could reach 100%. The trend of weight loss of piglets was effectively slowed down and growth performance recovered in GML treated groups. GML reduced the pathological damage of intestinal tract and the viral load in intestine and mesenteric lymph nodes. The levels of IL-8 and TNF-α in the blood of group As were inhibited by GML in a dose-dependent manner when compared with group C. Our study suggests that GML has potential anti-PEDV effects *in vivo*.

KEYWORDS

porcine epidemic diarrhea virus, monoglyceride laurate, clinical signs, pathological changes, viral load, cytokines

Introduction

PEDV is a single-stranded positive-stranded RNA virus with a capsid, a member of the family Coronaviridae and the genus of Alpha Coronavirus, and has been mutating since 2010, with outbreaks in China, USA, Korea and other countries (1–3). PEDV can cause infection in pigs of all ages, mainly in lactating piglets and fattening pigs. Affected piglets often show signs of acute watery diarrhea, vomiting and dehydration, with high mortality rates (4). Surviving piglets have slow growth, reduced feed remuneration and lower economic efficiency.

Currently, PEDV is mainly transmitted through direct or indirect contact and aerosols. PEDV mutates rapidly and is difficult to prevent and control with traditional commercial vaccines. Therefore, there is an urgent need to develop a more safe and effective anti-PEDV drugs.

In recent years, the clinical use of bioactive substances with antiviral activity as additives or in combination formulations has provided new ideas for virus diseases control. GML is a synthetic ester of glycerol and lauric acid that is readily absorbed and rapidly metabolized by oxidation (5). It can rapidly supply energy to the animal body. It has antiviral, anti-inflammatory, immunomodulatory and growth enhancing properties in piglets (6). *In vitro* studies have shown that low concentrations of GML can inhibit ASFV activity under liquid conditions, and that high doses of GML can significantly reduce the infectivity of ASFV in feed (7). Zhang (8) found that the addition of GML to the diet could reduce the viremia and immunosuppression in sows with PRRS. In addition, studies have shown that GML has a strong resistance and destructive effect on vesicular viruses, such as herpes simplex virus, influenza virus, etc., both *in vivo* and *in vitro* (9). A recent study found that the addition of a certain percentage of alpha-monolaurin to the feed of sows can cut off vertical transmission of PEDV (10). These results suggest that GML may be a new additive for the prevention and control of PED.

The aim of this study was to evaluated the anti-PEDV effect of GML by studying the clinical signs, pathological changes, viral load and cytokine production in piglets infected with PEDV. Different doses of GML have been used as feed additive to elucidate the protective effects of GML on PEDV infected piglets to provide new ideas for the treatment of this dangerous viral infectious disease.

Materials and methods

Materials and reagents

PEDV SC strain was identified and preserved by the Animal Biotechnology Center of Sichuan Agricultural University; Total of 25 piglets were purchased from Chengdu Wangjiang Agricultural and Livestock Technology Co Ltd; GML was prepared by Guangdong Nuacid Biotechnology Co Ltd; PrimeScript™ RT kit (PerfectRealTime), DNA/RNA extraction kit, TB Green® PremixExTaq™ (TliRNaseHPlus) were purchased from Takara (Dalian) Engineering Co Ltd.

Animals and grouping

Pigs were reared in the experimental condition according to the environment of a large-scale farm to prevent stress and infection with fullness a full-price feed and water. The piglets were observed for 7 days and no abnormalities were found before experiment. The piglets (6-week-old) were divided into 5 groups ($n = 5$). A1, A2 and A3 were the low, medium and high dose treatment groups respectively, B was the blank control group and C was the virus control group. According to the previous experimental experience, the challenge dose of 10TCID50 was selected, and 30 mL of liquid was orally administered to each pig in groups A and C. Group B received 30 mL normal saline orally. On the 1st, 2nd and 3rd day after challenge, 5, 10 and 20 g of GML were added and mixed into the diet of groups A1, A2 and A3, respectively, while groups B and C were given the same weight of normal saline as GML. Detailed information is shown in Table 1.

Clinical symptoms observation and sample collection

All pigs were examined daily for signs of diarrhea, depression, and anorexia signs. The body weight of each pig was measured at 1 days (6 weeks of age) and once a day thereafter until the end of the study (7 days). The average piglets weight (APW; kilograms) was analyzed at 7th day. Blood was collected from all pigs at 0 and 3 days in 5 ml serum separator tubes. The blood was centrifuged at 2,000 g for 10 min and serum aliquots were stored at −80°C until the cytokine testing. Feces were collected from day 1 to day 7 and dissolved in 1.5-ml centrifuge tubes with PBS. Feces were stored at −80°C until viral load testing.

All pigs were executed on the 7th day of the virus attack and intestinal tissues and mesenteric lymph nodes were immediately collected and stored at −80°C. Some of the intestinal tissues and mesenteric lymph nodes were fixed with 4% paraformaldehyde.

Histopathological observations

The 4% paraformaldehyde-fixed ileum tissue and mesenteric lymph nodes was dehydrated, permeabilized, embedded, sectioned, dewaxed, rehydrated, stained and sealed, and HE-stained sections were prepared to observe histopathological changes under a light microscope and photographed.

RT-qPCR quantification of PEDV load

Piglet intestinal tissues, mesenteric lymph nodes and feces were collected, ground and mixed thoroughly with 3.0 mL PBS and centrifuged at 12,000 r/min for 3 min. Total RNA from the small intestine,lymph nodes and feces were extracted using RNA iso Plus (Takara, Dalian, China) reagent. Then cDNA was synthesized using PrimeScript® RT reagent kit with gDNA Eraser (Takara, Dalian, China).

TABLE 1 Information on piglet grouping, attack and dosing.

Grouping	Number	Toxic dose	Toxic dose (infusion)	Sample No. 1	Time point of administration
A_1	5	10^5 $TCID_{50}$	30 mL	5 g	
A_2	5	10^5 $TCID_{50}$	30 mL	10 g	
A_3	5	10^5 $TCID_{50}$	30 mL	20 g	Days 1, 2 and 3 after the attack
B	5	Virus-free cytosol	30 mL	Sanitary saline	
C	5	10^5 $TCID_{50}$	30 mL	Sanitary saline	

TABLE 2 RT-qPCR reaction system of PEDV ORF1 gene.

Primer F	0.5 μL
Primer R	0.5 μL
ddH2O	3 μL
SYBR Green Premix Ex Taq II	5 μL
cDNA	1 μL
Total	10 μL

Real-time quantitative PCR was performed using SYBR® Premix Ex Taq™ (Tli RNaseHPlus) (Takara, Dalian, China). The relative viral load levels were evaluated by detection of ORF1 gene expression of PEDV. The upstream primer for detection of PEDV ORF1 gene was AAATGGGAAGTCGGCAGA, and the downstream primer sequence was GTTTTGTTGTGGCGGTAG. The reaction system is shown in Table 2; the reaction procedure was pre-denaturation at 95°C for 30 s; 95°C for 5 s; 60°C for 30 s; 40 cycles were performed. Melting curve: 95°C, 65°C increasing 0.5°C per second to 95°C.

Cytokine assay

The systemic cytokine profile (i.e., IFN-α, IFN-γ, IL-1β, IL-6, IL-8, IL-10 and TNF-α) was evaluated in serum samples collected at 0, and 3 days p.i.All cytokine tests were performed according to the kit (Elabscience) instructions.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Significant differences were determined using one-way analysis of variance (ANOVA) and multiple samples were analyzed in SPSS 20.0 (IBM Corp., Armonk, NY, USA) using Duncan's method and considered statistically significant at $p < 0.05$. One-way analysis of variance (ANOVA) was conducted with the Graph Pad Prism 5 software; $P < 0.05$ was considered a statistically significant difference.

Results

Clinical symptoms

As shown in Table 3, one pig died in group A1 on 3th day after the virus attack. No piglets died in the group A_2, the group A_3 and the group B. In the group C, two piglets died on second day, one on the fourth day and the other on the sixth day after infection, and only one piglet survived until 7 d. Two days after PEDV infection, piglets in group A appeared clinical symptoms such as diarrhea, anorexia and depression, and recovered after GML administration. The symptoms were similar in groups A_2 and A_3. Group B showed no significant changes.

Change in piglet weight

The piglets in group B showed normally body weight gain. The piglets in group C continued to lose weight until death (Figure 1). The piglets in group As lost a slight amount of body weight in the first 3 days after the virus attack. But these piglets started to recover bodyweight gain after the treatment of GML. The results showed that GML was effective in slowing down the weight loss of piglets and subsequently restoring their growth performance.

Necropsy and histopathological examination were performed

The intestinal cavity of the piglets in group C was filled with yellow contents and gas, the intestinal wall was thin, translucent and elastic, and the mesenteric lymph nodes were obviously enlarged, showing typical symptoms of PEDV. Histopathological examination (ileum) showed that there were no obvious abnormalities in intestinal structure and mesenteric lymph nodes between group B and group A. Histopathological examination showed that the intestinal morphology of group B was normal and neatly arranged. The intestinal villi, goblet cells and other structures were clearly visible (Figure 2A), and

TABLE 3 Statistics of clinical symptoms in each group after the attack.

Grouping	Symptoms	Time after attack (days)						
		1	2	3	4	5	6	7
A1	Diarrhea	0/5	4/5	4/4	3/4	3/4	1/4	0/4
	Depressed spirit	0/5	5/5	4/4	4/4	4/4	4/4	1/4
	Anorexia	0/5	2/5	2/4	2/4	1/4	0/4	0/4
A2	Diarrhea	0/5	4/5	5/5	3/5	3/5	1/5	0/5
	Depressed spirit	0/5	5/5	5/5	5/5	5/5	3/5	3/5
	Anorexia	0/5	3/5	3/5	1/5	1/5	0/5	0/5
A3	Diarrhea	0/5	4/5	5/5	3/5	3/5	1/5	0/5
	Depressed spirit	0/5	5/5	5/5	5/5	5/5	3/5	3/5
	Anorexia	0/5	3/5	2/5	1/5	0/5	0/5	0/5
B	Diarrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Depressed spirit	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Anorexia	0/5	0/5	0/5	0/5	0/5	0/5	0/5
C	Diarrhea	0/5	3/5	3/3	2/2	2/2	1/1	1/1
	Depressed spirit	0/5	3/5	3/3	2/2	2/2	1/1	1/1
	Anorexia	0/5	3/5	3/3	2/2	2/2	1/1	1/1

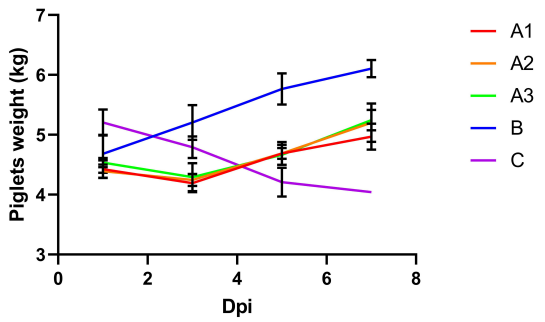


FIGURE 1
After challenge, group A was treated with 5, 10 and 20 g GML on day 1, 2 and 3, respectively; group B was not challenged or given drugs; group C was not given drugs after challenge, and the weight of piglets in different groups changed with time. Different colors represented different groups. The body weight of piglets in group A decreased from day 1 to day 3 and increased from day 4 to day 7. The weight of piglets in group B increased over time. The weight of piglets in group C decreased over time.

the lymph nodes were normal (Figure 2D). In group A1, the intestinal villi were slightly damaged, but other structures were normal (Figure 2B). The number of inflammatory cells such as plasma cells and macrophages increased slightly (Figure 2E). No obvious pathological changes were found in groups A2 and A3. In group C, the intestinal villi were ruptured or atrophied, shortened, partially damaged, and some intestinal epithelial cells were necrotic and exfoliated, accompanied by inflammatory cell infiltration (Figure 2C). Lymph node inflammatory cells were increased and the tissue was edematous (Figure 2F).

PEDV load in intestinal tissues and feces

As shown in Figure 3A, the fecal viral load in group A peaked on day 1 and then decrease. No PEDV was detected in group B. In group C, the viral load continued to increase to its peak until death; the viral load in both tissues in group A was approximately equal and much lower than that in group C (Figure 3B). The results suggest that GML can reduce the viral load effectively in the feces, intestine and mesenteric lymph nodes of PEDV-infected piglets.

Cytokine assay

As shown in Figure 4, pro-inflammatory cytokines were significantly increased in Group A and Group C on the third day after infection of PEDV. Compared with group B, the levels of IFN-γ, IL-1β and IL-6 increased significantly in group A, while the dose of GML has a negative correlation with the increasing trend of IL-1β and IL-6. Compared with group C, the levels of IL-8 and TNF-α were significantly lower than group A. GML decreased the levels of IL-1β, IL-6 and IL-10 in a dose dependent manner.

Discussion

PEDV is an acute, highly contagious and highly lethal virus to pigs, causing huge economic losses to the pig industry. Currently, PEDV is mutating at a rapid rate and is on an outbreak trend, with widespread epidemics in China (10).

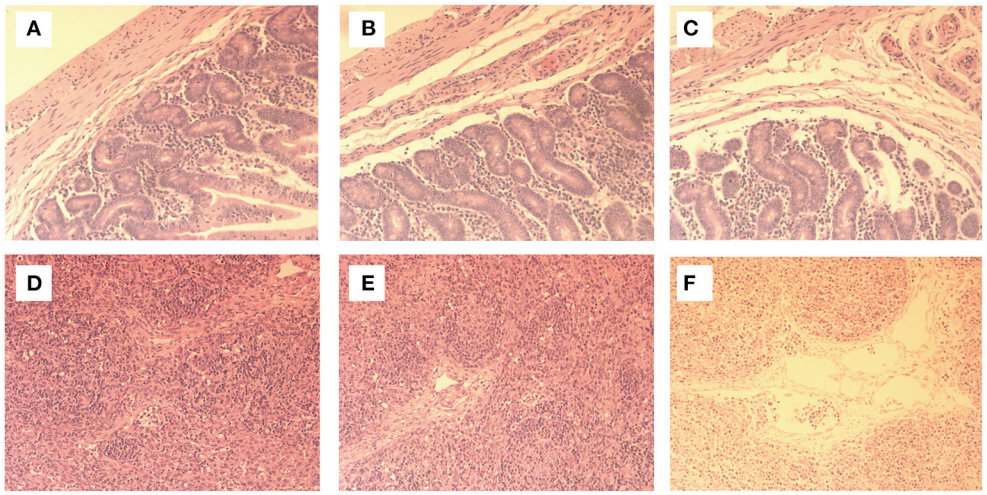


FIGURE 2
On the 7th day of the experiment, the intestines and mesenteric lymph nodes of the piglets were sectioned and fixed and made into tissue sections for observation. Histopathological examination showed that the morphology of group B was normal and neatly arranged, with intestinal villi, goblet cells and other structures clearly visible **(A)**. The morphology of lymph nodes was normal, without histopathological changes **(D)**. In group A1, the intestinal villi were slightly damaged and the other structures were normal **(B)**. Some lymph node cells were enlarged **(E)**. There were no obvious pathological changes in A2 and A3 groups. In group C, intestinal villi were broken, atrophic and shortened, and some intestinal villi were damaged. Some intestinal epithelial cells were necrotic and exocytic, accompanied by inflammatory cell infiltration **(C)**, increased inflammatory cells in lymph nodes, and tissue edema **(F)**.

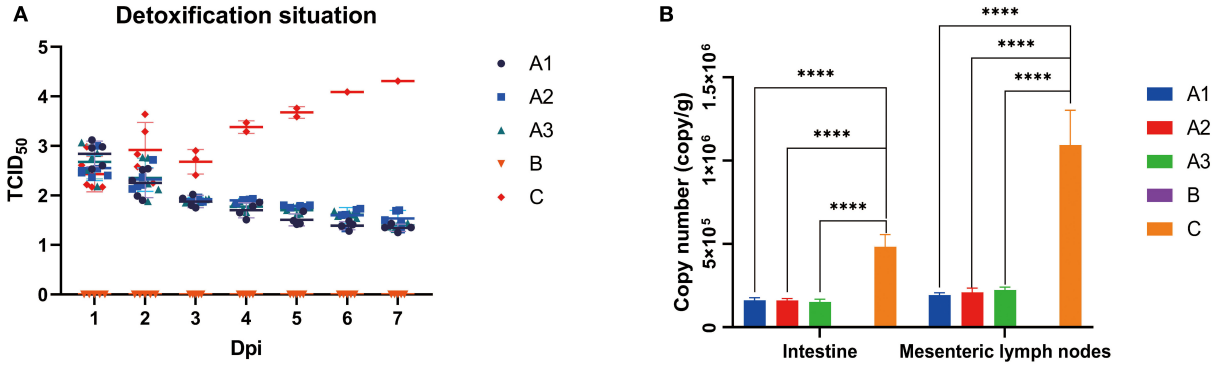


FIGURE 3
(A) shows the copy number of viruses detected by fluorescence quantification in feces of piglets in groups A, B and C on days 1–7. The copy number of viruses in groups A1, A2 and A3 decreased gradually with little difference over time, the copy number of viruses in group B increased gradually over time, and the copy number of viruses in group C was always 0. **(B)** shows the average virus copy number detected by fluorescence quantification in intestinal and mesenteric lymph nodes of piglets in group A, B and C after 7 days of challenge. In the small intestine, there was no significant difference in virus copy number between group A and group B, which was always 0, while the virus copy number in intestinal and mesenteric lymph nodes in group C was larger. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Existing PEDV vaccines hardly provide effective protection for piglets. Interferons are useful in the early stages of PEDV infection, but the high cost of treatment makes it not a good option (11). Studies indicate that laurate monoglycide has excellent antiviral effect and is a potential anti-PEDV drug (12). At the same time, as a natural active substance, it can be widely used, with non-toxic, harmless, and no residue characteristics. In this study, the results indicated that GML can reduce mortality, weight loss, virus load, pathological injury and inflammation of PEDV infected piglets.

Li et al. (13) showed that the addition of 0.4% α-GML could improve fat metabolism and growth performance of weaned piglets. Lan et al. (14) showed that the addition of 1,000 mg/kg α-GML to the diet significantly increased the average daily weight gain of weaned piglets, indicating that α-GML could improve the growth performance of piglets. Our study also

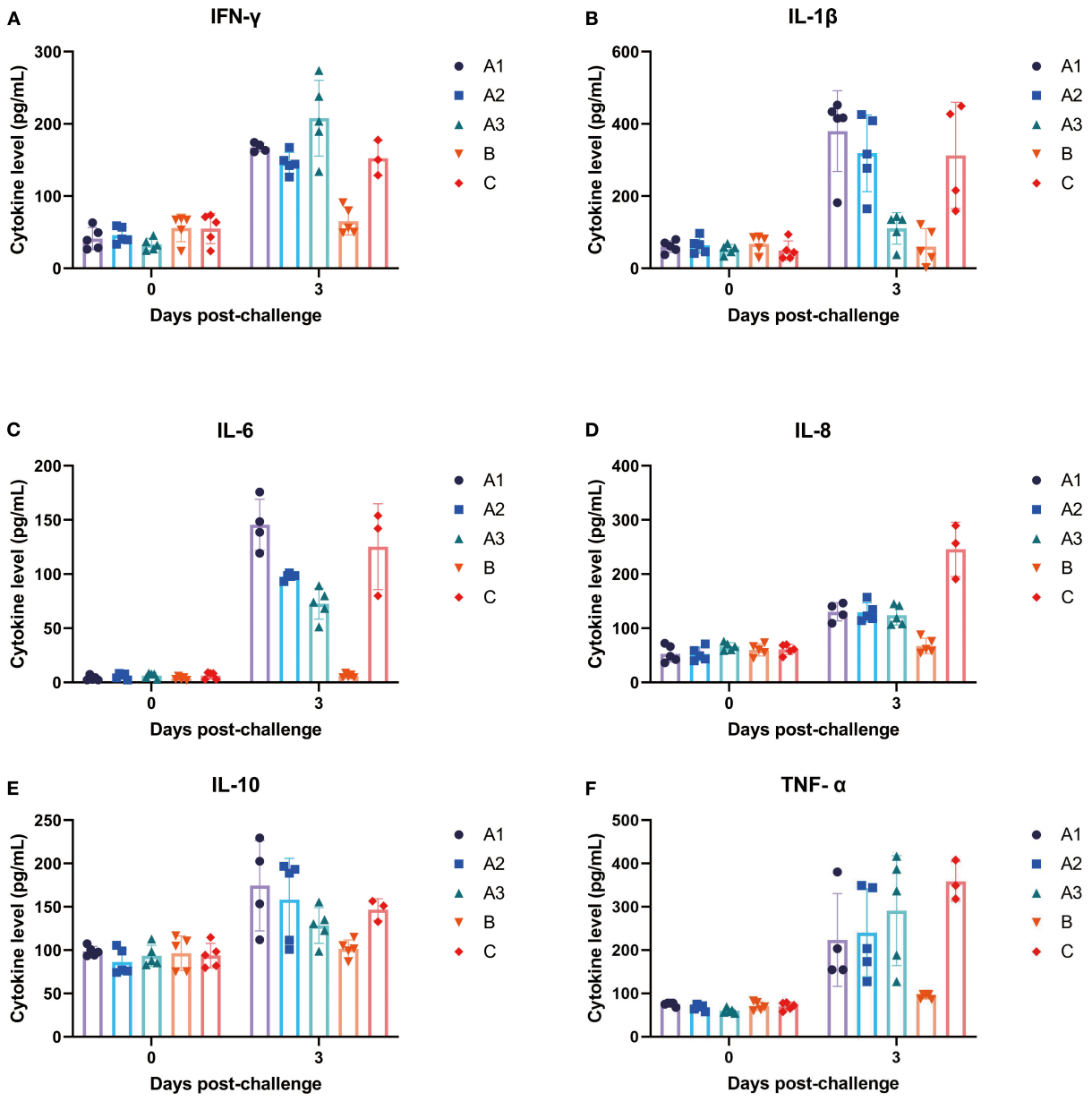


FIGURE 4
The changes of different cytokines in piglets at day 0–3 were detected. **(A)** IFN-γ, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8, **(E)** IL-10, and **(F)** TNF-α.

showed that GML was able to slow down the weight loss, restore their growth performance, and increase survival rate for PEDV infected piglets. The results of pathological changes also showed that GML could inhibit PEDV-induced intestinal epithelial cell necrosis.

PEDV infection mediates the body's production of SIgA, which plays an important role in intestinal mucosal immunity. Lin (15) studies have shown that the addition of α-GML to the diet can increase the titer of IgA in the colostrum of sows after vaccination and effectively prevent and control epidemic diarrhea in piglets. In addition, GML can significantly increase the level of SIgA in the ileum of piglets (16). In this trial, the viral load in the feces, intestine and mesenteric lymph nodes of piglets was significantly reduced in the low, medium and high dose groups of GML. This suggests that GML inhibits the proliferation of PEDV *in vivo*, probably because GML can insert into the virus capsule and disrupt the capsule structure of the virus (17), which directly reduces the infection titer of the virus and stimulates the production of SIgA to neutralize the virus, indirectly reducing the transmission and replication of PEDV.

Studies have shown that the addition of 1,000 mg/kg of α-GML to the basal diet significantly increased the relative abundance of the thick-walled phylum, while reducing the number of the phylum Bacteroides and Campylobacter, improving the intestinal structure and reducing the rate of diarrhea in weaned piglets (18). The results showed that the treatment of α-GML significantly increased the relative abundance of thick-walled bacteria and reduced the number of Bacteroides and Campylobacter. Jiang (19) found that the 150 mg/kg GML increased the relative abundance of intestinal butyric acid producing bacteria and the expression of propionic acid and butyric acid, which in turn reduced the permeability. The addition of GML to low protein diets increased the levels of the intestinal tight junction proteins claudin-1, occludin and ZO-1 (16). This is an effective way to ensure the integrity of the intestinal barrier in piglets. In this study, the intestinal morphology of piglets in the high and medium dose groups was intact, which is consistent with the findings of previous studies, indicating that different doses of GML have the ability to promote piglets' resistance to PEDV. We speculate that this may be related to the ability of GML to alter the structure of the intestinal flora, promote the expression of some intestinal tight junction proteins, reduce intestinal permeability and improve the biological and mechanical barriers of the intestinal mucosa.

Zhang et al. (20) showed that GML reduced the production of pro-inflammatory factors such as IL-2, IFN-γ, TNF-α and IL-10 induced by TCR, and the anti-inflammatory effect was dose-dependent within a certain range (18). Also they showed that high concentrations of GML reduced TNF-α levels in the blood of piglets. In the present study, a similar phenomenon was found, with the levels of TNF-α and IL-10 in the blood of piglets in the high-dose treatment group significantly reduced, and the inflammatory cell infiltration was suppressed. This indicated that GML was effective in alleviating PEDV-induced intestinal inflammation, while the elevated levels of IFN-γ were different from the results of previous studies, which may be related to the different levels of activation of the animals' own immune systems or caused by other ingredients in the samples. The effects of the components alone and in combination are unclear, and the existence of synergistic effects needs to be further explored.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the Sichuan Provincial Laboratory Animal Management Committee [License No: SYXK (chuan) 2019-187]. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

ZL, HT, YH, and LZha did the experiment. ZX and JL made important contributions to the analysis and manuscript compilation. HC and XZ conducted data analysis. LZhu and ZL wrote the first draft and revised the manuscript. XS helped with the analysis through constructive discussions. All authors proposed the research concept, directed the research activities, contributed to the article, and approved the submitted version.

Funding

This study was financially supported by the Sichuan Provincial Department of Science and Technology Rural Area Key R&D Program (Project Number: 2020YFN0147), the Sichuan Province's 14th Five-Year Plan Sichuan Pig Major Science and Technology Project (Project Number: 2021ZDZX0010), the Research and Demonstration of Key Technologies for Prevention and Control of Major Vertically Transmitted Diseases in Breeding Pigs (Project Number: 2022YFN0007).

Acknowledgments

The authors appreciate all the helps from our colleagues and collaborators.

Conflict of interest

Author XZ, JL, HC, and YH were employed by Innovation Center of Guangdong Nuacid Biotechnology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Wang L, Byrum B, Zhang Y. New variant of porcine epidemic diarrhea virus, United States, 2014. *Emerg Infect Dis.* (2014) 20:917–9. doi: 10.3201/eid2005.140195

2. Li W, Li H, Liu Y, Pan Y, Deng F, Song Y, et al. New variants of porcine epidemic diarrhea virus, China, 2011. *Emerg Infect Dis.* (2012) 18:1350–3. doi: 10.3201/eid1803.120002

3. Lee S, Lee C. Outbreak-related porcine epidemic diarrhea virus strains similar to US strains, South Korea, 2013. *Emerg Infect Dis.* (2014) 20:1223–6. doi: 10.3201/eid2007.140294

4. Jung K, Saif LJ, Wang Q. Porcine epidemic diarrhea virus (PEDV): an update on etiology, transmission, pathogenesis, and prevention and control. *Virus Res.* (2020) 286:198045. doi: 10.1016/j.virusres.2020.198045

5. Ansart MC, Girard-Globa A. Influence of dietary lipids on serum apoproteins AI and B and on intestinal synthesis of apo AI in the early weaning of piglets: comparison of saturated, unsaturated, and medium-chain triglycerides. *Am J Clin Nutr.* (1990) 51:47–52. doi: 10.1093/ajcn/51.1.47

6. He F, Liu H. Research and application of medium chain fatty acid triglyceride in animal production [J]. *Animal husbandry and veterinary medicine*. (2018) 50:137–40.

7. Jackman JA, Hakobyan A, Zakaryan H, Elrod CC. Inhibition of African swine fever virus in liquid and feed by medium-chain fatty acids and glycerol monolaurate. *J Anim Sci Biotechnol.* (2020) 11:114. doi: 10.1186/s40104-020-00517-3

8. Zhang X. Clinical evidence of alpha-monolaurate glycerides on the prevention and control of blue ear disease virus. *Pig Farming Today.* (2017) 4:103–5.

9. Joshi S, Kaushik V, Gode V, Mhaskar S. Coconut oil and immunity: what do we really know about it so far? *J Assoc Physicians India.* (2020) 68:67–72. doi: 10.1089/act.2006.12.310

10. Liu Y, Yao Y, Li R, Han W, Shi T, Wang Y, et al. Genetic variation analysis of porcine epidemic diarrhea virus in some regions of southwest China from 2020 to 2021. *China Vet Anim Husb.* (2022) 49:2279–90.

11. Zhao X, Yuan X, Jiang P, Li H, Wang Y. Trial and clinical application of recombinant porcine alpha interferon for the control of epidemic diarrhea disease in piglets. *Zhejiang Agric Sci.* (2017) 58:291–3. doi: 10.16178/j.issn.0528-9017.20170233

12. Jiang Z, Yang M, Du Z, Zhang H, Feng F. Antibacterial and antiviral properties of monoglycerides of lauric acid and their applications in foodstuffs. *J Chin Cereals Oils*. (2015) 30:142–6.

13. Li T, Wang C, Wan Z, Fang Y, Zhu A. Effect of coconut oil and glycerol monolaurate on productive performance and fecal microorganisms of weaned piglets. *Grain Feed Industry*. (2013) 6:54–7.

14. Lan J, Guo X, Tang J, Jia N, Zhang L, Xu Y, et al. Effect of α-monolaurin glycerides on growth performance, fecal microorganisms and serum immune factors in weaned piglets. *J Anim Nutr.* (2020) 32:1136–42. doi: 10.3969/j.issn.1006-267x.2020.03.020

15. Lin G. Diagnosis, treatment and preventive and control measures of epidemic diarrhea in pigs in a large-scale pig farm. *Fujian Anim Husb Vet Med.* (2018) 40:40–2.

16. Cui Z, Wang X, Hou Z, Liao S, Qi M, Zha A, et al. Low-protein diet supplemented with medium-chain fatty acid glycerides improves the growth performance and intestinal function in post-weaning piglets. *Animals.* (2020) 10:1852. doi: 10.3390/ani10101852

17. Hyldgaard M, Sutherland DS, Sundh M, Mygind T, Meyer RL. Antimicrobial mechanism of monocaprylate. *Appl Environ Microbiol.* (2012) 78:2957–65. doi: 10.1128/AEM.07224-11

18. Li L, Wang H, Zhang N, Zhang T, Ma Y. Effects of α-glycerol monolaurate on intestinal morphology, nutrient digestibility, serum profiles, and gut microbiota in weaned piglets. *J Anim Sci*. (2022) 100:skac046. doi: 10.1093/jas/skac046

19. Jiang Z. *Study on the Mechanism of Action of Monoglycerides of Lauric Acid on Growth and Health Based on Microbiomics and Metabolomics*. [dissertation/doctoral thesis]: Hangzhou: Zhejiang University (2018).

20. Zhang MS, Sandouk A, Houtman JC. Glycerol monolaurate (GML) inhibits human T cell signaling and function by disrupting lipid dynamics. *Sci Rep.* (2016) 6:30225. doi: 10.1038/srep30225

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 07 November 2022
DOI 10.3389/fvets.2022.972326

OPEN ACCESS

EDITED BY
Dayou Shi,
South China Agricultural University, China

REVIEWED BY
Juan Liu,
Southwest University, China
Jingui Li,
Yangzhou University, China

*CORRESPONDENCE
Hualin Fu
fuhl2005@sohu.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 18 June 2022
ACCEPTED 12 September 2022
PUBLISHED 07 November 2022

CITATION
Li D, Wang Y, Liu N, Chen S, Liu H, Wang P, Yu Z, Shu G, Lin J, Zhang W, Peng G, Zhao L, Tang H, Zhang K, Wen B and Fu H (2022) Modified Sijunzi granule decreases post-weaning diarrhea in Rex rabbits *via* promoting intestinal development. *Front. Vet. Sci.* 9:972326. doi: 10.3389/fvets.2022.972326

Modified Sijunzi granule decreases post-weaning diarrhea in Rex rabbits *via* promoting intestinal development

Dongbo Li[1†], Yueli Wang[1†], Ning Liu[2†], Shiqi Chen[1], Hanzhong Liu[2], Ping Wang[2], Zhiju Yu[2], Gang Shu[1], Juchun Lin[1], Wei Zhang[1], Guangneng Peng[1], Ling Zhao[1], Huaqiao Tang[1], Kai Zhang[2], Bin Wen[2] and Hualin Fu[1]*

[1]Department of Pharmacy, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, China, [2]Sichuan Academy of Grassland Sciences, Chengdu, China

Traditional Chinese medicine (TCM) formulas can be adjusted on the basis of TCM basic theory to achieve the best curative effect, especially for diseases with complex pathogenesis, such as post-weaning diarrhea (PWD). Shugan Jianwei Sijunzi decoction (SJ-SJZD) can be recognized as modified Sijunzi Decoction (SJZD) supplemented with *Astragalus mongholicus Bunge, Bupleurum chinense DC, Citrus* × *aurantium L., and Crataegus pinnatifida Bunge (fruit)* in a fixed dosage ratio. The inactive ingredients were subsequently added to make granule, which was Shugan Jianwei Sijunzi granule (SJ-SJZG). Previous studies have confirmed the antagonism of SJ-SJZG to PWD. However, the mechanism of SJ-SJZG protective effects on small intestine in weaned Rex rabbits remained unclear. Animals were randomly divided into negative control (NC), low dose (LD), medium dose (MD), high dose (HD), and positive control (PC). SJ-SJZG significantly increased the intestinal length and the jejunum villi length. The SIgA level was statistically increased in duodenum and jejunum with the ELISA. Immunohistochemical detection showed that SIgA protein expression was also increased significantly in jejunum. Meanwhile, the relative expression of Zo1 in duodenum and jejunum of SJ-SJZG group increased significantly. SJ-SJZG significantly increased the relative expression of occludin in duodenum and jejunum as well. Moreover, real-time PCR results showed a significant increase in GLUT2 and SGLT1 relative expression in ileum. SJ-SJZG could also obviously enhance the expression of GLUT2 in jejunum and the expression of SGLT1 in duodenum. In conclusion, SJ-SJZG had been proven to be effective in promoting the development of small intestine and improving the immunity of small intestine. Moreover, SJ-SJZG could ensure the integrity of mucosal barrier and increase the ability of intestine to absorb glucose in small intestine.

KEYWORDS

modified Sijunzi granule, post-weaning diarrhea, SIgA, intestinal mucosal structure, tight junction protein, glucose transporter

Introduction

Rex rabbit, as a great economic return animal, has excellent fur and grows rapidly (1). However, Rex rabbits are prone to diarrhea and even death after weaning, which is called post-weaning diarrhea (PWD), reducing their breeding and affecting economic benefits (2). It is due to the incomplete intestinal development of weaned Rex rabbits. When Rex rabbits were weaned, due to the lack of breast milk antigen, the intestinal immunity decreased rapidly (3). Moreover, the tight junction between the small intestinal epithelial cells of weaned rabbits was not as close as adult rabbits. The intestinal wall was very thin and the intestinal permeability is high, which lead to the destruction and invasion of intestinal mucosa by incomplete digestion products and toxins (3). The villi and epithelial cells of the small intestine of weaned Rex rabbits were further damaged, which exacerbated the symptoms of diarrhea and led to death.

Sijunzi Decoction (SJZD), as a traditional Chinese medicine (TCM) prescription, was well-known for treating disorders of digestive function manifested by poor appetite, indigestion, and diarrhea and mostly used to treat spleen (Qi) deficiency. In TCM theory, the Qi deficiency will lead to a lack of biochemical source and blood, poor food digestion and absorption, and decline of body immunity, leading to functional diarrhea and other gastrointestinal diseases, which are also the symptoms of stress-induced diarrhea in weaned rabbits. Modern research had confirmed the mechanism of SJZD in the treatment of spleen deficiency (4). Recent studies had also shown that SJZD could regulate the expression of intestinal immune factor genes and proteins (5). Besides that, researchers revealed that SJZD may promote intestinal epithelial restitution after wounding (6). Hence, SJZD had great potential in the treatment of diarrhea symptoms of weaned Rex rabbits. Accumulated studies had shown that SJZD can enhance intestinal immunity, promote intestinal development, and restore intestinal barrier function (7, 8).

Moreover, SJZD has also been modified to own wider pharmacological actions based on the original major formula and TCM theory to fit different clinical demands. All modified SJZD used SJZD as a major formula and combined it with the other TCMs that possess synergistic or additive activity to promote the function of the SJZD. For example, supplementary SJZD can improve the digestion and absorption of small intestine and promote the expression of small intestinal growth factor (9); Qilan SJZD can increase the mRNA expression level of small intestinal cytokines and improve the level of cellular immunity (10); modified SJZD can enhance intestinal absorption of nutrients (11).

In the present study, we added *Astragalus mongholicus Bunge, Bupleurum chinense DC, Citrus × aurantium L.*, and *Crataegus pinnatifida Bunge (fruit)* to the formula of the original SJZD, *Codonopsis pilosula (Franch.) Nannf*, *Atractylodes Lancea (Thunb.) DC*, *Wolfiporia cocos*, *Glycyrrhiza uralensis Fisch. ex DC* (Preparata), as a modified SJZD, which is called Shugan Jianwei Sijunzi Decoction. Shugan Jianwei Sijunzi granule (SJ-SJZG) was used as a granulated extract obtained by adding inactive ingredients to the hot water extract of a mixture of the above eight crude herbs.

Previous studies had shown that the protective effect of SJ-SJZG on PWD in Rex rabbits was confirmed by growth performance, diarrhea frequency, and mortality, but the mechanisms were unclear (12, 13). To further explore the development effect of SJ-SJZG on weaned Rex rabbits, especially on intestinal development, we investigated the effects of SJ-SJZG on the morphological structure of small intestine, mucosal immunity, and the gene expression of tight junction protein and glucose transporter in weaned Rex rabbits, aiming to provide a theoretical basis for the research of SJ-SJZG and its application in production.

Materials and methods

Chemicals and reagents

Glutamine was purchased from Huana Chemical Co., Ltd. (China). HE staining (hematoxylin and eosin) was purchased from Thermo Fisher Co., Ltd. (America). SIgA ELISA kits were purchased from Nanjing Jiancheng Bioengineering Institute. SIgA rabbit polyclonal antibody was obtained from Youning Weisheng Technology Co., Ltd. (Shanghai). FastKing RT kits were purchased from Tiangen Biotech Co., Ltd. (Beijing). The other reagents were purchased from Chengdu Chron Chemicals Co., Ltd. (China).

Animals

A total of 160 healthy Sichuan White Rex rabbits, half male and half female, aged 45 days and weighing about 850 g were provided by Rex Rabbit Research Institute of Sichuan Academy of grassland sciences. The animals were provided with basal diet and tap water at liberty and maintained in cages under controlled conditions (23 ± 2°C, 12-h light/dark cycle). All experiments and procedures were carried out according to the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China. The composition and nutrient levels of the basal diet are listed in

Abbreviations: PWD, post-weaning diarrhea; TCM, traditional Chinese medicine; SJZD, Sijunzi Decoction; SJ-SJZD, Shugan Jianwei Sijunzi decoction; SJ-SJZG, Shugan Jianwei Sijunzi granule; NC, negative control; LD, low dose; MD, medium dose; HD, high dose; PC, positive control. HPLC, high-performance liquid chromatography; SIgA, secretory immunoglobulin A; H&E, histopathological examination.

TABLE 1 The composition and nutrient levels of basal diet (air-dry basis) %.

Items	Content	Nutrient levels	Content
Ingredients		Digestible energy (DE, MJ/kg)	10.21
Corn	20.0	Crude protein (CP)	16.00
Bran	22.8	Crude fiber (CF)	14.29
Peanut vine	40.1	EE	3.02
Soyben meal	14.6	Met+Cys	0.46
NaCl	0.5	Lys	0.56
$CaHPO_4$	1.0	Ca	0.58
Premix	1.0	P	0.62
Total	100	I (mg/kg)	0.22

The premix provided following per kilogram of diet: Lys 1.5 g; VA 8,000 IU; VD 3 1,000 IU; VE 50 mg; Fe 100 mg; Cu 50 mg; Mg 150 mg; Zn 50 mg; Mn 30 mg; Se 0.1 mg. Nutrition levels were calculated values.

Table 1. After 7 days of adaptive feeding, animals were randomly divided into five groups, 32 in each group, including negative control (NC), low dose (LD), medium dose (MD), high dose (HD), and positive control (PC). The NC group was fed with the basic diet above. SJ-SJZG (0.5, 1, and 2%) was administrated in LD, MD, and HD groups, respectively, as a dietary supplement for 30 consecutive days. Similarly, for PC group, SJ-SJZG was replaced by 0.8% glutamine. In each group, six rabbits were randomly selected, weighted, and sacrificed on the 15th day after treatment, respectively. The intestinal length and relative weight were measured after washing by physiological saline. Then, two samples of duodenum, jejunum, and ileum were collected, respectively, fixed with 4% paraformaldehyde, or preserved in liquid nitrogen. On the 30th day of the experiment, another six rabbits in each group were killed at random, and the sampling was as above. All animal procedures were in accordance with the national standard, Laboratory Animal-Requirements of Environment and Housing Facilities (GB14925-2001), the Sichuan Agricultural University Institutional Animal Care and Use Committee under permit number CSQ-2018203003. The detailed experimental design is shown in Figure 1.

Preparation of SJ-SJZG

SJ-SJZG was prepared according to the method of the previous studies but a little improvement in our research group (12). In brief, eight herbs, Codonopsis pilosula (Franch.) Nannf, Atractylodes Lancea (Thunb.) DC, Wolfiporia cocos, Glycyrrhiza uralensis Fisch. ex DC (Preparata), Astragalus mongholicus Bunge, Bupleurum chinense DC, Citrus × aurantium L., and Crataegus pinnatifida Bunge (fruit) with a dosage ratio of 9:9:9:6:10:6:6:6, were mixed and wetted with distilled water (1:8, w/v) for 2 h. Then, we boiled the herbs for 3 h, filtered it with multi-layer gauze, and collected the filtrate. The herbs were added with water (1:6, w/v) and boiled for 3 h twice, filtered with multi-layer gauze similarly. All the filtrates were mixed and concentrated for granulation. The extracts were granulated by adding inactive ingredients and wetting agents. The concentration of SJ-SJZG was regarded as each gram of granules contained one gram of raw plant drugs. All the above herbs were purchased from Sichuan C&Y Traditional Chinese Medicine CO., LTD.

High-performance liquid chromatography (HPLC)

The SJ-SJZG was analyzed using high-performance liquid chromatography (HPLC) with Corona ultra-detection (CAD) in Agilent HPLC system. In brief, 5 g SJ-SJZG was dissolved in 10 mL 70% methanol solution and diluted 10 times. The solution was subjected to centrifugation at 1,500 rpm and 15 min, and the supernatant was filtered through a 0.22-μm filter. Subsequently, 20 μL supernatant was injected into HPLC system for analysis. The chromatographic conditions were as follows: Reverse-phase column (Inertsil ODS-3, 5 μm, 4.6 mm× 250 mm I.D.) connected with a guard column (C18, 5 μm, 4.6 mm×10 mm I.D.). The elution flow rate was 1.0 mL/min with a mobile phase gradient of A-B (A: H_2O/H_3PO_4 = 1,000 mL/2 mL; B: CH_3CN), which was varied as follows: 0min, 90% A, 10% B; 9∼29 min, 90∼85% A, 10∼15% B; 29∼37 min, 85∼82% A, 15∼18% B; 37∼46 min, 82% A, 18% B; 46∼51 min, 82∼80%A, 18∼20% B; 51∼72 min, 80∼72% A, 20∼28% B; 72∼82 min, 72% A, 28% B; 82∼87 min, 72∼70% A, 28∼30% B; 87∼92 min, 70∼67% A, 30∼33% B; 92∼99 min, 67∼50% A, 33∼50% B; 99∼110 min, 50% A, 50% B; 110∼115 min, 50∼47% A, 50∼53% B; 115∼118 min, 47∼30% A, 53∼70% B; 118∼130 min, 30% A, 70% B; 0min, 90% A, 10% B; 130∼135 min, 30∼90% A, 70∼10% B; 135∼140 min, 90% A, 10% B. The injection volume was 20 μl, and the UV detection wavelength was set at 285 nm for saikoside A, liquiritin, and hesperidin and 250 nm for glycyrrhizic acid. In the quantitative analysis of active components of SJ-SJZG, the concentrations of saikoside A, liquiritin, hesperidin, and glycyrrhizic acid were 0.645, 0.652, 1.161, and 0.398 mg/g in SJ-SJZG, respectively (Figure 2).

Histopathological examination (H&E) staining

The small intestine tissues fixed with 4% paraformaldehyde were washed with running water for 30 min. Subsequently, they were put into the pathological embedding plastic basket for dehydration to transparent with gradient alcohol and finally embedded in paraffin. The tissues were sliced into 5 μm thick slices by the slicer (Ultra-Thin Semiautomatic Microtome,

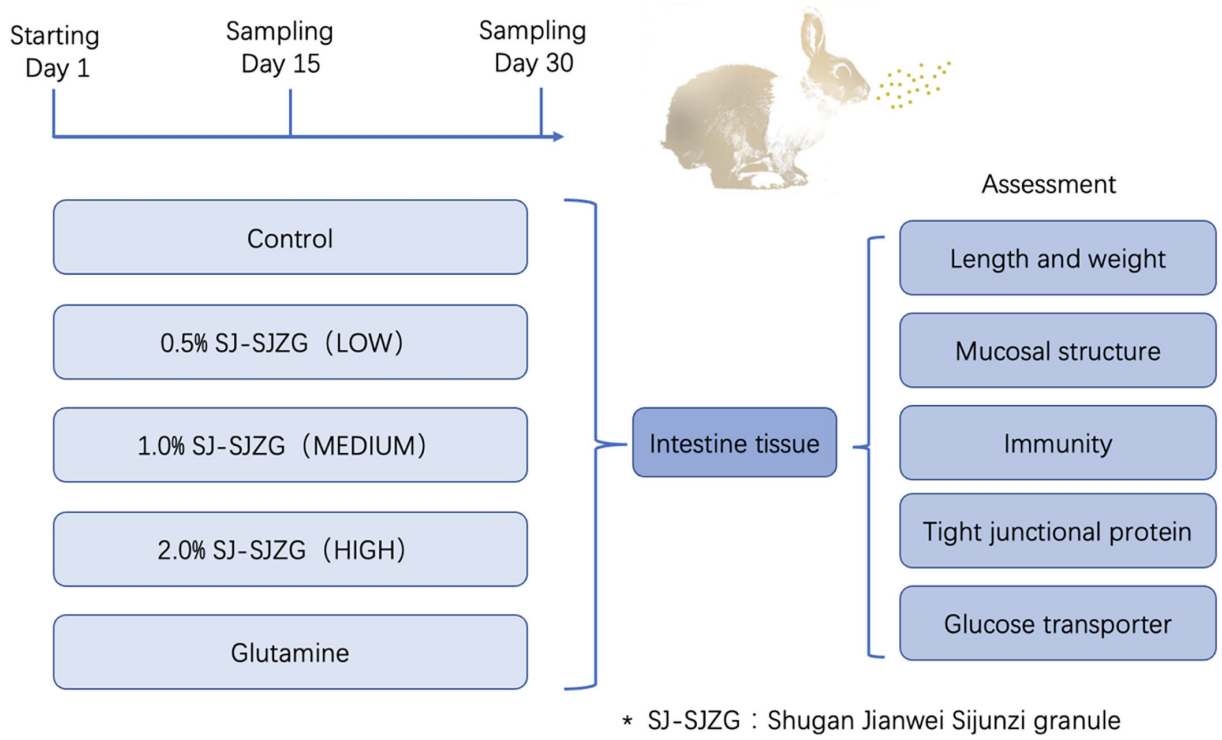


FIGURE 1
Experimental design of this study.

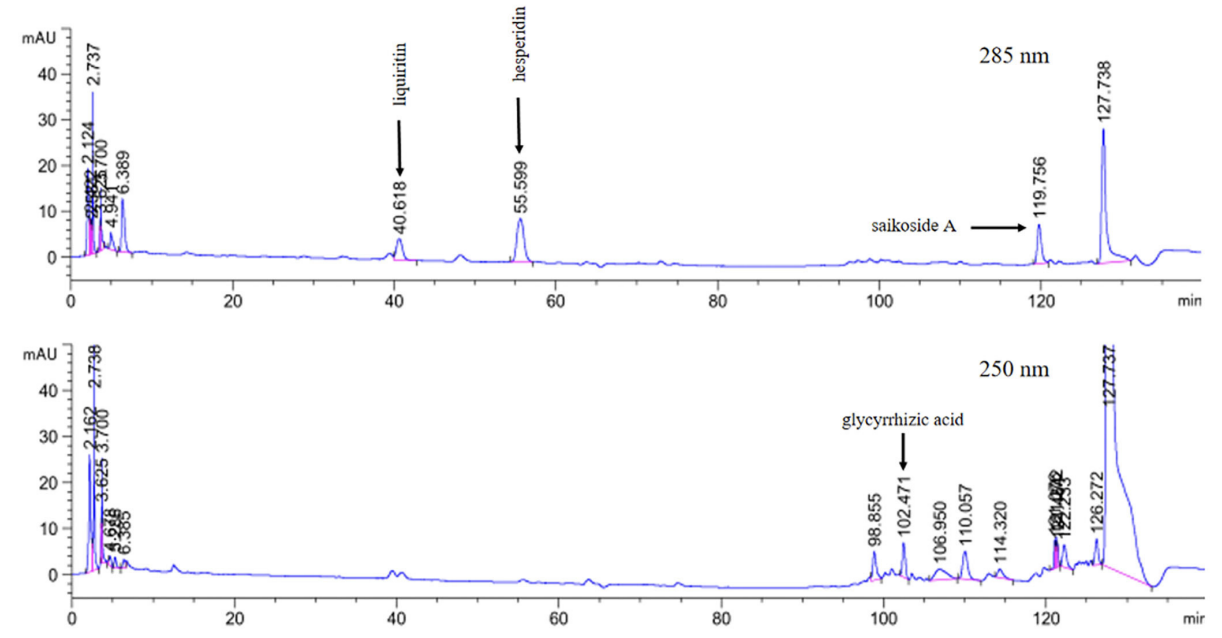


FIGURE 2
Qualitative and quantitative analysis of SJ-SJZG. The retention times of liquriritin, hesperidin, and saikoside A at 285 nm were 40.62, 55.60, and 119.76 min respectively; the retention times for glycyrrhizic acid at 250 nm was 102.47 min.

RM2235, from Leica, Germany), flattened in warm water, fished on the slide, and baked at 60°C for 2h. Then, the sections were placed in xylene to remove the paraffin and stained with hematoxylin and eosin. Finally, the tissues were dehydrated with gradient alcohol, put in xylene making them transparent, and sealed with resin glue.

Histomorphology

The slides were observed under the CX22 microscope (Leica, Germany), and the whole tissue slice was browsed completely by DM 1000 Leica microscopic imaging system. Image pro Plus 6.0 (Media Cybernetics, America) was used to measure the length of villi and the depth of crypt, and at least 10 intact villi and corresponding crypts in intestinal tissue were measured. Their ratio was calculated as follows (Equation 1).

$$R_{\left(\frac{V}{C}\right)} = \frac{L_{villi}}{D_{crypt}} \quad (1)$$

Where L_{villi} and D_{crypts} stand for the length of villi and the depth of crypt, respectively.

Immunohistochemical staining

The abundance of SIgA was assessed for paraffin-embedded slides after the sections were dewaxed. Endogen biotin and non-specific signals were blocked with appropriated reagents. Antigen retrieval was carried out in a microwave oven (two cycles for 5 min each at 780 W, in citrate buffer, pH 6.0, twice washed in PBS for 5 min each). Then, the treated slides were overnight incubated with primary antibodies at −4°C in a humidified chamber, washed in PBS, and visualized by biotinylated secondary antibodies followed by staining by DAB kit for 2 min and washed in distilled water, and finally counterstained with hematoxylin, dehydrated, transparentized, and sealed. At least 10 fields of view from each sample by BA400 Digital (Motic China Group CO., LTD.) were analyzed with the imaging system for each protein of interest. All tissues were observed under 100 times of each slice, and then, three visual fields were selected to collect 400 times of microscopic images, respectively. The integrated optical density (IOD) and area of all collected images are measured by Image pro Plus 6.0, and the mean density of each image is calculated. The average optical density of three images is used to calculate the average, and the average optical density of each sample is obtained.

ELISA

The value of SIgA on the intestinal mucosa was assayed by ELISA kits according to the manufacturer's instructions. The absorbance value of the sample was determined by Varioskan Flash (Thermo Scientific). The data were processed by ELISA scale. The logistic curve (four-parameter) model was used to fit the standard curve between the concentration and the OD value of the standard, and the regression equation is obtained. Then, the OD value of each well sample was brought into the regression equation to calculate the SIgA level.

Tissue RNA extraction and qRT-PCR assay

Total RNA was isolated from samples of jejunum, ileum, and colon with TRIzol regent (Tiangen Biotech, Beijing) and then treated with DNase I (Tiangen Biotech, Beijing) according to the manufacturer's instructions. Subsequently, we tested the integrity, purity, and concentration of RNA. Fastking cDNA first-strand synthesis Kit, Super real premix plus, 2 × Taq PCR mastermix, and 5 × RNA loading buffer were purchased from Beijing Tiangen Biochemical Technology Co., Ltd. The PCR reaction process was as follows: 95°C for 2 min, followed by amplification in 40 cycles of 95°C for 5 s, 15 s at 60°C, and 20 s at 72°C, and then 65°C and 95°C for 5 s, using the C1000 Touch^{TM} Thermal Cycler Real-Time System (Bio-Rad). Referring to the data of the National Center for Biotechnology Information (NCBI) database, fluorescent quantitative-specific primers were designed by primer 5.0 software. All primers are synthesized by Thermo Fisher Technology Co., Ltd. and are presented in Table 2. GAPDH was used as an internal control to normalize the expression of target gene transcripts.

Statistical analysis

The one-way analysis of variance (ANOVA) was used to analyze experimental data by SPSS 25.0 software. All values were presented as the mean ± SD, and $P < 0.05$ was considered statistically significant.

Results

Growth-promoting effects of SJ-SJZG on small intestine

The length and relative weight of small intestine, reflecting development degree and functional strength, were measured in the two periods, Day 15 and Day 30. The results indicated that SJ-SJZG could increase the small intestinal length of weaned Rex rabbits. The HD group significantly increased the small intestinal length of weaned Rex rabbits. Compared with NC group, it increased by 12.74% at Day 15 and 12.96% at Day 30, respectively, and the same trend was observed

TABLE 2 Primer sequences for qPCR.

Gene	Geneback		Primer sequences (5′to3′)	bp	Temperature°C
Claudin1	DQ_993356	F	GGAAGATGATGAGGAGCAA	77	59°C
		R	AGCCCAGCCAGTGAAAA		
Occludin	XM_008262318	F	CTTGCCTGGGACAGAACCTA	121	59°C
		R	AGCCATAACCGTAGCCGTAA		
Zo1	XM_008269782	F	GACTGATGCGAAGACGTTGA	117	59°C
		R	GCAGAATGGATGCTGTCAGA		
SGLT1	EU_414633	F	TGTTCCGCAGGGACACTAA	75	59°C
		R	GGGATCAGGACGTAAAGAGG		
GLUT2	XM_01734	F	AGGCACTGTCCACCACC	161	59°C
		R	GTCTCCAAGCCACCCAC		
GAPDH	NM_001082253	F	TGCCACCCACTCCTCTA	163	59°C
		R	AGTAAGAGCCCTCAAACCACCGG		

in PC group. The relative weight of small intestine in SJ-SJZG groups also increased. However, there was no significant difference between SJ-SJZG groups and NC group due to the rapid growth of Rex rabbits. All the above results are shown in Table 3.

Effects of SJ-SJZG on intestinal mucosal structure

Histopathological examination was commonly used to evaluate the integrity of intestinal tissue. Collected at Day 15 and Day 30, the tissue samples of duodenum, jejunum, and ileum were made into sections for H&E staining. The 400× microscopic images are shown in Figure 3. The results of H&E staining assay demonstrated that in NC and LD groups, the part of intestinal villi shed, and the intestinal villi of intestine were shorter. The proper layer was wider, and the connective tissue of proper layer loosened. However, the results of HD and PC groups showed that the intestinal structure was integrity, stage was clear, and intestinal villus was neatly arranged in all groups. The tissue structure of the samples in MD group was normal, with no obvious histopathological damage found, and the mucosal epithelium showed cell abscission and necrosis occasionally. As shown in Tables 4–6, our studies showed that SJ-SJZG could increase the length of villi, the depth of crypt, and their radio, which is mentioned as $R_{(V/C)}$, and there was a dose-dependent effect in the LD, MD, and HD dose groups. Moreover, the villi length and $R_{(V/C)}$ were remarkably risen in jejunum in HD group at Day 15, and $R_{(V/C)}$ of PC group also increased significantly compared with the NC group at Day 15 (Table 5). As a whole, SJ-SJZG had better protection on intestinal mucosal mucosa at Day 15. The intestinal development of Rex rabbits in Day 15 was not sufficient compared the Rex rabbits at Day 30. The young rabbits could fully reflect the development and protection of SJ-SJZG on intestinal mucosal structure.

Promotion effects of SJ-SJZG on intestinal mucosal immunity

SIgA, as the most important antibody in intestinal mucosal immunity, was resistant to various bacterial pathogens. We tested the content of SIgA in the small intestine of weaned Rex rabbits by ELISA. At the same time, we located and quantitatively analyzed SIgA by immunohistochemical staining. The SIgA level in intestinal mucosa was significantly increased in HD group of duodenum and jejunum at Day 15 and Day 30 (Figures 4A,B). For the PC group, the SIgA content also increased significantly in duodenum at Day 15 and Day 30 (Figures 4A,B). However, there was no significant SIgA content in ileum between all groups. In addition, SIgA level in all groups had tendency that increased with time. Immunohistochemical results showed that the SIgA positive cell was mainly distributed in the cytoplasm (Figure 5A). Immunohistochemical detection showed that SIgA protein expression of jejunum was increased significantly in HD and NC group at Day 30 (Figure 5B). The results were consistent with the results of ELISA.

SJ-SJZG enhanced intestinal barrier function and increased the relative expression of tight junction protein, Zo1, Claudin1, and Occludin

The barrier function of intestine was closely related to tight junction protein. Zo1, claudin1, and occludin were all important members in the tight junction protein family. The expression

TABLE 3 Effects of SJ-SJZG on intestinal length and relative weight in weaned Rex rabbits.

Time	Item	Group				
		NC	LD	MD	HD	PC
Day 15	Intestinal length(cm)	273.67 ± 16.42^{a}	274.33 ± 36.87^{ab}	303.07 ± 24.96^{ab}	308.53 ± 33.03^{b}	305.00 ± 28.81^{ab}
Day 30		282.80 ± 27.57^{a}	289.40 ± 29.48^{ab}	306.53 ± 36.94^{ab}	319.47 ± 25.92^{b}	319.00 ± 17.70^{b}
Day 15	Relative weight(g/kg)	29.87 ± 5.30	31.90 ± 4.84	32.33 ± 2.35	33.21 ± 4.85	33.70 ± 4.02
Day 30		29.47 ± 1.19	31.63 ± 3.54	32.44 ± 3.11	31.28 ± 1.61	31.53 ± 4.75

Data are expressed as mean ± SD, (n = 6). In the same row, values with no letter or the same letter superscripts means no significant difference (P > 0.05), while with different small letter means no significant difference (P < 0.05). The same as below.

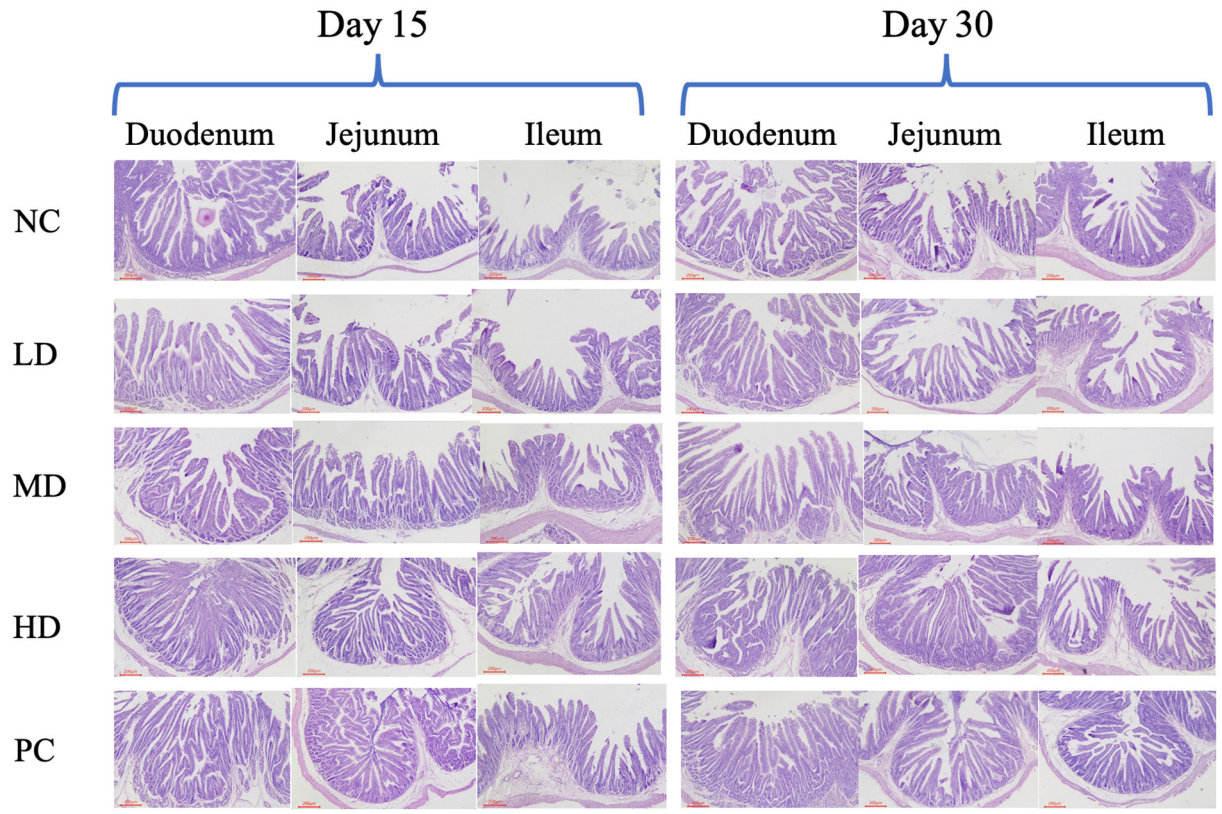


FIGURE 3
Intestinal tissue morphology was observed by H&E staining (HE × 400).

of Zo1 in duodenum and jejunum of HD group and PC group increased significantly (Figures 6A,B). Glutamine could increase the expression of claudin1 in duodenum and jejunum (Figures 6C,D). Similarly, SJ-SJZG and glutamine significantly increased the expression of occludin in duodenum and jejunum, and the expression level increased in a dose-dependent manner (Figures 6E,F).

SJ-SJZG enhanced the relative expression of glucose transporter, GLUT2, and SGLT1

GLUT2 and SGLT1 were two glucose transporters on intestinal mucosal epithelial cells. The results showed that SJ-SJZG could significantly increase the expression of GLUT2 in

TABLE 4 Effects of SJ-SJZG on $R_{(V/C)}$ in duodenum of weaned Rex rabbits.

Time	Item	Group				
		NC	LD	MD	HD	PC
Day 15	L_{villi} (μm)	648.94 ± 46.31	659.42 ± 21.11	682.06 ± 76.20	701.86 ± 40.39	698.93 ± 37.18
Day 30		722.12 ± 47.55	745.91 ± 37.83	754.38 ± 44.28	762.19 ± 38.63	772.96 ± 55.47
Day 15	D_{crypt} (μm)	100.53 ± 6.62	98.50 ± 7.68	97.39 ± 1.28	94.00 ± 8.41	96.74 ± 9.39
Day 30		92.93 ± 4.49	92.87 ± 5.14	92.52 ± 4.95	91.14 ± 5.01	90.14 ± 8.13
Day 15	$R_{(V/C)}$	6.46 ± 0.32	6.72 ± 0.50	7.01 ± 0.87	7.48 ± 0.28	7.27 ± 0.78
Day 30		7.79 ± 0.70	8.04 ± 0.51	8.17 ± 0.60	8.39 ± 0.86	8.59 ± 0.29

Data are expressed as mean ± SD (n = 6).

TABLE 5 Effects of SJ-SJZG on $R_{(V/C)}$ in jejunum of weaned Rex rabbits.

Time	Item	Group				
		NC	LD	MD	HD	PC
Day 15	L_{villi} (μm)	453.29 ± 27.38^{a}	457.91 ± 14.00^{a}	487.47 ± 14.25^{ab}	499.71 ± 21.04^{b}	492.58 ± 27.44^{ab}
Day 30		502.08 ± 21.98	507.90 ± 28.78	511.70 ± 26.05	520.39 ± 51.60	526.21 ± 42.03
Day 15	D_{crypt} (μm)	110.79 ± 7.44	108.76 ± 7.23	108.72 ± 6.47	107.84 ± 3.04	106.77 ± 5.35
Day 30		92.46 ± 4.06	93.18 ± 2.77	89.94 ± 3.02	90.65 ± 6.49	91.51 ± 7.21
Day 15	$R_{(V/C)}$	4.10 ± 0.25^{a}	4.23 ± 0.38^{ab}	4.50 ± 0.37^{ab}	4.63 ± 0.07^{b}	4.62 ± 0.17^{b}
Day 30		5.43 ± 0.09	5.46 ± 0.46	5.69 ± 0.38	5.77 ± 0.85	5.78 ± 0.73

Data are expressed as mean ± SD (n = 6). $R_{(V/C)}$, Ratio of villus length to crypt depth.

jejunum and ileum, presenting dose dependence (Figures 7A,B). Unlike GLUT2, the expression of SGLT1 caused by SJ-SJZG was mainly concentrated in duodenum and ileum. The expression of SGLT1 in duodenum of MD group and HD group was significantly higher than NC group. The relative expression of SGLT1 in ileum of high-dose group was also significantly higher (Figures 7C,D).

Discussion

Early weanling animals often arise ablactation hyperirritability, which causes diarrhea. Antibiotic therapy, probiotic treatments, and nutrients supplement had been discussed to prevent PWD. However, PWD, as a comprehensive unhealthy state, could not be treated from a single method. Given the multifactorial and complex pathogenesis of PWD, multimodal interventions, such as the use of TCM formulas, may have potential to prevent and/or treat this poor condition. In the present study, we demonstrated the developmental effects of SJ-SJZG on small intestine in weaned Rex rabbits. Mechanistically, we found that SJ-SJZG could not only increase the villi length and $R_{(V/C)}$ to promote the development of the small intestine, but also strengthen immune function by SIgA level boost. Moreover, epithelial cells arranged closely along the intestinal mucosal membrane owing to the expression of intestinal tight protein increased, especially in jejunum. Finally, SJ-SJZG may regulate glucose absorption by enhancing the relative expression of glucose transporter.

According to the theory of TCM, the different combinations of TCMs will be customized for treatment to various clinical symptoms. SJZD, as an ancient Chinese medicine prescription, was also modified by the addition of other TCMs. For example, network pharmacology indicated that the prescription of SJZD plus *Astragalus* was effective in treating chronic atrophic gastritis (14). Similarly, researchers added three to six other TCMs, which included *Astragalus, Citrus* × *aurantium L.*, and *Crataegus pinnatifida Bunge*, to SJZG for patients with spleen and stomach Qi deficiency syndrome (15). Jiawei SJZD, another formula containing SJZD plus *Bupleurum chinense DC, Astragalus, Citrus* × *aurantium L.*, and so on, significantly reduced the side effects of patients after chemotherapy for colon cancer and improved the immune function of patients (16). Similar to the modified SJZD above, SJ-SJZG contained *Astragalus mongholicus Bunge, Bupleurum chinense DC, Citrus* × *aurantium L.*, and *Crataegus pinnatifida Bunge (fruit)* and the formula of the original SJZD. *Astragalus mongholicus Bunge* could regulate intestinal barrier and treat Qi deficiency (17). It combination with Codonopsis pilosula can also treat colitis (18). *Bupleurum chinense DC* also had a protective effect on small intestinal injury (19). *Citrus* × *aurantium L.* and *Crataegus pinnatifida Bunge(fruit)*, as the partners

TABLE 6 Effects of SJ-SJZG on $R_{(V/C)}$ in ileum of weaned Rex rabbits.

Time	Item	Group				
		NC	LD	MD	HD	PC
Day 15	L_{villi} (μm)	420.22 ± 4.64	423.36 ± 14.79	443.51 ± 29.12	452.29 ± 18.26	447.20 ± 38.64
Day 30		472.49 ± 41.22	473.00 ± 48.95	473.51 ± 44.29	483.02 ± 19.43	485.07 ± 40.62
Day 15	D_{crypt} (μm)	112.69 ± 5.67	111.40 ± 6.18	107.61 ± 5.32	107.36 ± 3.63	107.77 ± 2.80
Day 30		87.26 ± 3.85	86.92 ± 5.90	87.83 ± 9.86	85.25 ± 2.09	86.30 ± 5.91
Day 15	$R_{(V/C)}$	3.74 ± 0.21	3.80 ± 0.08	4.12 ± 0.24	4.22 ± 0.30	4.15 ± 0.41
Day 30		5.42 ± 0.50	5.48 ± 0.89	5.44 ± 0.89	5.67 ± 0.36	5.63 ± 0.53

Data are expressed as mean ± SD (n = 6).

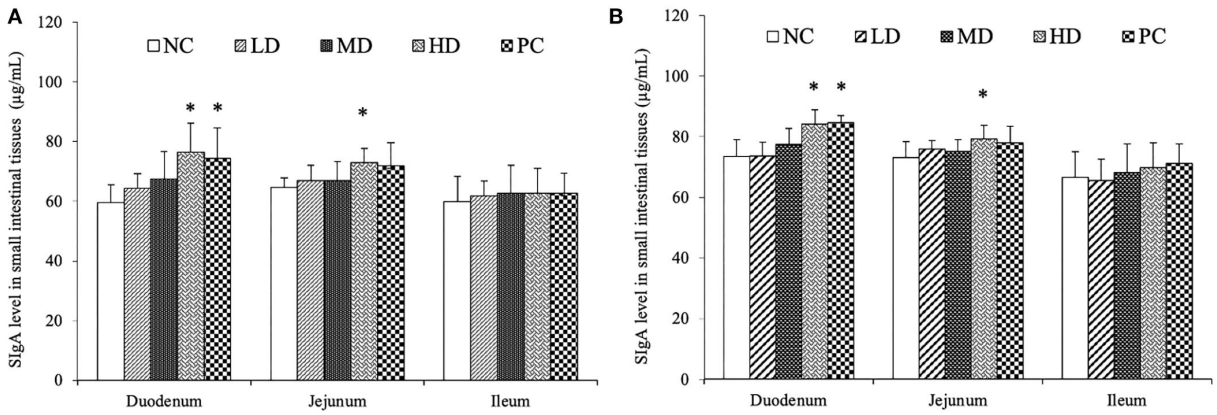


FIGURE 4
SIgA content in small intestinal tissues by Elisa, Day 15 **(A)** and Day 30 **(B)**. The data were presented as means ± S.E.M ($n = 6$). Significant difference with NC group was designated as $^*p < 0.05$.

of regulating Qi and strengthening stomach in TCM, had been reported to prevent intestinal diseases and protect the intestinal barrier (20, 21). The four herbs and SJZD all had protective effects on digestive tract, but there was no report of combination therapy for PWD. We proposed to add the above four TCMs into SJZD to form modified SJZD for the first time and then made it into granules for taking conveniently, which is called SJ-SJZG. The main bioactive ingredients of SJ-SJZG were saikoside A, liquiritin, hesperidin, and glycyrrhizic (Figure 2). Saikoside A, an extract from *Bupleurum chinense DC,* possessed several pharmacological activities, including anti-oxidant, anti-tumor, and protecting intestinal function (19, 22). Hesperidin, from *Citrus* × *aurantium L.*, a well-known extract in TCM, had shown the various pharmacological effects of hesperidin, such as anti-inflammatory and anti-oxidation, promoting gut health and improving immunity against infections (23, 24). Liquiritin and glycyrrhizic, both from *Glycyrrhiza uralensis Fisch*, exhibited anti-inflammatory activity, which enhanced intestinal motility (25, 26). To sum up, whether from the TCM-based theory in nourishing Qi or the pharmacological activity of main components, SJ-SJZG had potential in the protection or treatment of PWD.

Our previous studies had confirmed SJ-SJZG had a beneficial effect of PWD which not only reflected in diarrhea rate and growth performance, but also the utilization of protein, carbohydrate, and lipid nutrition in body (12). However, the SJ-SJZG mechanism of reducing PWD needs to be investigated further. Therefore, we made a profound study on SJ-SJZG in preventing or protecting PWD. Moreover, glutamine was used as the positive control group. As an important conditionally indispensable amino acid, glutamine presented an important role in promoting immune function, the expression of genes related to intestinal health when the animal had been in earlier age or during stress (27). It had been confirmed that glutamine protected the intestinal health on weaned rabbits (28). Meanwhile, available evidence had suggested that dietary supplementation with glutamine improved the gene expression

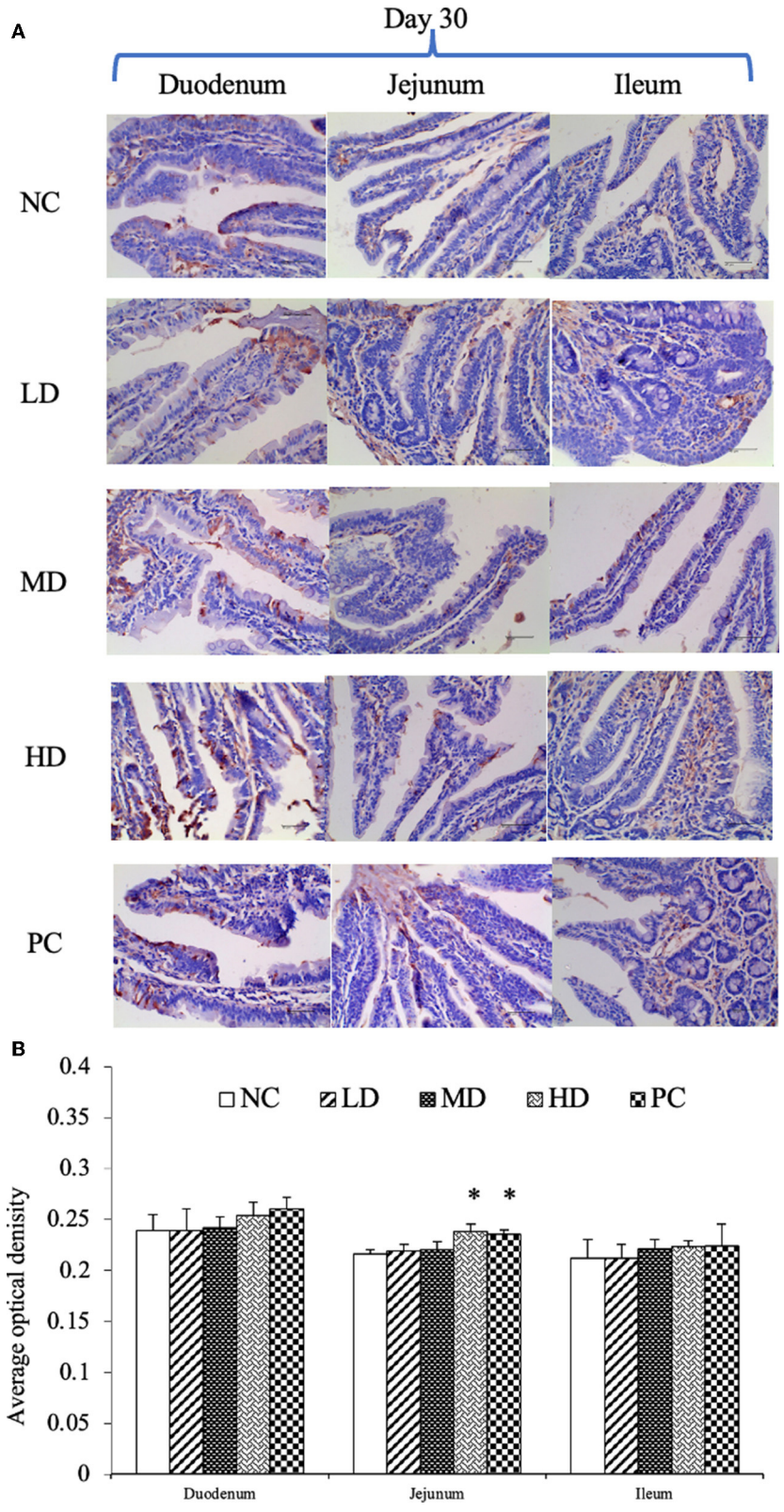


FIGURE 5
Immunohistochemical results of SIgA at Day 30 **(A)**. Average optical density of SIgA expression on small intestinal mucosa **(B)**. The data were presented as means ± S.E.M. ($n = 6$). Significant difference with NC group was designated as *$p < 0.05$.

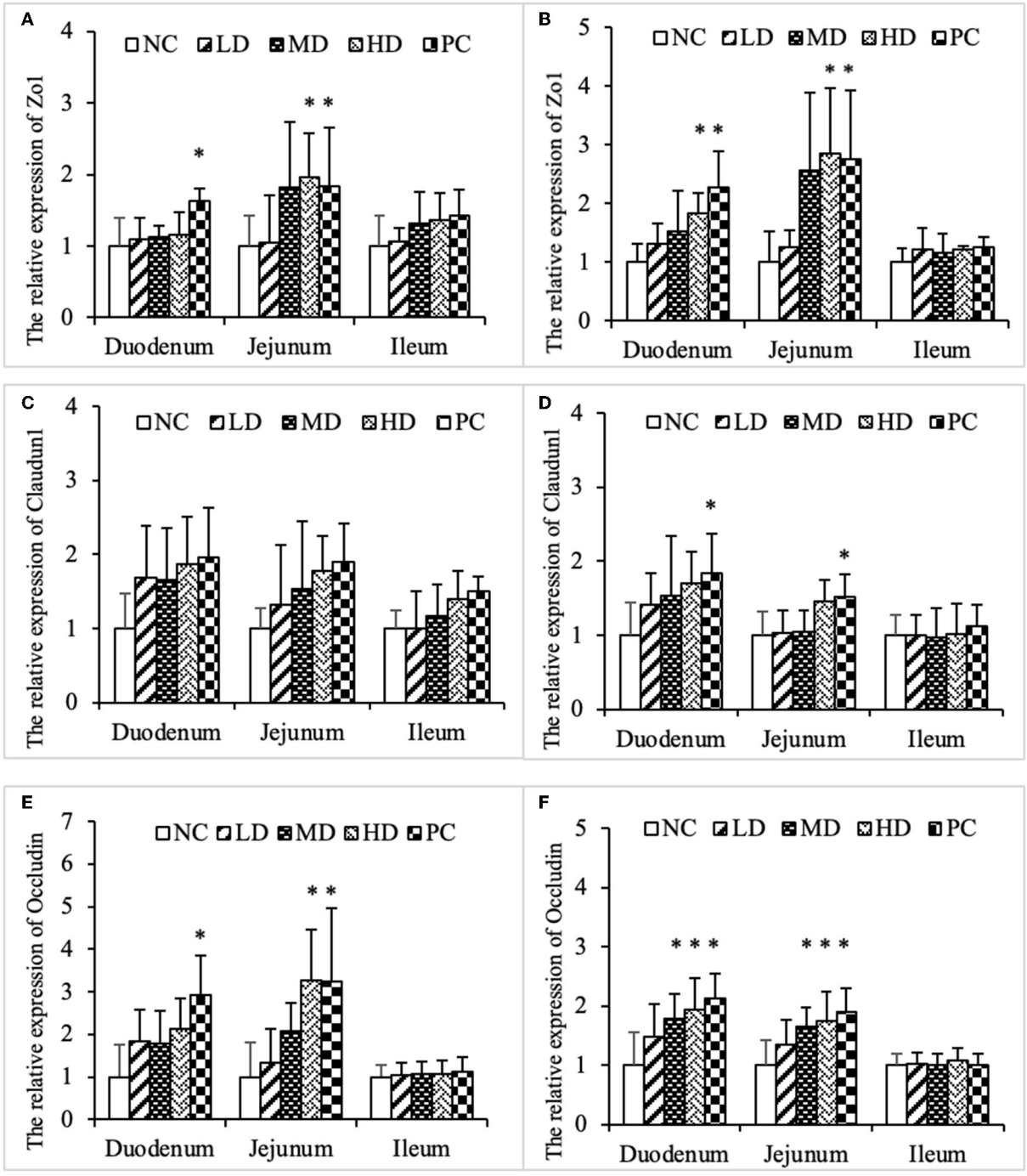


FIGURE 6
Effects ofSJ-SJZG on the relative expression of tight junction protein in small intestine mucosa. **(A)** Zo1, Day 15. **(B)** Zo1, Day 30. **(C)** Claudin1, Day 15. **(D)** Claudin1, Day 30. **(E)** Occludin, Day 15. **(F)** Occludin, Day 30. The data were presented as means ± S.E.M. ($n = 6$). Significant difference with NC group was designated as $^{*}p < 0.05$.

and immune performance on small intestinal mucosa in Rex rabbits (29). Previous studies have reported that diet supplemented with 0.8% glutamine could increase the height of small intestinal villi and reduce the depth of recess in weaned rabbits (30).

The small intestine was an important site of nutrient absorption. The longer the epithelial villi, the more the epithelial cells. The shallower the crypt depth, the higher the rate of epithelial cell maturation. The villi and crypt represented absorptive function of small intestine. Our experiment showed

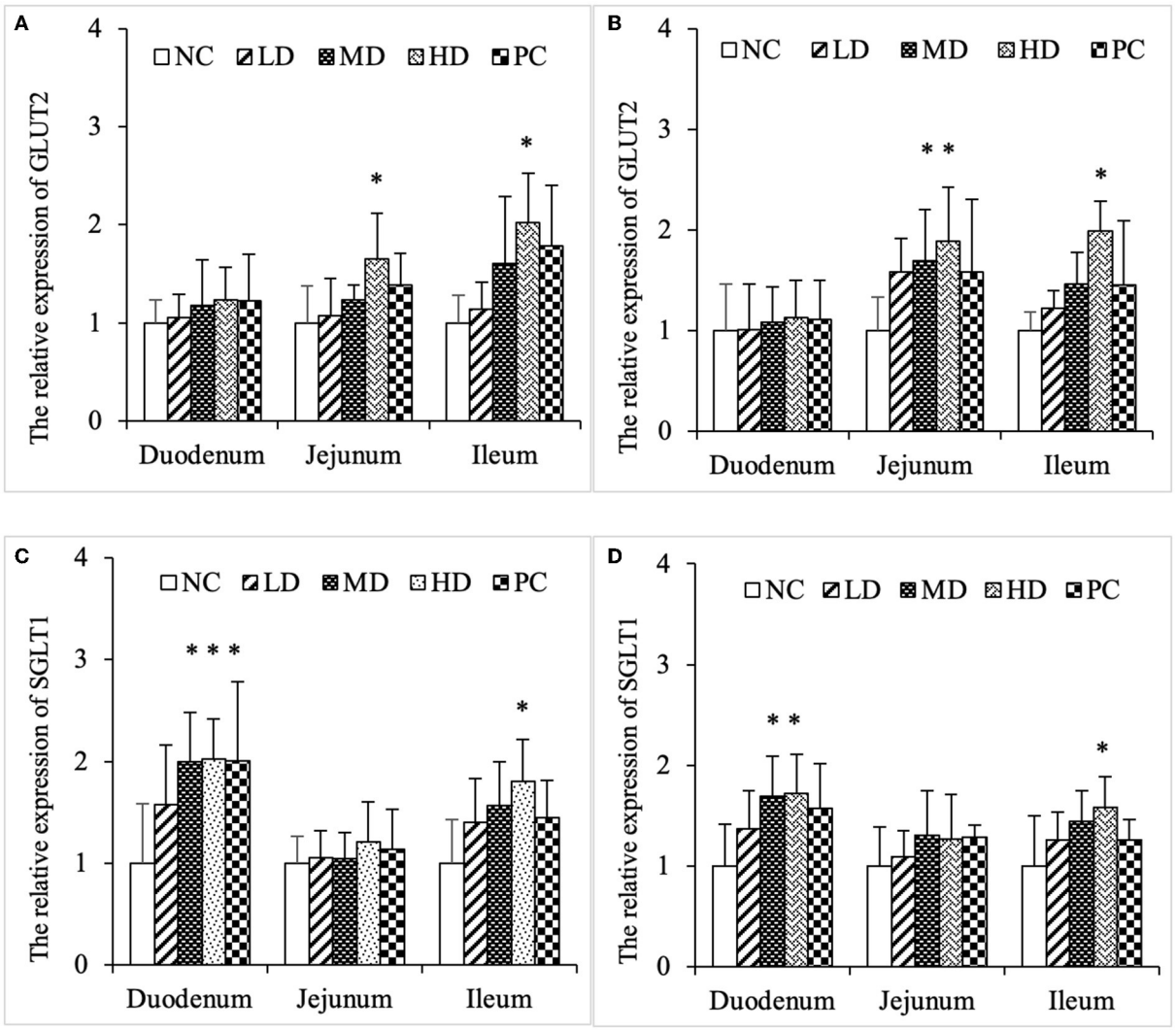


FIGURE 7
Effects of SJ-SJZG on the relative expression of glucose transporters in small intestinal mucosa. **(A)** GLUT2, Day 15. **(B)** GLUT2, Day 30. **(C)** SGLT1, Day 15. **(D)** SGLT1, Day 30. The data were presented as means ± S.E.M. ($n = 6$). Significant difference with NC group was designated as $^{*}p < 0.05$.

that both SJ-SJZG and glutamine showed the effect of increasing $R_{(V/C)}$, especially in the jejunum (Tables 4–6). SJ-SJZG had the similar function as glutamine in protecting the integrity of small intestinal mucosa and promoting the development of small intestine. Available evidence had suggested that SIgA promoted the clearance of antigens and pathogenic microorganisms from the intestinal lumen by blocking their access to epithelial receptors, entrapping them in mucus, and facilitating their removal by peristaltic and mucociliary activities. Meanwhile, SIgA also had the capacity to directly quench bacterial virulence factors (31). In this study, we observed that SJ-SJZG significantly increased SIgA content and protein expression (Figures 4, 5). Interestingly, both the SIgA and $R_{(V/C)}$ results showed that SJ-SJZG had the best effect on the jejunum. The protective and immunological effects of SJ-SJZG on the small intestine were mainly focused on the jejunum. SJ-SJZG could protect the integrity of jejunal mucosal epithelial cells by improving immunity in the jejunum. The intestinal barrier function referred to the sum of structure and function that can prevent harmful substances from crossing the intestinal mucosa to enter other tissue organs and blood circulation in the body. The permeability of the intestinal mucosa, as the physical barrier of the intestine, was closely related to the expression amount of tight junction proteins (32). Our study showed that SJ-SJZG increased the relative expression of tight junction protein, especially Zo1 and occludin. It was different from glutamine elevating the expression of the relative expression of Zo1, claudin1, and occludin simultaneously (Figure 6).

As two glucose protein transporters with different functions, GLUT2 was responsible for passive forward concentration transport while the SGLT1 was responsible for active reverse concentration transport. According to our research, SJ-SJZG could increase the passive glucose transport capacity of jejunum and ileum by increasing the protein relative expression of GLUT2 (Figures 7A,B). SJ-SJZG can increase the active glucose transport capacity of duodenum and ileum by increasing the protein relative expression of SGLT1, but glutamine was mainly active in duodenum (Figures 7C,D). This indicated SJ-SJZG had a promoting effect on glucose absorption in the small intestine. It was conceivable that SJ-SJZG showed great potential to promote nutrient absorption in small intestine.

Conclusion

In summary, we explored the mechanism of SJ-SJZG on small intestine developmental effects in weaned Rex rabbits. SJ-SJZG had been proved to be effective in promoting the development of small intestine and improving the immunity of small intestine. Moreover, SJ-SJZG could ensure the integrity of mucosal barrier and increase the ability of intestine to absorb glucose in small intestine. Our research provided a reliable theoretical basis for reducing the occurrence of PWD by SJ-SJZG.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Sichuan Agricultural University Institutional Animal Care and Use Committee CSQ-2018203003.

Author contributions

DL: methodology, data curation, and writing—original draft preparation. YW: formal analysis and validation. NL: funding support and investigation. SC and HL: visualization. PW, ZY, GS, and JL: supervision. WZ, HT, and GP: software. KZ, BW, and LZ: writing—reviewing and editing. HF: project administration. All authors read and approved the final manuscript.

Funding

This work was financially supported by the Earmarked Fund for the China Agriculture Research System (CARS-43-A-3), Science & Technology Department of Sichuan Province (2016NYZ0046), and the basic scientific research operating expenses of Sichuan Science & Technology Department.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Kong GY. Current situation,problems and development direction of rex rabbit industry in China. *J Anim Sci Vet Med.* (2017) 36:106−9. doi: 10.3969/j.issn.1004-6704.2017.04.015

2. Zhi-Ping HE. Economic benefit analysis of rex rabbit breeding. *J Anim Sci Vet Med.* (2017) 36:44–5. doi: 10.3969/j.issn.1004-6704.2017.03.036

3. Liu H, Chaohui FU, Liu L, Fuchang LI. Effects of Dietary Glutamine Supplemental Level on Fur Quality and Intestinal Barrier of Rex Rabbits during Weaner to 3 Months of Age. *Chin J Anim Nutr.* (2017) 5:356–62. doi: 10.3969/j.issn.1006-267x.2017.05.041

4. Qy A, Sm B, Wn A, Hc A, Qza C. Label-free mass spectrometry-based proteomics for investigating the therapeutic mechanism of Sijunzi decoction on spleen deficiency syndrome – ScienceDirect. *Microchem J.* (2021) 164:105944. doi: 10.1016/j.microc.2021.105944

5. Yanmei WU, Xiande MA, Han X, Tan J, Guan H. Effect of Sijunzi decoction on IFN-gamma expression in small intestine tissue of spleen deficiency mice infected with candida. *J Liaoning Univ Tradit Chin Med.* (2019) 10:58–61.

6. Shi Y, Zhu H, Li R, Wang D, Zhu Y, Hu L, Chen W. Effect of polysaccharides from Sijunzi decoction on Ca 2+ related regulators during intestinal mucosal restitution. *Phytomedicine.* (2019) 58:152880. doi: 10.1016/j.phymed.2019.152880

7. Zhang D, Zhou L, Zhang Z, Wang Q, Wang P. Effects and mechanism of polysaccharide of sijunzi decoction on sIgA in intestine of immune suppression mice. *Tradit Chin Drug Res Clin Pharmacol.* (2010) 21:11–4. doi: 10.19378/j.issn.1003-9783.2010.01.005

8. Tao S, Yu X, Zhou Z. Effect of Sijunzi decoction on restoration of gut barrier after relief of intestinal obstruction in rabbits intestine. *Chin J Surg Integr Tradit West Med.* (2010) 16:319–23. doi: 10.3969/j.issn.1007-6948.2010.03.024

9. Xia XD, Meng-Chu HE, Shu YS, Yan-Ting HE, Feng SB Yu LI, et al. Effects of supplementary sijunzi decoction on digestive and absorptive function and expression of EGF and SS in small intestine of canine, J. *Gansu Agric Univ.* (2019) 46:1065—72. doi: 10.13432/j.cnki.jgsau.2019.04.003

10. Zhang DX, Yin WU, Song CF, Tian QC, Liu D, Kui-Hong MA, et al. Effect of Qilan sijunzi decoction on IL-2 And IFN-γ mRNA expression of Small Intestine in chickens. *Chin J Vet Med.* (2019) 1:85–9. Available online at: https://en.cnki.com.cn/Article_en/CJFDTotal-ZSYZ201901022.htm

11. Zi-Sheng HU, Ji-An LI, Duan HH, Yu-Bing XU, Liu HB, Surgery DO. Influence of early enteral nutrition combining with modified Sijunzi decoction on nutritional status in patients after abdominal surgery. *China Mod. Med.* (2014) 20:143-44, 149. Available online at: http://en.cnki.com.cn/Article_en/CJFDTotal-ZSYX201604021.htm

12. Liu N, Hualin FU, Liu H, Rui LI, Zhang K, Zhiju YU, et al. Effects of different chinese herbal compound on growth performance and serum biochemical indexes of weaned rex rabbits. *China Anim Husb Vet Med.* (2018) 2:422–28. doi: 10.16431/j.cnki.1671-7236.2018.02.017

13. Liu N, Rui LI, Hua-Lin FU, Liu HZ, Wang P, Wen B. Effects of compound chaiqi san on weaned rex rabbits' growth performance,serum biochemical indices and immune indexes. *J. Sichuan Agric. Univ.* (2017) 35:88-92, 123.

14. Tian G, Wu C, Li J, Liang B, Zhang F, Fan X, et al. Network pharmacology based investigation into the effect and mechanism of modified sijunzi decoction against the subtypes of chronic atrophic gastritis. *Pharmacol Res.* (2019) 144:158–66. doi: 10.1016/j.phrs.2019.04.012

15. Zhiqiang NI, Wang Y. Clinical study on jiawei sijunzi decoction on synergism and attenuation of chemotherapy for patients with spleen and stomach Qi deficiency syndrome after rectal cancer operation. *J Hunan Univ Chin Med.* (2019) 39:532–36. doi: 10.3969/j.issn.1674-070X.2019.04.021

16. Shuchan MO, Tian T, Zhongbiao WU, Department S. Effect of jiawei sijunzi decoction in treatment of colon cancer after chemotherapy. *Liaoning J Tradit Chin Med.* (2018) 45:571–74. doi: 10.13192/j.issn.1000-1719.2018.03.038

17. Chen XL, Liang XL, Kong XQ Ji MM, Liao ZG. Effect and molecular mechanism research of on inhibiting intestinal absorption of six alkaloids of Aconitum carmichaelii in spleen deficiency rats. *Chin Herb Med.* (2021) 4:117–24. doi: 10.1016/j.chmed.2021.07.001

18. Tang S, Liu W, Zhao Q, Li K, Gao X. Combination of polysaccharides from Astragalus membranaceus and Codonopsis pilosula ameliorated mice colitis and underlying mechanisms. *J Ethnopharmacol.* (2020) 264:113280. doi: 10.1016/j.jep.2020.113280

19. Shu G, Xu D, Ran C, Yin L, Amevor FK. Protective effect of dietary supplementation of Bupleurum falcatum L saikosaponins on ammonia exposure induced ileum injury in broilers. *Poult Sci.* (2020) 100:100803. doi: 10.1016/j.psj.2020.10.057

20. Guo C, Wang Y, Zhang S, Zhang X, Ding K. Crataegus pinnatifida polysaccharide alleviates colitis via modulation of gut microbiota and SCFAs metabolism. *Int J Biol Macromol.* (2021) 181:357–68. doi: 10.1016/j.ijbiomac.2021.03.137

21. He W, Liu M, Li Y, Yu H, Wang T. Flavonoids from Citrus aurantium ameliorate TNBS-induced ulcerative colitis through protecting colonic mucus layer integrity. *Eur J Pharmacol.* (2019) 857:172456. doi: 10.1016/j.ejphar.2019.172456

22. Xza B, Zl A, Sc A, Hang LA, Lin DA, Xf A, et al. new discovery: Total Bupleurum saponin extracts can inhibit the proliferation and induce apoptosis of colon cancer cells by regulating the PI3K/Akt/mTOR pathway. *J Ethnopharmacol.* (2021) 283:114742. doi: 10.1016/j.jep.2021.114742

23. Zhang Q, Song X, Chen X, Jiang R, Peng K, Tang X, et al. Antiosteoporotic effect of hesperidin against ovariectomy-induced osteoporosis in rats via reduction of oxidative stress and inflammation. *J Biochem Mol Toxicol.* (2021) 36:1456–60. doi: 10.1002/jbt.22832

24. Kamboh A, Zhu AW-Y. Individual and combined effects of genistein and hesperidin on immunity and intestinal morphometry in lipopolysacharide-challenged broiler chickens. *Poult Sci.* (2014) 93:2175–83. doi: 10.3382/ps.2014-03971

25. Yin L, Zhang Y, Shu Z, Dong L, Wu X, Chen J, et al. Chemical profile and anti-inflammatory activity of total flavonoids from Glycyrrhiza uralensis. *Fisch. (spring 2018).* (2017) 17:726-34. doi: 10.22037/IJPR.2018.2197

26. Yinghua LV, Huang X, Yang J, Zheng Q, Liu H. Effects of combinations of Gegenqinlian Decoction on small intestine movement in mice and the quantitative analysis. *Chin J Clin Pharmacol Ther.* (2016) 21:399–403.

27. He J, Feng GD, Ao X, Li YF, Qian HX, Liu JB, et al. Effects of L-glutamine on growth performance, antioxidant ability, immunity and expression of genes related to intestinal health in weanling pigs. *Livest Sci.* (2016) 189:102-9. doi: 10.1016/j.livsci.2016.05.009

28. Delgado R, Abad-Guamán R, Nicodemus N, Diaz-Perales A, García J, Carabao R, et al. Effect of pre- and post-weaning dietary supplementation with arginine and glutamine on rabbit performance and intestinal health. *BMC Vet Res.* (2019) 15:199. doi: 10.1186/s12917-019-1945-2

29. Gao YQ, Ren ZJ, Zhi-Gang HU, Jian ZL, Chen HL, Song B. Effect of dietary supplementation with different levels of glutamine on immune performance and mucin gene expression in ileum of young rex rabbit. *Chin J Vet Sci.* (2016) 36:655–60. doi: 10.16303/j.cnki.1005-4545.2016.04.21

30. Guo Z, Tang L, Congyan LI, Xie X, Ren Y, Kuang L, et al. Effects of glutamine on intestinal development of meat rabbits, Chin. *J Anim Nutr.* (2017) 29:3132–7.

31. Mantis NJ, Rol N, Corthésy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol.* (2011) 4:603–11. doi: 10.1038/mi.2011.41

32. Ma J, Shah AM, Wang Z, Hu R, Kong X. Comparing the gastrointestinal barrier function between growth-retarded and normal yaks on the Qinghai-Tibetan Plateau. *PeerJ.* (2020) 11:600516. doi: 10.7717/peerj.9851

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 08 November 2022
DOI 10.3389/fvets.2022.1045152

OPEN ACCESS

EDITED BY
Dayou Shi,
South China Agricultural University, China

REVIEWED BY
Wei Xu,
Zhejiang University, China
Dandan Hu,
Guangxi University, China
Kedsirin Sakwiwatkul,
Mahasarakham University, Thailand

*CORRESPONDENCE
Liting Cao
caoliting@swu.edu.cn

†These authors have contributed equally to this work and share first authorship

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 15 September 2022
ACCEPTED 13 October 2022
PUBLISHED 08 November 2022

CITATION
Zhou B, Huang H, Gui F, Bi S, Du H and Cao L (2022) Enhancement of intestinal mucosal immunity and immune response to the foot-and-mouth disease vaccine by oral administration of danggui buxue decoction. *Front. Vet. Sci.* 9:1045152. doi: 10.3389/fvets.2022.1045152

Enhancement of intestinal mucosal immunity and immune response to the foot-and-mouth disease vaccine by oral administration of danggui buxue decoction

Bingxin Zhou[1†], Huan Huang[1†], Fuxing Gui[1], Shicheng Bi[1,2,3], Hongxu Du[1,3,4] and Liting Cao[1,3,4*]

[1]Department of Traditional Chinese Veterinary Medicine, College of Veterinary Medicine, Southwest University, Chongqing, China, [2]Immunology Research Center, Medical Research Institute, Southwest University, Rongchang, Chongqing, China, [3]Chongqing Engineering Research Center of Veterinary Medicine, Chongqing, China, [4]Chi Institute of Traditional Chinese Veterinary Medicine, Southwest University, Chongqing, China

This study investigated the effect of Danggui Buxue decoction (DBD) on the immunity of an O-type foot-and-mouth disease (FMD) vaccine and intestinal mucosal immunity. SPF KM mice were continuously and orally administered DBD for 5 d and then inoculated with an O-type FMD vaccine. The contents of a specific IgG antibody and its isotypes IgG1, IgG2a, IgG2b, and IgG3 in serum and SIgA in duodenal mucosa were determined by ELISA at 1 and 3 W after the 2nd immunization. qRT-PCR was used to detect mRNA expression levels of IL-4, IL-10, IFN-γ, and IL-33 in the spleen, and mRNA expression levels of J-chain, pIgR, BAFF, APRIL, IL-10, IFN-γ and IL-33 in the duodenum. The results showed that compared with the control group, oral administration of DBD significantly increased levels of the anti-FMD virus (FMDV)-specific antibodies IgG, IgG1, and IgG2a in the serum of O-type FMD vaccine-immunized mice 1 W after the 2nd immunization ($P < 0.05$), upregulated mRNA expression levels of spleen lymphocyte cytokines IL-4 and IL-33 ($P < 0.05$), promoted the secretion of SIgA in duodenal mucosa ($P < 0.05$). The mRNA expression levels of J-chain, pIgR, BAFF, APRIL, IL-10, and IL-33 in duodenal tissues were upregulated ($P < 0.05$). This study indicates that DBD has a good promotion effect on the O-type FMD vaccine and the potential to be an oral immune booster.

KEYWORDS

Danggui Buxue decoction, foot-and-mouth disease vaccine, humoral immunity, intestinal mucosal immunity, immune enhancement

Introduction

Foot-and-mouth disease (FMD) is a highly contagious acute infectious disease caused by FMD virus (FMDV) and mainly affects cloven-hoofed animals such as pigs, cattle, goats, and camels (1). The incidence of FMD is extremely high and the transmission speed is extremely fast. FMDV is mainly transmitted through the digestive tract, respiratory tract, skin, and mucosa. Mucosal damage in the early stage of infection is highly susceptible to infection by other bacteria in the external environment (2). In today's intensive farming environment, once an individual gets sick, a group infection will soon break out and cause serious economic losses to the breeding industry (3). Presently, vaccination is the most important means for the prevention and control of FMD (4). Therefore, compulsory immunization with FMD vaccine was adopted in China to prevent and control the disease, which can control a possible pandemic of FMD to a certain extent (5). However, due to the uneven level of breeding health management in some areas, the phenomenon of poor immune effects of FMD vaccines has been reported from time to time (6). Therefore, it is necessary to develop an immune booster to improve the immune effect of the FMD vaccine.

Chinese herbal medicine comes from nature and has low toxicity and side effects. It also has a unique effect in coordinating the overall balance of the body and strengthening the disease-resistance ability. Recently, researchers have used modern science and technology to study traditional Chinese medicine and found that Chinese herbal medicine or effective active ingredients have significant immunomodulatory effects (7, 8). Danggui Buxue decoction (DBD) was created by Li Dongyuan, one of the four great scholars of the *Jin* and *Yuan* Dynasties. It is composed of *Radix Astragali* and *Angelica sinensis* and replenishes *Qi* and promotes the production of blood (9). Modern studies have confirmed that DBD can promote angiogenesis (10), improve bone marrow hematopoietic function (11), enhance non-specific immunity (12) and improve immune regulation in a pathological injury model (13). The intestinal tract is not only a place for digestion and absorption of nutrients but also has important immune functions. The intestinal mucosa is a key line of defense for the body to resist pathogen infection, and various defense mechanisms are conducted in the intestine, which play an important role in the establishment and maintenance of homeostasis between the host and the external environment (14). Chinese herbal medicine is a group of natural substances, which has the advantages of high safety, enhancing humoral and cellular immunity, non-toxic side effects, wide range of medication, etc. And it has been reported that a large number of herbal medicines or their extracts have been used as vaccine adjuvants, such as *Angelica sinensis* polysaccharide (15), ginsenoside Rg1 (16) and Qi-Wu Rheumatism Granule (17). Most Chinese herbal medicines are taken orally to exert effects and have a close relationship with the intestinal mucosal immune system. Therefore, this study was conducted to investigate the effect of oral DBD on the immune function of an O-type FMD vaccine and intestinal mucosal immunity in mice to provide a theoretical basis for the clinical application of traditional Chinese medicine.

Materials and methods

Reagents

Angelica sinensis and *Radix astragali* were purchased from Chongqing Rongchang Tongjunge pharmacy.

The O-type foot-and-mouth disease synthetic peptide vaccine (Peptide 2600+2700+2800) (20190408) was purchased from Shenlian Biomedicine (Shanghai, China) Co., Ltd. A mouse (FMDV-OIgG) ELISA kit (20190512), mouse FMDV-OIgG1, FMDV-OIgG2a, FMDV-OIgG2b, FMDV-OIgG3) ELISA kit (20190422), and mouse SIgA ELISA kit (20190612) were purchased from Shanghai Huzhen Industrial Co., Ltd. TRIzon Reagent (20190323), HiFi Script gDNA Removal RT Master Mix (30412), and 2xEs Taq Master Mix (50428) were purchased from Beijing Kangwei Century Co., Ltd. Eva GREEN (17E0.1-1075001) was purchased from Biotium, USA.

Preparation of danggui buxue decoction

One hundred grams of *Radix astragali* and 20 grams of *Angelica sinensis* were accurately weighed, soaked in 8 times distilled water for 30 min with high heat until boiling, and then simmered gently for 1 h. The liquid was filtered and the residue was added to the same amount of distilled water for an additional 1 h. The filtrate was filtered again and combined. The final concentration of liquid was equivalent to 2 g/m L of the original medicine. The medicinal liquid was separated into 2, 1, 0.5, 0.25, 0.125, and 0.0625 g/mL DBD and stored at 4°C for later use.

Animals

A total of 94 SPF KM mice weighing 20 ± 2 g were purchased from Chongqing Ensiweier Biotechnology Co., Ltd. All experimental mice were raised in an environment with a temperature of 20–25°C and a relative humidity of 60–80% and were freely allowed to feed and drink. After 1 W of adaptive feeding, the formal experiment began. This study was approved by the Laboratory Animal Ethical Review Committee of Southwest University (permit number IACUC-20211020-01), and all experimental animals were euthanized at the end of the study.

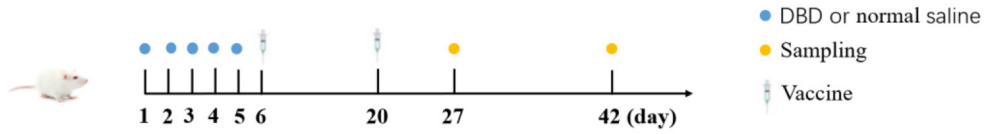


FIGURE 1
Grouping and processing in mice.

Experimental design

In experiment 1, 70 KM mice, half male and half female, were randomly divided into 7 groups (n = 10), which were 2, 1, 0.5, 0.25, 0.125 and 0.0625 g/mL DBD dose groups and control group. The dose was 0.25 mL/each, which was given by gavage for 5 d. The control group was given the same amount of normal saline. Twenty-four hours after the last dose, all experimental mice were subcutaneously injected with 0.2 mL of O-type FMD vaccine in the groin followed by a second immunization at 2 W later. The mice were weighed before immunization and 1 and 3 W after immunization, and blood samples were collected under the jaw at 1 and 3 W after immunization. All mice were sacrificed by neck removal at 3 W after immunization, and the spleen and thymus were aseptically harvested (Figure 1).

In experiment 2, 24 KM mice, half male and half female, were randomly divided into two groups (n = 12), which were the optimal dose group of DBD and the control group, respectively. The mice were treated according to the method of Experiment 1. All experimental mice were sacrificed by neck stripping 3 W after the 2nd immunization, and the spleen, duodenum, and mucosal flushing fluid were aseptically extracted (Figure 1).

TABLE 1 Sequences of primers for qRT-PCR.

Gene	Primer sequences (5′-3′)	bp
IL-4	F: CACAGCAACGAAGAACACCAC	131
	R: TCTGCAGCTCCATGAGAACAC	
IL-10	F: GTTGCCAAGCCTTATCGGA	132
	R: GCCGCATCCTGAGGGTC	
IL-33	F: GTCTCCTGCCTCCCTGAGTACATAC	128
	R: AGTAGCGTAGTAGCACCTGGTCTTG	
IFN-γ	F: TTGCCAAGTTTGAGGTCAACAA	126
	R: CGAATCAGCAGCGACTCCT	
APRIL	F: GTTAACATTACCTCCAAGGACTCT	115
	R: TTCCAGTGTCCCAGACTCGTA	
BAFF	F: GTTGCCAAGCCTTATCGGA	114
	R: GCCGCATCCTGAGGGTC	
pIgR	F: GGGTTGCCACATCCTGCCAAG	131
	R: CACACCAGTACCAGCCTTCATCTTC	
J-chain	F: CTCTGATCCCACCTCCCCACTG	150
	R: TCAGGAACACCATCGTCTTCATTGC	
β-actin	F: TATGCTCTCCCTCACGCCATCC	129
	R: GTCACGCACGATTTCCCTCTCAG	

Determination of the immune organ index

The spleen and thymus of all experimental mice were accurately harvested, and the blood stains on the surface were washed with sterilized saline. Then, excess water on the organ surface was aspirated with clean filter paper and weighed.

Calculation formula: Organ index = organ weight (mg)/mouse weight (g).

Detection of serum-specific antibody and duodenal SIgA

The serum and duodenal mucosa supernatant were obtained by separating the collected blood and duodenal mucosa rinse solution at 3,000 rpm/min for 10 min. The content of FMDV-specific antibody IgG in serum at 1 W and 3 W after the 2nd immunization, specific antibody isotypes IgG1, IgG2a, IgG2b, and IgG3 in serum, and SIgA in duodenal mucosal rinse solution 3 W after the 2nd immunization were detected according to ELISA kit instructions.

RNA extraction and qRT-PCR

Total RNA was extracted from the spleen and duodenum using TRIzol Reagent and reverse transcribed into cDNA using HiFi Script gDNA Removal RT Master Mix and 2xEs Taq Master Mix. The mRNA expression levels of IL-4, IL-10, IFN-γ, and IL-33 in the spleen and APRIL, BAFF, pIgR, J-chain, IL-10, and IL-33 in the duodenum were detected by real-time PCR using Eva GREEN (Biotium17E0.1-1075001) with mouse β-actin as the internal reference. The results are expressed as $2^{-\Delta\Delta ct}$, and the primer sequences are shown in Table 1.

10.3389/fvets.2022.1045152

TABLE 2 Effect of oral administration of DBD on the mean body weight in mice ($n = 10$).

Group	Pre-immunization	2 W after 1st immunization	2 W after 2nd immunization	3 W after 2nd immunization
0.0625 g/ml	20.3 ± 0.58	28.1 ± 1.09	34.7 ± 0.86	43.0 ± 0.63
0.125 g/ml	20.1 ± 0.53	28.3 ± 1.24	33.8 ± 0.51	42.7 ± 1.28
0.25 g/ml	20.1 ± 0.55	29.0 ± 0.96	34.2 ± 0.95	42.3 ± 0.94
0.5 g/ml	19.4 ± 0.48	29.0 ± 1.14	33.7 ± 0.52	42.1 ± 0.86
1 g/ml	20.4 ± 0.34	29.3 ± 1.04	33.3 ± 0.65	42.6 ± 1.31
2 g/ml	20.1 ± 0.41	28.8 ± 0.81	33.2 ± 0.61	43.8 ± 0.77
control	19.2 ± 0.55	29.7 ± 0.73	33.9 ± 0.82	43.3 ± 0.99

TABLE 3 Effect of DBD on the organ index in mice ($n = 10$).

Group	Spleen index	Thymus index
0.0625 g/ml	2.25 ± 0.17	4.48 ± 0.30
0.125 g/ml	2.50 ± 0.17	4.37 ± 0.32
0.25 g/ml	2.57 ± 0.11	4.64 ± 0.15
0.5 g/ml	2.97 ± 0.16	5.48 ± 0.23
1 g/ml	2.42 ± 0.13	5.27 ± 0.19
2 g/ml	2.70 ± 0.19	5.78 ± 0.26
control	2.67 ± 0.16	5.37 ± 0.17

Statistics analysis

Data are expressed as the mean ± standard error (SE) and were analyzed by one-way ANOVA *post-hoc* Duncan's method and *t*-test with IBM SPSS Statistics 22.0 software. Differences with $P < 0.05$ were considered statistically significant.

Results

Body weight and immune organ index

As depicted in Tables 2, 3, the body weight (Table 2), spleen index, and thymus index (Table 3) of the experimental mice were not significantly affected by different doses of DBD compared with the control group ($P > 0.05$).

Serum IgG and its isotypes

As shown in Figure 2, the serum anti-FMDV-specific antibody IgG content in each dose group was significantly increased at 1 W after the 2nd immunization ($P < 0.05$). The 0.5 g/mL and 2 g/mL dose groups significantly increased the content of IgG and IgG1 serum-specific antibodies ($P < 0.05$), and the 0.5 g/mL dose group simultaneously promoted the secretion of serum IgG2a ($P < 0.05$) 3 W after the 2nd immunization. There was no significant difference in antibody content among the other dose groups ($P > 0.05$).

mRNA expression levels of splenic-associated lymphocyte cytokines

As depicted in Figure 3 and compared with the control group, the mRNA expression levels of IL-4 and IL-33 in the spleen were significantly increased by oral DBD ($P < 0.05$), and the mRNA expression levels of IL-10 and IFN-γ were not different from those of the control group ($P > 0.05$).

SIgA content of duodenal mucosal flushing fluid

The effect of DBD on the SIgA content of duodenal mucosal flushing fluid is shown in Figure 4. The results suggest that 0.5 g/mL DBD significantly increased the intestinal SIgA antibody content ($P < 0.05$).

Relative mRNA expression of immune-related genes in the intestinal mucosa

After oral administration of DBD, the mRNA expression levels of J-chain, pIgR, BAFF, APRIL, IL-10, and IL-33 in intestinal tissues were significantly increased compared with those in the control group ($P < 0.05$). Additionally, the expression of IFN-γ was decreased, but there was no significant difference compared with the control group ($P > 0.05$) (Figure 5).

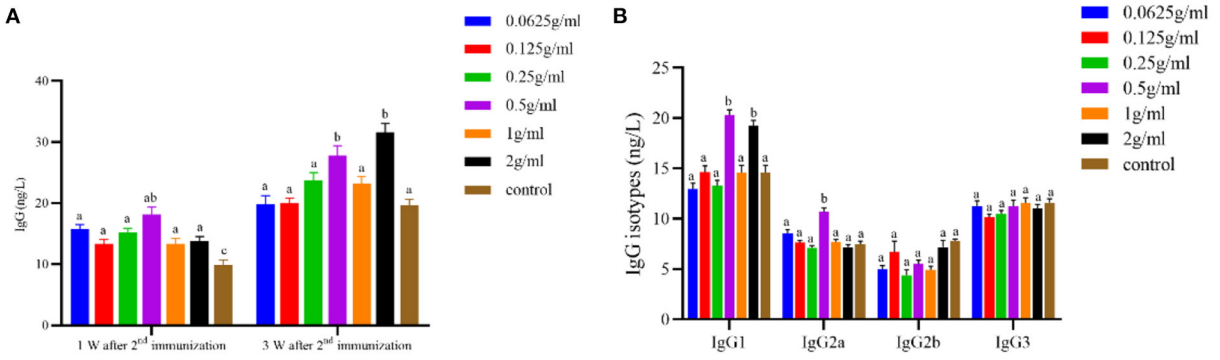


FIGURE 2
Effects of orally administered DBD on serum FMDV-specific antibody content in mice. **(A)** The concentration of IgG antibody. **(B)** The concentration of IgG isotypes antibody. [a,b]Values in the same column with different superscript letters differ significantly at $P < 0.05$.

Discussion

Most modern pharmacological studies on the immunomodulatory effect are conducted by regulating non-specific immunity or evaluating the therapeutic effect of pathological models, while there are few studies on specific immunity (18). Therefore, this trial explored the effects of gastric infusion DBD on specific immunity. The organ index is the ratio of an organ to its weight in a test animal, which can reflect the strength of its function to a certain extent. The spleen, thymus, and liver are the most important immune organs in mammals, and the quality of animal immune organs is increased by their proliferation caused by cell growth and division. Therefore, the weight is proportional to the immune function of the body (19, 20). In this study, it was found that the organ index of mice after gavage of different doses of DBD was not significantly different from that of the control group, but the 0.5 g/ml and 2 g/ml dose groups were superior to the control group. The humoral immune response of the body mainly includes IgG, IgM, IgA, IgD and IgE. And IgG is the main effector molecule of humoral immune response. The level of IgG antibody in serum can be used as an indicator of the success of vaccination. According to the differences in the antigenicity of the γ chain in IgG molecules, IgG in mice has four isotypes of antibodies, namely, IgG1, IgG2a, IgG2b, and IgG3. The results of this test showed that the oral administration of DBD could improve the antibody level of FMDV antigen, which was consistent with the results of Sun et al. (21).

When FMDV infects animals, both humoral and cellular immune functions resist the pathogen. This study found that DBD can increase the specific antibody level and specific antibody of the mouse class level, which suggests that after lavage DBD, the change in the antibodies may be related to the change in immune cells and immune active substances in the body. During the body's cellular immune response, immune cells affect the production of antibodies by secreting cytokines, which also inhibit the proliferation of related cells (22). Although the mechanism of antibody generation is extremely complex, numerous studies have shown that specific cytokines can promote certain types of antibody subclasses switching and

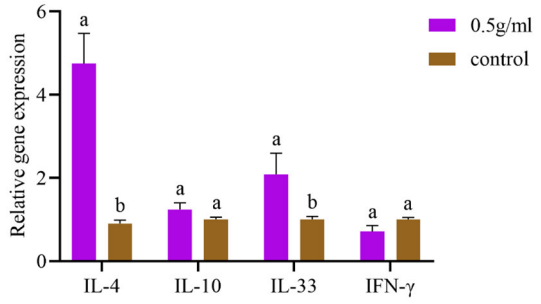


FIGURE 3
Effects of orally administered DBD on spleen cytokine mRNA expression in mice. [a,b]Values in the same column with different superscript letters differ significantly at $P < 0.05$.

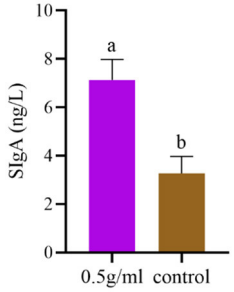


FIGURE 4
Effects of orally administered DBD on intestinal SIgA antibody content in the duodenum of mice. [a,b]Values in the same column with different superscript letters differ significantly at $P < 0.05$.

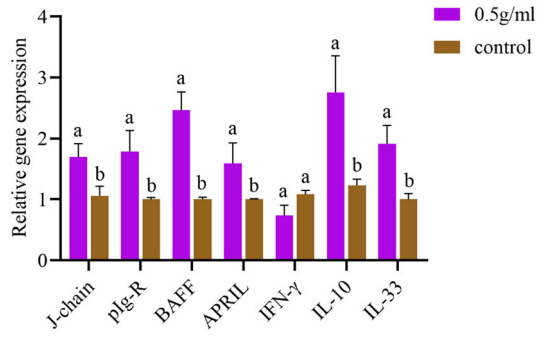


FIGURE 5
Effects of orally administered DBD on the mRNA expression of cytokines in the duodenum of mice. [a,b]Values in the same column with different superscript letters differ significantly at $P < 0.05$.

further to inhibit other antibody subclasses (23). CD4+ cells are divided into Th1 and Th2 subsets. Th1 subsets mainly express IFN-γ and IL-2, which are involved in cellular immunity to endogenous cell infection, while Th2 subsets express IL-4 and IL-10, which participate in allergic reactions and humoral immunity against parasitic infection (24). IFN-γ is an important B cell type switching factor that can induce B cells to secrete antigen-specific IgG2a (25). IL-4 can induce antigen-dependent IgG2a and IgE production (26) and enhance IgG1-mediated humoral immunity and NK-cell killing (27). In this study, the mRNA expression of IL-4 in the spleen was significantly increased after oral administration of DBD, but IL-10 and IFN-γ were not significantly changed, which may be due to the balance regulation of Th1 and Th2 cytokines in mice by DBD. Thus, DBD affects the distribution of antibodies and the synergistic inhibitory effect of IL-4 on IFN-γ (28).

The intestinal mucosa is the body's first line of defense against infection, which can induce effective mucosal immunity and generate a systemic immune response. Many studies have confirmed that oral administration of Chinese herbal medicine can enhance intestinal mucosal immune function (29, 30). As an important effector factor of mucosal immune response, SIgA can block the adhesion of pathogens to the mucosa, neutralize toxins and other bioactive antigens, and prevent a variety of microorganisms and mucosal antigens from entering intestinal epithelial cells. It does not require complement to form immune complexes with viruses in the respiratory tract, digestive tract and other parts to be excreted. Therefore, the concentration of SIgA antibody is commonly used clinically to evaluate the mucosal immune effect. The concentration of the SIgA is commonly used to evaluate the mucosal immune effect (31, 32). Studies have examined the immune-enhancing effect of *atractylodes* polysaccharide (33) and found that after gavage of *atractylodes* polysaccharide, the serum-specific IgG antibody and intestinal total SIgA content of mice vaccinated with FMD vaccine were significantly increased, which was similar to the results of this experiment. This suggests that the enhancement of the serum IgG response by Chinese herbal medicine is related to the enhancement of intestinal mucosal immunity. The IgA monomer produced by IgA+ plasma cells and the 15 kDa disulfide bond (J-chain) are connected in the cytoplasm and constantly aggregate to form the polymeric immunoglobulin receptor (pIgA). pIgA and the 80 kDa polymeric immunoglobulin receptor (pIgR) are combined and translocated to the surface of epithelial cells to form SIgA. However, PIgR can delay the degradation of SIgA without affecting the affinity of the antigen (34). Intestinal SIgA secretion is mediated by T cell-dependent and non-T cell-dependent immune regulation. In the T cell-dependent pathway, the interaction between antigen-presenting cells and Th2 cells releases Th2 cytokines, which promote the proliferation of IgA+ B cells and their differentiation into IgA-secreting plasma cells. Studies have shown that the dependence of T cells on mouse intestinal SIgA is also important (35) because mature B cells express IgA only after class switch recombination (CSR). In the T cell-independent response, B cells can be activated by triggering toll-like receptors, B cell receptor (BCR) antigen compounds or activated by DC-presented natural antigens, and the activation of the B cell activating factor of the TNF family (BAFF) and proliferation inducing ligand (APRIL) are important factors for IgA type switching and recombination (36, 37). IL-10 promotes the proliferation and differentiation of B cells into IgA-secreting plasma cells and also acts as an anti-inflammatory cytokine to inhibit NKT cell-mediated colitis and protect intestinal mucosa by regulating the expression of CD1d in intestinal epithelial cells (38, 39). The above discussion is involved, which needs to be further improved and explored its mechanism by our laboratory.

Conclusion

In conclusion, oral DBD can improve the immune function of mice immunized with the O-type FMD vaccine by improving serum-specific antibodies and the intestinal mucosal immune response, which could boost the immune system and improve the immune effect of the FMD vaccine.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by Laboratory Animal Ethical Review Committee, Southwest University, Chongqing, China. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BZ and LC conceived and designed the experiments. BZ, SB, and HD performed the experiments and collected and analyzed the data. HH and FG wrote the article, while HH, FG, and LC revised the article. All authors read and approved the final manuscript.

Funding

This research was supported by Chongqing Research Program of Basic and Frontier Technology (cstc2020jcy-jmsxmX0418) and Fundamental Research Funds for the Central Universities (SWU120002).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Diaz-San Segundo F, Medina GN, Stenfeldt C, Arzt J, de Los Santos T. Foot-and-mouth disease vaccines. *Vet Microbiol.* (2017) 206:102–12. doi: 10.1016/j.vetmic.2016.12.018

2. Pega J, Bucafusco D, Di Giacomo S, Schammas JM, Malacari D, Capozzo AV, et al. Early adaptive immune responses in the respiratory tract of foot-and-mouth disease virus-infected cattle. *J Virol.* (2013) 87:2489–95. doi: 10.1128/JVI.02879-12

3. Carpenter TE, O'Brien JM, Hagerman AD, McCarl BA. Epidemic and economic impacts of delayed detection of Foot-And-Mouth disease: a case study of a simulated outbreak in California. *J Vet Diagn Invest.* (2011) 23:26–33. doi: 10.1177/104063871102300104

4. Ren HR, Jin Z, Pei X, Li MT, Wang YM, Zhang J. Assessment of immunization procedures for foot-and-mouth disease in large-scale pig farms in China based on actual data and dynamics. *Anim Dis.* (2022) 2:45–60. doi: 10.1186/s44149-021-00035-3

5. Li W, Shan AT, Yuan FY, Zhou DN, Zhao HZ, Liu ZW, et al. Immune purification and effect evaluation of foot and mouth disease in large-scale breeding farms. *Chin J Vet Sci.* (2021) 41:2299–303. doi: 10.16303/j.cnki.1005-4545.2021.12.01

6. Tian Q, Zhou Y, Zhang MH, Zhou KG, Cheng ZT, et al. R and GAM analysis for environment factors influencing on the immune effect of FMD, PRRS and CSF in pigs in Guizhou province. *Chinese J Preventive Vet.* (2020) 42:690–695. doi: 10.3969/j.issn.1008-0589.201908030

7. Chen ZJ, Liu LJ, Gao CF, Chen WJ, Vong CT, Yao P, et al. Astragali Radix (Huangqi): a promising edible immunomodulatory herbal medicine. *J Ethnopharmacol.* (2020) 258:112895. doi: 10.1016/j.jep.2020.112895

8. Wang D, Liu Y, Zhao W. The adjuvant effects on vaccine and the immunomodulatory mechanisms of polysaccharides from traditional Chinese medicine. *Front Mol Biosci.* (2021) 8:655570. doi: 10.3389/fmolb.2021.655570

9. Yang FX, Wang Y, Xia PF, Yang RJ, Wang YX, Zhang J, et al. Review of chemical constituents, pharmacological effects and clinical applications of Danggui buxue decoction and prediction and analysis of its Q-markers. *China J Chin Materia Med.* (2021) 46:2677–85. doi: 10.19540/j.cnki.cjcmm.20200828.201

10. Shi XQ, Zhu ZH, Yue SJ, Tang P, Chen YY, Pu ZJ, et al. Integration of organ metabolomics and proteomics in exploring the blood enriching mechanism of Danggui Buxue Decoction in hemorrhagic anemia rats. *J Ethnopharmacol.* (2020) 261:113000. doi: 10.1016/j.jep.2020.113000

11. Wang X, Bei H, Du R, Chen Q, Wu F, Chen J, et al. Metabolomic analysis of serum reveals the potential effective ingredients and pathways of Danggui Buxue Tang in promoting erythropoiesis. *Complement Ther Med.* (2020) 48:102247. doi: 10.1016/j.ctim.2019.102247

12. Li C, Zhu F, Wang S, Wang J, Wu B. Danggui Buxue Decoction ameliorates inflammatory bowel disease by improving inflammation and rebuilding intestinal mucosal barrier. *Evid Based Complement Alternat Med.* (2021) 2021:8853141. doi: 10.1155/2021/8853141

13. Deng P, Li X, Wei Y, Liu J, Chen M, Xu Y, et al. The herbal decoction modified Danggui Buxue Tang attenuates immune-mediated bone marrow failure by regulating the differentiation of T lymphocytes in an immune-induced aplastic anemia mouse model. *PLoS ONE.* (2017) 12:e0180417. doi: 10.1371/journal.pone.0180417

14. McCright J, Ramirez A, Amosu M, Sinha A, Bogseth A, Maisel K. Targeting the gut mucosal immune system using nanomaterials. *Pharmaceutics.* (2021) 13:1755. doi: 10.3390/pharmaceutics13111755

15. Gu P, Wusiman A, Zhang Y, Cai G, Xu S, Zhu S, et al. Polyethylenimine-coated PLGA nanoparticles-encapsulated *Angelica sinensis* polysaccharide as an adjuvant for H9N2 vaccine to improve immune responses in chickens compared to Alum and oil-based adjuvants. *Vet Microbiol.* (2020) 251:108894. doi: 10.1016/j.vetmic.2020.108894

16. Yuan D, Yuan Q, Cui Q, Liu C, Zhou Z, Zhao H, et al. Vaccine adjuvant ginsenoside Rg1 enhances immune responses against hepatitis B surface antigen in mice. *Can J Physiol Pharmacol.* (2016) 94:676–81. doi: 10.1139/cjpp-2015-0528

17. Xu Q, Zhou Y, Zhang R, Sun Z, Cheng LF. Antiarthritic activity of Qi-Wu Rheumatism Granule (a Chinese Herbal Compound) on complete Freund's adjuvant-induced arthritis in rats. *Evid Based complement Alternat Med.* (2017) 2017:1960517. doi: 10.1155/2017/1960517

18. Zeng Y, Zhang SY, Hu GY. Research progress of Danggui Buxue Decotion. *Lishizhen Med Materia Medica Res.* (2016) 27:422–4. doi: 10.3969/j.issn.1008-0805.2016.02.062

19. Fu YF, Jiang LH, Zhao WD, Meng XN, Huang SQ, Yang J, et al. Immunomodulatory and antioxidant effects of total flavonoids of *Spatholobus suberectus* Dunn on PCV2 infected mice. *Sci Rep.* (2017) 7:8676. doi: 10.1038/s41598-017-09340-9

20. Li ZH, Zhang JQ, Wang T, Zhang JF, Zhang LL, Wang T. Effects of capsaicin on growth performance, meat quality, digestive enzyme activities, intestinal morphology, and organ indexes of broilers. *Front Vet Sci.* (2022) 9:841231. doi: 10.3389/fvets.2022.841231

21. Sun YX. Immunological adjuvant effect of a water-soluble polysaccharide, CPP, from the roots of *Codonopsis pilosula* on the immune responses to ovalbumin in mice. *Chem Biodivers.* (2009) 6:890–6. doi: 10.1002/cbdv.200800154

22. Kelley KW, Bluthé RM, Dantzer R, Zhou JH, Shen WH, Johnson RW, et al. Cytokine-induced sickness behavior. *Brain Behav Immun.* (2003) 17:112–8. doi: 10.1016/S0889-1591(02)00077-6

23. Stavnezer J. Antibody class switching. *Adv Immun.* (1996) 61:79–146. doi: 10.1016/S0065-2776(08)60866-4

24. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* (1986) 136:2348–57.

25. Coutelier JP, van der Logt JT, Heessen FW, Vink A, van Snick J. Virally induced modulation of murine IgG antibody subclasse. *J Exp Med.* (1988) 168:2373–8. doi: 10.1084/jem.168.6.2373

26. Faquim-Mauro EL, Coffman RL, Abrahamsohn IA, Macedo MS. Cutting edge: mouse IgG1 antibodies comprise two functionally distinct types that are differentially regulated by IL-4 and IL-12. *J Immunol.* (1999) 163:3572–6. doi: 10.1093/intimm/12.12.1733

27. Stavnezer J. Immunoglobulin class switching. *Curr Opin Immunol.* (1996) 8:199–205. doi: 10.1016/S0952-7915(96)80058-6

28. Wurster AL, Rodgers VL, Satoskar AR, Whitters MJ, Young DA, Collins M, et al. Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon γ-producing Th1 cells. *J Exp Med.* (2002) 196:969–77. doi: 10.1084/jem.20020620

29. Shi YX, Yan JK, Wang XM, Zhang SL, Yu JJ. Effect of the Chinese herbal extract on the number of intraepithelial lymphocytes and goblet cells and the level of IL-2 in the intestine of stressed mice. *Chin Vet Sci.* (2011) 41:85–8. doi: 10.16656/j.issn.1673-4696.2011.01.015

30. Zhai L, Li Y, Wang W, Wang Y, Hu S. Effect of oral administration of ginseng stem-and-leaf saponins (GSLS) on the immune responses to Newcastle disease vaccine in chickens. *Vaccine.* (2011) 29:5007–14. doi: 10.1016/j.vaccine.2011.04.097

31. Li Y, Jin L, Chen T. The Effects of secretory IgA in the mucosal immune system. *Biomed Res Int.* (2020) 2020:2032057. doi: 10.1155/2020/2032057

32. Mostov KE, Deitcher DL. Polymeric immunoglobulin receptor expressed in MDCK cells transcytoses IgA. *Cell.* (1986) 46:613–21. doi: 10.1016/0092-8674(86)90887-1

33. Xie F, Sakwiwatkul K, Zhang C, Wang Y, Zhai L, Hu S. Atractylodis macrocephalae Koidz. polysaccharides enhance both serum IgG response and gut mucosal immunity. *Carbohydr Polym.* (2013) 91:68–73. doi: 10.1016/j.carbpol.2012.07.083

34. Woof JM, Kerr MA. The function of immunoglobulin A in immunity. *J Pathol.* (2006) 208:270–82. doi: 10.1002/path.1877

35. Uematsu S, Fujimoto K, Jang MH, Yang BG, Jung YJ, Nishiyama M, et al. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5(J). *Nat Immunol.* (2008) 9:769–76. doi: 10.1038/ni.1622

36. Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, et al. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science.* (2001) 293:2111–4. doi: 10.1126/science.1061964

37. Xu W, He B, Chiu A, Chadburn A, Shan M, Buldys M, et al. Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. *Nat Immunol.* (2007) 8:294–303. doi: 10.1038/ni1434

38. Olszak T, Neves JF, Dowds CM, Baker K, Glickman J, Davidson NO, et al. Protective mucosal immunity mediated by epithelial CD1d and IL-10. *Nature.* (2014) 509:497–502. doi: 10.1038/nature13150

39. Cerutti A. The regulation of IgA class switching. *Nat Rev Immunol.* (2008) 8:421–34. doi: 10.1038/nri2322

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 15 November 2022
DOI 10.3389/fvets.2022.1005616

OPEN ACCESS

EDITED BY
Dayou Shi,
South China Agricultural University, China

REVIEWED BY
Yasmine Hasanine Tartor,
Zagazig University, Egypt
Kun Li,
Nanjing Agricultural University, China

*CORRESPONDENCE
Daniel Buldain
dbuldain@fcv.unlp.edu.ar

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 28 July 2022
ACCEPTED 31 October 2022
PUBLISHED 15 November 2022

CITATION
Buldain D, Gortari Castillo L, Buchamer AV, Bandoni A, Marchetti L and Mestorino N (2022) *In vitro* synergistic interaction between *Melaleuca armillaris* essential oil and erythromycin against *Staphylococcus aureus* isolated from dairy cows. *Front. Vet. Sci.* 9:1005616. doi: 10.3389/fvets.2022.1005616

In vitro synergistic interaction between *Melaleuca armillaris* essential oil and erythromycin against *Staphylococcus aureus* isolated from dairy cows

Daniel Buldain[1,2]*, Lihuel Gortari Castillo[1,2], Andrea Verónica Buchamer[1], Arnaldo Bandoni[3,4], Laura Marchetti[1] and Nora Mestorino[1]

[1]Laboratorio de Estudios Farmacológicos y Toxicológicos (LEFyT), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, Argentina, [2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), La Plata, Argentina, [3]Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Cátedra de Farmacognosia, Buenos Aires, Argentina, [4]Instituto de Química y Metabolismo del Fármaco (IQUIMEFA), CONICET-Universidad de Buenos Aires, Buenos Aires, Argentina

Staphylococcus aureus frequently causes subclinical mastitis around the world with a high impact on the milk industry and public health. Essential oils (EO) are recognized antimicrobials that can be synergistic with antibiotics. The main objective of this study was to evaluate the essential oil (EO) of *Melaleuca armillaris* as an adjuvant of erythromycin (ERY) for the alternative treatment of bovine mastitis caused by *S. aureus*. The Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC) of EO, ERY, and its combinations were established against *S. aureus* at different pHs (7.4, 6.5 and 5.0), emulating extra and intracellular conditions. Sensitive ($N = 3$) and resistant ($N = 3$) strains to ERY and *S. aureus* ATCC 29213 as control were used. Math models were applied to describe the antibacterial activity of EO and combinations EO-ERY. The EO was bactericidal against all the strains independently of the pH with a slight improvement in acid conditions. The synergism between EO and ERY was estimated by the Fractional Inhibitory Concentration Index (FIC) and by mathematical modeling of the bacterial killing data. Synergism was observed with ERY, where combinations had bactericidal activity also even with pH modification. *M. armillaris* EO is an interesting adjuvant for ERY, being a promissory option for further analysis of intracellular efficacy against *S. aureus*.

KEYWORDS

***Melaleuca armillaris*, essential oil, erythromycin, synergism, *Staphylococcus aureus*, mastitis**

10.3389/fvets.2022.1005616

Introduction

Bovine mastitis is a pathology that commonly affects dairy cattle, being a contagious disease with a great impact on milk industry profitability (1). Sick animals must be treated appropriately to guarantee both their welfare and ability to produce high quality milk (2). However, using antimicrobials can present disadvantages such as partially low cure rates and the presence of residues in milk that could favor the emergence of resistant microorganisms. This implies the necessity to perform studies in finding alternative treatments (3). The innovative and alternative treatments may include reducing the use of antimicrobials, replacing them with more effective and safer treatments, or replacing their application with other types of compounds (2).

Staphylococci are frequently isolated in bovines with mastitis (4) and, particularly, *Staphylococcus aureus* has great relevance in this disease (5), being one of the main causative agents of intramammary infections in dairy cows worldwide (6). *S. aureus* can be found in the skin of the mammary gland and teat lesions, so the main reservoir is the infected udders, where the microorganisms adapt, survive, and grow (6). This microorganism can form biofilms, grow in cell cytoplasm, and cause persistent bacteraemia or chronic infection, or it can remain quiescent and reactivates months or years later. On the other hand, if the bacterial population density in the infectious focus is high, *S. aureus* can become resistant to most of the antibiotics used in monotherapy (7). Ideally antimicrobials used for the treatment of *S. aureus* infections should get into phagocytic cells and remain inside for an adequate time, not be metabolized in cells, have significant antimicrobial activity at acid pH, be able to be administered through the teat canal, and have good distribution in the mammary gland (8). Macrolides and β-lactams, among others, are antimicrobials commonly used for the treatment of bovine mastitis caused by *S. aureus* (9, 10).

Macrolides are considered bacteriostatic agents, although they can exert a bactericidal effect under certain conditions. These antimicrobials act on the 50S ribosomal subunit and interfere with protein synthesis, and they are particularly active against Gram-positive bacteria and mycoplasmas (11, 12). Macrolides have wide distribution in the body, prolonged elimination half-life, activity against important microbial pathogens. They can reach high intracellular concentrations (10) and decreases it antimicrobial activity at pH 5.0 (11, 13). Intracellular activity of most macrolides is low (14), it seems to be associated with inactivation due to acidic pH of the phagolysosome (pH near 5) where they are located (15). These antibiotics lose approximately 90% of their activity for each pH unit that decreases (16). However, new macrolides such as azithromycin present intracellular activity *in vivo* against *S. aureus* (13, 14).

Erythromycin (ERY) is a macrolide recommended for the treatment of bovine mastitis caused by Gram-positive cocci (9) and reach high concentrations in milk after parenteral or intramammary administration (17). It belongs to the group of antimicrobials classified as critically important for human medicine (18) and the European Medicines Agency (EMA) recommends avoiding irresponsible and unnecessary use in animal production (19).

Currently, the therapeutic tool available against bovine mastitis continues being the intramammary administration of antibiotics. However, this is associated with the problem of antimicrobial resistance, being necessary to seek new alternative treatment approaches. Plant antibacterial agents can act as important sources of new antibiotics and compounds targeting bacterial virulence, which can be used alone or in combination with existing drugs (20). Consequently, medicinal plants are becoming an excellent natural product resource for future antibacterial therapy. The use of phytotherapeutic resources is aimed to satisfy a market necessity, which prefers healthier products that guarantee less environmental impact and that allow us to face the problem of antimicrobial resistance to conventional therapeutic products. Combination therapy, which combines conventional antibiotics with natural products, represents a promising strategy to deal with antibiotic resistance in the future. There are numerous reports dating back to the use of medicinal plants in ethnoveterinary medicine for bovine mastitis treatment, however, it is essential to standardize the extracts and compounds used to guarantee their efficacy (20).

Essential oils (EOs) are mixtures of volatile compounds isolated from plants, and their main chemical components possess a wide range of potential bacterial inhibitors. They can act as bacteriostatic or bactericides. Their active molecules have a great variety of target sites, mainly in the plasma membrane and in the cytoplasm, and in certain situations, they completely alter the morphology of cells. Effects of EOs generally lead to destabilization of the phospholipid bilayer, destruction of plasma membrane function and composition, loss of vital intracellular components, and inactivation of enzyme mechanisms (21). On the other hand, EOs have the potential to act as synergistic adjuvants of antibiotics, decreasing the concentration of antimicrobials to inhibit a microorganism (22–25).

Melaleuca armillaris EO had antimicrobial activity against *S. aureus* isolated from cows when it was applied alone (26, 27) and in combination with antibiotics like cloxacillin (28) and rifaximin (29). Therefore, the objective of this study was to combine this EO with ERY evaluating their interaction against *S. aureus* at different pH emulating pH conditions at the extra and intracellular level.

Materials and methods

Melaleuca armillaris essential oil extraction and characterization

The EO was obtained by steam distillation of fresh leaves and herbaceous branches of *M. armillaris* plants from the surroundings of Coronel Brandsen town (latitude 35°06′18.9″S and longitude 58°10′57.0″W), Buenos Aires, Argentina. A sample of the *M. armillaris* plants used was deposited in the LPAG Herbarium of the Faculty of Agrarian and Forestry Sciences, UNLP (30). As was described in previous works (27–29), the EO was dried with sodium sulfate anhydrous, filtered with a cotton funnel, and stored at 4°C in an amber glass bottle. EO composition was characterized by gas chromatography combined with mass spectrometry and flame ionization detection (GC-MS-FID; Agilent, Agilent Technologies, Santa Clara, CA, USA) (28), and the physicochemical characteristics were determined also (27).

Staphylococcus aureus strains and susceptibility against ERY

S. aureus ATCC 29213 reference strain and six wild-type *S. aureus* strains, isolated according to the National Mastitis Council procedure (31), from subclinical mastitis Holstein cows were used. Sampling was carried out following the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (32) and was approved by the Institutional Committee (CICUAL) of the Faculty of Veterinary Sciences, National University of La Plata (47.3.15J). Strains were identified phenotypically as a Gram-positive, coagulase-positive, catalase-positive, β-hemolytic, Voges Proskauer-positive, fermentation of trehalose, mannitol, and maltose. The susceptibility was checked by disk diffusion test using erythromycin disk 15 μg disk (Britania, Argentina) and *S. aureus* ATCC 25923 as quality control (33). Also we evaluated susceptibility to penicillin (10 IU), oxacillin (1 μg), clindamycin (2 μg), rifampin (5 μg), ciprofloxacin (5 μg) from Oxoid, England; cefoxitin (30 μg), gentamicin (10 μg), tetracycline (30 μg), azithromycin (15 μg), cefoperazone (30 μg), cephalexin (30 μg), enrofloxacin (5 μg), vancomycin (30 μg) from Britania, Argentina.

Minimum inhibitory and bactericidal concentration (MIC and MBC) of ERY and EO

The MIC of EO and ERY was performed by microdilution in broth using 96-well polystyrene microtiter plates with Mueller Hinton broth (MHB) (Biokar Diagnostics, France). A 0.5% of Tween 80 (Biopack, Argentina) was added to enhance the EO dissolution. The broth pH was adjusted to 7.4, 6.5 and 5.0 by addition of hydrochloric acid 1N (Anedra, Argentina), emulating pH conditions of extracellular and intracellular level sites. The ERY (Parafarm, Argentina) range of concentrations analyzed (applying a scheme of two-fold serial dilution) were between 1024 and 0.007 μg/mL. EO concentrations tested were between 50 and 0.1 μL/mL. In both cases each well was inoculated with a final bacterial concentration of 5×10^5 CFU/mL. Microplates were incubated at 35°C for 18–24 h. MIC was established as the lowest concentration which inhibits the bacterial growth. Each determination was done in triplicate. Positive and negative controls contained MHB with Tween 80 (0.5%) were included in the test.

The MBC was determined by inoculation spreading of 25 μL from each well showing no evident bacterial growth (after establishing the MIC) in nutritive agar plates for colony counting after incubation at 35°C for 18–24 h. The MBC was established as the first antimicrobial concentration which produce the fall of 99.9% from the initial inoculum.

Antimicrobial activity of combinations ERY/EO

The MIC of ERY-EO combinations at pH 7.4, 6.5 and 5.0 was established by the checkerboard technique (34) against the same strains used before. Thus, presence or absence of synergism was analyzed.

The design of the microtiter plate consisted of a row with two-fold serial dilution of EO and a column with two-fold serial dilution of ERY (antimicrobials MIC control). The intermediate wells had ERY/EO combinations in different proportions. The bacterial inoculums of *S. aureus* were 5×10^5 CFU/mL per well and the incubation was carried out at 35°C for 18–24 h. The MIC was established as the combination that inhibit the bacterial growth.

The results interpretation was similar for the MIC of individual antimicrobials but considering it as a mix. The fractional inhibitory concentration index (FIC) was determined by the following equation:

$$FIC = \frac{(A)}{(MIC)\,a} + \frac{(B)}{(MIC)\,b}$$

A synergistic effect exists if FIC $\leq$ 0.5, partial synergism (PS) if 0.5 $<$ FIC $<$ 1, indifference or addition (I) if 1 $\leq$ FIC $<$ 2, and antagonism (A) if FIC $\geq$ 2 (34, 35). Also, the same formula was applied to evaluate the synergism in terms of MBC, considering the same cutoff.

Time-kill assay and antibacterial activity index of ERY, EO and combinations ERY-EO

Time kill-assays for the 3 *S. aureus* strains resistant to ERY, exposing them to different amounts of the antimicrobial alone, and combined with EO at pH 7.4, 6.5 and 5.0 were performed. The concentrations were selected based on the MIC obtained in each case. For EO-ERY combinations 0.5 MIC, 1 MIC, 2 MIC, 4 MIC and 8 MIC were used. In the case of ERY alone 0.5 MIC, 1 MIC, 2 MIC and 4 MIC at pH 7.4 and 0.5 MIC, 1 MIC and 2 MIC at pH 6.5 and 5.0, respectively, were assayed. The reason of this was the high MIC value (particularly in acid conditions) for these strains making difficult the solubilization of the antibiotic in the culture media.

A tube for each condition to be evaluated containing a volume of 1 mL including Mueller-Hinton Broth with 0.5% Tween 80 (pH 7.4, 6.5, and 5.0), antimicrobial, and a bacterial concentration of 5×10^5 CFU/mL was prepared. Also, a positive (without antimicrobial) and a negative (without antimicrobial and inoculums) control were included. Tubes were incubated at 35°C and the bacterial plate count was performed sampling at 0, 2, 4, 8, 12, and 24 h after incubating at 35°C by 24 h. The experiment was performed in triplicate for each strain. With the data obtained Log_{10} (CFU/mL) *vs.* time graphs were constructed and the antibacterial activity index (*E*) was evaluated. *E* index was defined as the difference in Log_{10} between the bacterial count (CFU/mL) at the initial time (nt-0) and at the end of the assay (nt-24): $E = (\text{nt-24}) - (\text{nt-0})$. Three theoretical breakpoints to establish the bacteriostatic effect ($E = 0$), bactericidal effect ($E = -3$), and effect of virtual eradication of bacteria ($E = -4$) (36).

Then the *E* index *vs.* ERY concentration was graphed to compare what happens in presence and absence of EO. Wild type resistant strains were grouped according to the MIC, obtaining two groups of three strains for each one (resistant and sensitive), using the mean of triplicates for each strain.

Math modeling of *E* index

The *E* index values *vs.* ERY concentration data was mathematically modeled using a sigmoid model similar to a maximum response model (27, 37, 38). In this way we could analyzed deeply the effect of EO in the antimicrobial activity of ERY against *S. aureus* under the different conditions evaluated. The model equation was:

$$E = E_0 - (E_{max}.C^{\gamma})/(C_{50}^{\gamma} + C^{\gamma})$$

Where *E* is the index *E* (Log_{10} CFU/mL) for a concentration C (μg/mL), E_0 is the index *E* in the absence of the antimicrobial (Log_{10} CFU/mL), E_{max} is the maximum reduction in Log_{10} of E_0, C_{50} (μg/mL) is the concentration that causes 50% of the reduction of the E_{max}, and γ is the coefficient of sigmoidicity. The experimental data were fitted with the nonlinear least squares regression model using Sigma Plot software (Sigma Plot 12.0, 2011).

The C_{50} of ERY was compared at the 3 pHs by using the one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test with a level of significance established at $p < 0.05$. On the other hand, the C_{50} of ERY was compared with the same parameter obtained in the combinations with EO by means of the t test for unpaired data with a level of significance established at $p < 0.05$.

TABLE 1 MIC and MBC values of ERY for all the strains evaluated at pH 7.4, 6.5, and 5.0.

Strain	pH 7.4		pH 6.5		pH 5.0	
	MIC ERY μg/mL	MBC ERY μg/mL	MIC ERY μg/mL	MBC ERY μg/mL	MIC ERY μg/mL	MBC ERY μg/mL
ATCC 29213	0.5	32	1	> 256	8	>256
SA13	0.5	32	1	256	8	>256
SA96	0.5	32	1	256	8	>256
SA139	0.5	32	1	256	8	>256
SA78A	1,024	>1,024	>1,024	>1,024	>1,024	>1,024
SA79A	1,024	>1,024	>1,024	>1,024	>1,024	>1,024
SA86B	1,024	>1,024	>1,024	>1,024	>1,024	>1,024

Results

We worked with 3 wild type strains sensitive to ERY (SA13, SA96 and SA139), 3 wild type strains resistant to ERY (SA78A, SA79A and SA86B), and the reference strain ATCC 29213. Isolates SA78A, SA79A, and SA86B were also resistant to penicillin, azithromycin, and clindamycin. MICs and MBCs of ERY for all strains are shown in Table 1. We found that MIC of ERY was very low against *S. aureus* sensitive strains compared to resistant ones (0.5 and 1,024 μg/mL, respectively). In acid conditions the antibiotic was less effective, suffering a substantial loss of potency. This was particularly evident by the increment of MIC up to 2 times at pH 6.5, and 16 times at pH 5.0 compared to the MIC at pH of 7.4 for sensitive strains. Regarding resistant isolates, the loss of potency led to MIC values higher than 1,024 μg/μL. The MBC/MIC ratio for sensitive strains at pH 7.4 was about of 64, 256 at pH 6.5 and at pH 5.0 it was >32 (since the MBC was established as >256 μg/mL). For ERY resistant strains, the MBC was established as >1,024 μg/mL, showing an increase respect to MIC at pH 7.4. At pH 5.0 these values were also determined as >1,024 μg/mL. In this way, the extreme loss of potency suffered by this antibiotic in acidic media is clearly confirmed.

10.3389/fvets.2022.1005616

TABLE 2 MIC and MBC values of EO for all the strains evaluated at pH 7.4, 6.5, and 5.0.

Strain	pH 7.4		pH 6.5		pH 5.0	
	MIC EO µL/mL	MBC EO µL/mL	MIC EO µL/mL	MBC EO µL/mL	MIC EO µL/mL	MBC EO µL/mL
ATCC 29213	25	50	25	50	12.5	25
SA 13	12.5	25	12.5	25	6.25	12.5
SA 96	12.5	25	12.5	25	6.25	12.5
SA 139	12.5	25	12.5	25	6.25	12.5
SA 78A	12.5	50	6.25	50	3.1	25
SA 79A	12.5	50	6.25	50	3.1	25
SA 86B	12.5	50	6.25	50	3.1	25

TABLE 3 Results of synergism tests of EO/ERY combinations at pH 7.4, 6.5, and 5.0 in terms of MIC (FIC evaluation).

Strain	pH 7.4		pH 6.5		pH 5.0	
	MIC EO/ERY (µL/mL)/(µg/mL)	FIC	MIC EO/ERY (µL/mL)/(µg/mL)	FIC	MIC EO/ERY (µL/mL)/(µg/mL)	FIC
ATCC 29213	12.5/0.125	0.75	3.1/0.25	0.37	3.1/2	0.5
SA 13	6.25/0.125	0.75	3.1/0.25	0.5	3.1/2	0.75
SA 96	6.25/0.125	0.75	3.1/0.25	0.5	3.1/2	0.75
SA 139	6.25/0.125	0.75	3.1/0.25	0.5	3.1/2	0.75
SA 78A	6.25/64	0.56	3.1/128	<0.62	1.5/128	<0.62
SA 79A	6.25/64	0.56	3.1/128	<0.62	1.5/128	<0.62
SA 86B	6.25/64	0.56	3.1/128	<0.62	1.5/128	<0.62

In the case of EO, MIC and MBC (shown in Table 2) were slightly lower with the media acidity for all strains. The ratio MBC/MIC was 2 for the reference and sensitive wild type strains independently of the pH. For resistant wild type strains, this relation was 4 at pH 7.4 and the double at pH 6.5 and 5.0.

In Table 3 are listed the combinations of ERY and EO which presented lower FIC values. It is possible to observe synergism and partial synergism, depending on the strain and pH condition. The decrease of ERY concentration necessary to produce bacteria inhibition was important for resistant strains in presence of EO.

We also evaluated the synergism considering a similar index that FIC but in terms of the bactericidal combinations found. So, in Table 4 we show the EO/ERY combinations which had bactericidal activity and the respective fractional bactericidal concentration index (FBC). With these results, we also can mention that the combination EO/ERY has a synergic or partially synergic effect depending on the strain or pH condition, but it is more relevant concerning the analysis of inhibitory concentrations. At pH 7.4 the 3 resistant isolates presented FIC values of 0.56, in which the amount of antibiotic necessary for inhibition decreased 16 times (1,024 vs. 64 µg/mL) in the presence of 6.25 µL/mL of EO (MIC of EO decreased by half). At pH 6.5, the FIC coefficient was <0.65 (we could not establish it exactly since the MIC of the antibiotic only in this condition was determined as >1,024 µg/mL). In this case, the MIC of the antibiotic decreased at least 8 times (>1,024 to 128 µg/mL) but in the presence of 3.1 µL/mL of the essence, that is, the EO decreased its MIC by half. Finally, at pH 5.0 the situation was very similar to that observed at pH 6.5, since the MIC of ERY was reduced from >1,024 to 128 µg/mL, with the difference that the amount of plant extract was even less. The concentration of EO in the mixture at this pH was 1.5 µL/mL. Something similar was observed with the FBC index, but the synergic effects seem to be more important analyzing the bactericidal effect respect the inhibition.

Once the MIC of ERY alone and combined with EO was established we perform time-kill assays for resistant strains. Figure 1 shows the effect of different ERY concentrations at pH 7.4, 6.5 and 5.0, where even at high concentrations the bacteriostatic effect prevails. Analyzing the bacterial death

TABLE 4 Results of synergism tests of EO/ERY combinations at pH 7.4, 6.5, and 5.0 in terms of MBC.

Strain	pH 7.4		pH 6.5		pH 5.0	
	MBC EO/ERY (μL/mL)/(μg/mL)	FBC	MBC EO/ERY (μL/mL)/(μg/mL)	FBC	MBC EO/ERY (μL/mL)/(μg/mL)	FBC
ATCC 29213	6.25/0.5	0.14	6.25/2	<0.13	12.5/4	<0.52
SA 13	3.1/1	0.16	6.25/2	0.26	6.25/4	<0.52
SA 96	3.1/1	0.16	3.1/4	0.13	6.25/4	<0.52
SA 139	3.1/1	0.16	6.25/2	0.26	6.25/8	<0.53
SA 78A	25/64	<0.56	25/64	<0.56	12.5/64	<0.56
SA 79A	25/64	<0.56	25/64	<0.56	12.5/64	<0.56
SA 86B	25/64	<0.56	25/128	<0.63	12.5/64	<0.56

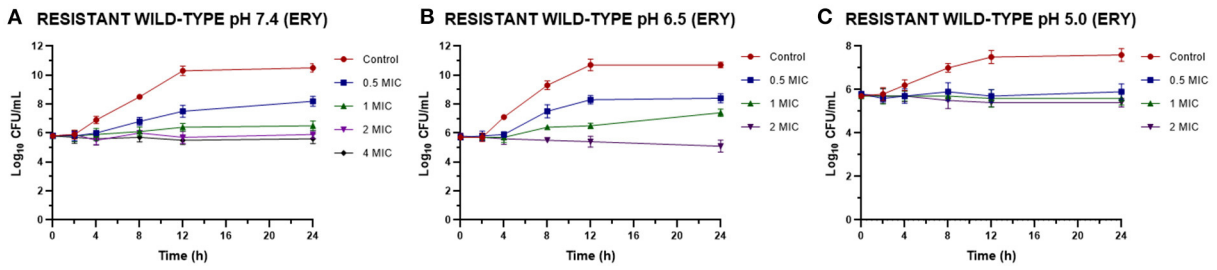


FIGURE 1
Time-kill curves for ERY against *S. aureus* resistant strains ($n = 3$, using the mean of triplicates for each strain) at pH 7.4 **(A)**, 6.5 **(B)**, and 5.0 **(C)** [minimum inhibitory concentration (MIC) = 1,024 μg/mL at pH 7.4 and 2,048 μg/mL at pH 6.5 and 5.0].

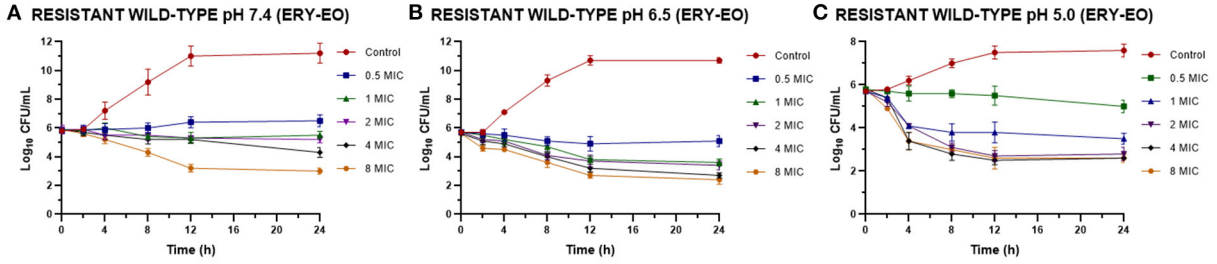


FIGURE 2
Time-kill curves for ERY/EO combinations against *S. aureus* resistant strains ($n = 3$, using the mean of triplicates for each strain) at pH 7.4 **(A)**, 6.5 **(B)**, and 5.0 **(C)** [minimum inhibitory concentration (MIC) = 64 μg/mL at pH 7.4 and 128 μg/mL at pH 6.5 and 5.0].

curves for *S. aureus* (resistant strains) against ERY (Figure 1), it can be observed again how, at the concentrations evaluated, the antimicrobial acts in a bacteriostatic way, since after 24 h of contact it was not possible to obtain a significant drop in the bacterial count, regardless of the pH and the sensitivity profile of the strains. The incidence of pH is observed in the amount of antibiotic necessary to achieve the same effect, since the concentrations in all cases increase with the acidification of the culture medium.

However, the addition of EO allow a drop in the bacterial count during the time evaluated, even with lower ERY amounts (Figure 2).

The antibacterial index *E* was graphed vs. ERY concentrations at pH 7.4, 6.5 and 5.0 for the reference strain (Figure 3), sensitive strains (Figure 4) and resistant strains (Figure 5). In these figures is possible to observe again the synergic effect of the addition of EO in ERY activity, also considering the change of pH.

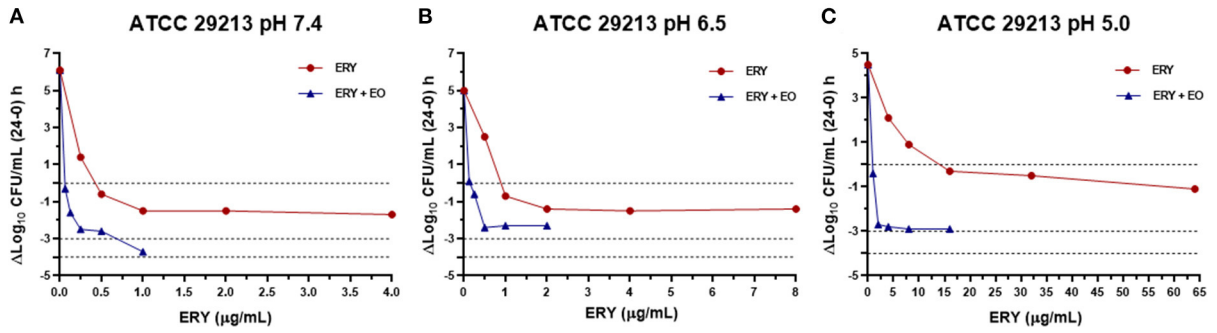


FIGURE 3
Graphic representation of the antibacterial effect (E: ΔLog_{10} CFU/mL 24–0 h) of ERY alone and combined with EO against *S. aureus* ATCC 29213 at pH 7.4 **(A)**, 6.5 **(B)**, and 5.0 **(C)**.

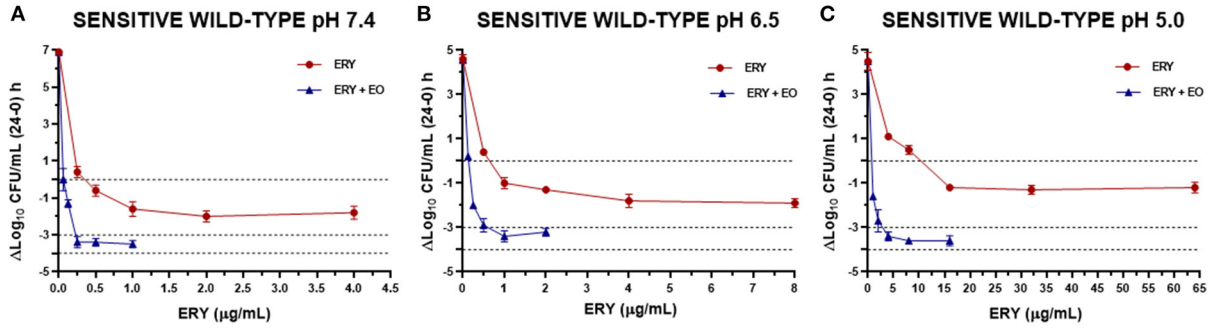


FIGURE 4
Graphic representation of the antibacterial effect (E: ΔLog_{10} CFU/mL 24–0 h) of ERY alone and combined with EO against *S. aureus* sensitive strains grouped (using the mean for each strain) at pH 7.4 **(A)**, 6.5 **(B)**, and 5.0 **(C)**.

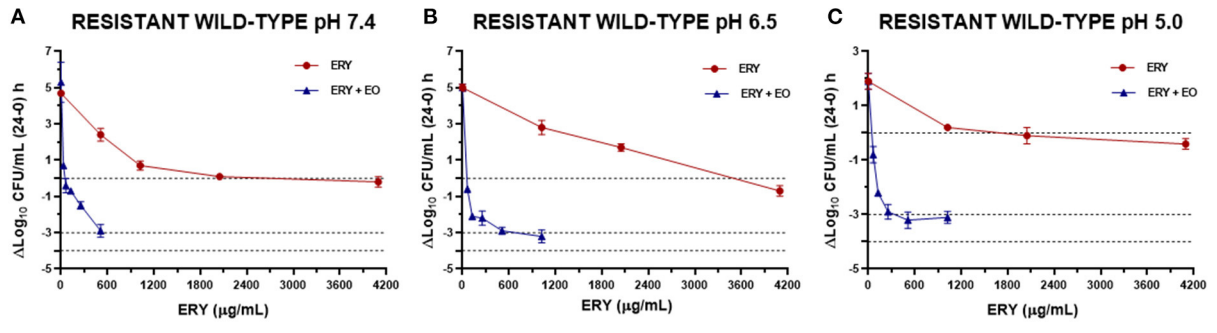


FIGURE 5
Graphic representation of the antibacterial effect (E: ΔLog_{10} CFU/mL 24–0 h) of ERY alone and combined with EO against *S. aureus* resistant strains grouped (using the mean for each strain) at pH 7.4 **(A)**, 6.5 **(B)**, and 5.0 **(C)**.

The antibacterial effect *E vs.* ERY concentration curves at the 3 pHs evaluated were mathematically modeled. The parameters obtained for ERY alone are shown in Table 5 and the same parameters for the combination with EO are described in Table 6. For the reference strain and isolates sensitive to this macrolide, it was possible to optimally adjust the model with R close to 1 in all the evaluated conditions. On the other hand, when evaluating resistant strains using only ERY, the data corresponding to pH 6.5 and 5.0 did not show good adjustments (R close to 0). This is probably because of a lower number of

TABLE 5 Parameters estimated by applying the sigmoid model to evaluate the antibacterial activity index for *S. aureus* at pH 7.4, 6.5, and 5.0 vs. erythromycin.

ERY	ATCC 29213			Sensitive strains			Resistant strains		
pH	7.4	6.5	5.0	7.4	6.5	5.0	7.4	6.5	5.0
R	1.00	1.00	0.99	0.99	0.99	0.99	1.00	–	–
E_{max} (Log_{10} CFU/mL)	7.78	6.47	5.72	9.05	6.54	5.94	4.87	–	–
ϒ	2.15	3.47	1.25	1.17	1.47	1.28	2.38	–	–
C_{50} (μg/mL)	0.21	0.57	5.16	0.12[a]	0.32[a]	3.85[b]	543.17	–	–
E_0 (Log_{10} CFU/mL)	6.11	5.02	4.48	6.87	4.59	4.33	4.67	–	–

Sigmoid model. E_0, E-index without erythromycin; E_{max}, maximum reduction in bacterial count; C_{50}, concentration of erythromycin necessary to achieve 50% of the maximum antibacterial effect; ϒ, sigmoidicity coefficient. Wild strains were grouped by minimal inhibitory concentration (MIC) similarity. For the strains 78A, 79A, and 86B at pH 6.5 and 5.0 it was not possible to carry out the modeling, the model yielded R adjustment values close to zero. Different lowercase letters between the pHs mean statistical differences ($p < 0.01$). Values without significant differences have the similar letter (a, b).

TABLE 6 Parameters estimated by applying the sigmoid model to evaluate the antibacterial activity index for *S. aureus* at pH 7.4, 6.5, and 5.0 vs. erythromycin in the presence of EO.

EO-ERY	ATCC 29213			Sensitive strains			Resistant strains		
pH	7.4	6.5	5.0	7.4	6.5	5.0	7.4	6.5	5.0
R	0.99	0.99	1.00	0.99	0.99	0.99	0.99	0.99	1.00
E_{max} (Log_{10} CFU/mL)	10.69	7.62	7.38	10.79	7.94	8.08	13.85	8.26	5.12
ϒ	0.64	1.35	3.89	1.27	1.99	1.61	0.39	1.19	1.87
C_{50} (μg/mL) of ERY	0.03	0.08	0.84	0.04[a]	0.11[a]	0.53[b]	189.50[c]	33.59[d]	61.15[e]
E_0 (Log_{10} CFU/mL)	6.11	5.01	4.48	6.87	4.59	4.34	5.34	4.97	1.89

Sigmoid model. E_0, E index in the absence of antimicrobials; E_{max}, maximum reduction in bacterial count; C_{50}, concentration of erythromycin (in the presence of EO) necessary to achieve 50% of the maximum antibacterial effect; ϒ, sigmoidicity coefficient. Wild strains were grouped by minimal inhibitory concentration (MIC) similarity. Different lowercase letters between the pHs mean statistical differences ($p < 0.05$). Values without significant differences have the similar letter (a, b, c, d, e).

points in the curve due 4 and 8 MIC were not evaluated for dissolution problems. For all strains confronted with ERY, it is notorious how the C_{50} increases with the decrease in pH, the increase at pH 5.0 being very significant ($p < 0.001$). In turn, the presence of EO allows the C_{50} of ERY to be significantly lower than when used alone at the 3 pHs evaluated ($p < 0.0001$). This parameter at pH 7.4 decreases from 543.167 to 189.503 μg/mL with 12.5 μL/mL of EO. For resistant isolates with 4 MIC at pH 6.5 and 2 MIC at pH 5.0, bactericidal effects are achieved. While at pH 7.4 a greater amount of both is required to achieve this effect. An explanation for this fact could be that ERY resistant strains appear to be more susceptible to pH lower than 7.4, where E_{max} is lower for the control without antimicrobials of resistant strains compared to the sensitive and reference strains.

Discussion

Our results shows that ERY acts in a bacteriostatic way and the *M. armillaris* EO is bactericidal, since if this ratio is >4 it is indicative that a compound is bacteriostatic and bactericidal if it is lower than 4 (39).

The combination of *M. armillaris* EO and ERY was interesting, particularly in the case of wild *S. aureus* strains resistant to this macrolide. We previously demonstrate the EO antimicrobial activity against *S. aureus* and the efficacy as adjuvant of cloxacillin (28) and rifaximin (29). The strains resistant to ERY evaluated in this work (SA78A, SA79A and SA86B) had very high MIC values (1,024 μg/mL), losing antimicrobial activity in acid conditions as was mentioned before. The combination with EO allowed to obtain mixtures whose FIC was close to 0.5, establishing partial synergism. Small amounts of *M. armillaris* EO were able to decrease significantly the concentration required to inhibit these *S. aureus* strains with high resistance to ERY.

There are few studies about synergism of ERY and plant extracts. Synergism against *S. aureus* has been found when combining this antibiotic with the *Lippia alba* EO (40) and the extract of *Indigofera suffruticosa* (41). Magi et al. (42) found that ERY is synergistic with carvacrol (terpene found in some essential oils) against Streptococci.

There are several resistance mechanisms developed by *S. aureus* that affect the activity of macrolides (11, 43). In first place, due to the modification of the target site by methylation or mutation, preventing the binding of the antibiotic to its ribosomal target. A second mechanism involves the efflux of the antibiotic, and a third the inactivation of the drug. Modification of the ribosomal target confers broad-spectrum resistance to

macrolides while efflux and inactivation affect only some of these molecules (44).

It is difficult to establish the mechanism by which *M. armillaris* EO and ERY exerts a synergistic effect. The large number of compounds found in the extract could have effect on different bacterial targets. The EO composition was previously described (28). The 1.8-cineole (main component found in the *M. armillaris* EO) can disintegrate the cell membrane and reducing the cytoplasm, causing damage to the structure of *S. aureus* (45). Regarding the other components of this EO, it has been postulated that α-Pinene, Terpinen-4-ol, sabinene, β-Myrcene and α-Terpinene would also be involved in the interaction with the cell membrane, where they dissolve in the phospholipid bilayer aligning between fatty acid chains. This physical distortion of the structure would cause expansion and destabilization of the membrane, increasing its fluidity, which in turn would increase passive permeability (46). A possible explanation for the synergy between the EO and ERY would be that destabilization in the membrane, cell wall and an eventual decrease in the activity of efflux pumps would increase the arrival of antibiotics into the bacterial cell interior, therefore it would become even more concentrated and facilitate interaction with the site of action at the ribosomal level.

Piatkoswka et al. (43), who studied strains of *S. aureus* resistant to ERY, said that resistance was the consequence of a strong decrease in the permeability of the cell wall to ERY. According to these authors, this variant of resistance mechanisms turns out to be the most efficient, creating the most resistant strains, with a MIC value $>1{,}024\,\mu g/mL$. The highly resistant strains did not present a large accumulation of the macrolide at intracellular level by destabilizing the cell membrane, so the barrier that stops their entry would be in the bacterial wall. Among other behaviors observed, cells from highly resistant strains tended to form larger and more stable aggregates, indicating that they differ in cell wall composition from less resistant ones. It is then possible that the activity of EO has implications on the cell wall.

An important aspect that presented differences was the change in bacterial death curves shapes. All resistant strains exposed to ERY alone at the 3 pH conditions presented growth curves that correspond to a bacteriostatic antibiotic, which was also reflected in that the MBC/MIC ratios were in all cases >4 (as mentioned above this is common for bacteriostatic antimicrobials).

With the addition of EO to the culture medium, bactericidal effects were obtained. This was also reflected in the E-index analysis. Like was observed for other classes of antibiotics like cloxacillin (28) and rifaximin (29), the E-Index *vs.* antibiotic concentration, show how the curve is shifted toward lower concentrations of ERY in the presence of EO. On the other hand, it is clearly stating how the addition of the plant extract favors the scope of the bactericidal effect. Again, the acidification of the culture medium presents an effect like that described above with the analysis of MIC values for the antibiotic.

Using the sigmoid model similar to the maximum response model to adjust the antibacterial index data as a function of ERY concentration, it is possible to observe what happens with the maximum effect (E_{max}) and the necessary concentration (C_{50}) to achieve 50% of E_{max}. There are no publications that report mathematical modeling of the antimicrobial activity of natural products combined with antibiotics. We have previously published the mathematical modeling of the activity of the *M. armillaris* EO on *S. aureus* (27). In this work we establish the usefulness of this type of models to compare the addition of adjuvants in the activity of antimicrobials. It is clear how the C_{50} of erythromycin decreases because of EO addition, reinforcing the results of synergism observed by previously analyzing the FIC and FBC indices.

Considering that macrolides can concentrate at the intracellular level, mainly within macrophages and polymorphonuclear leukocytes (47), the MIC and MBC of ERY combined with EO could be reached at the subcellular level. It has been reported that ERY can accumulate between 4 and 38 times more at the intracellular level than in the extracellular environment in macrophages, 8 times in polymorphonuclear neutrophils and 6 to 12 times in epithelial cells (48, 49). Therefore, it is feasible to reach these levels, transforming its combination with *M. armillaris* EO into a good alternative to evaluate for the treatment of *S. aureus* at the intracellular level.

There are few *in vivo* studies with EOs, Byung-Wook et al. (50) treated clinical mastitis in cows with *Origanum vulgare* EO, resulting in a decrease in *S. aureus* infection without causing swelling, redness, pain, and increased temperature in the udder. There are some EO-based products on the market for intramammary application, such as Phyto-Mast. This is recommended for intramammary use in lactation and drying. Thyme (*Thymus vulgaris*) EO is the antimicrobial active component and when used in cattle it did not present any irritating and inflammatory effect (51). Regarding residues in milk (taking thymol as marker), they were only detected 12 h after intramammary administration to goats (52) and cows (53). These findings allow us to consider the feasibility of administering EOs intramammary in the future as part of the treatment against mastitis. It is interesting to take advantage of the secondary metabolites produced by plants with pharmacological potential in the control of bovine mastitis in the context of both the problem of bacterial resistance and in the search for organic productions free of chemical residues. However, it is essential to standardize the extracts to ensure the quality and efficacy of the formulations.

Conclusions

The *M. armillaris* EO was synergic with erythromycin. The MICs and MBCs decreased with the addition of small amounts of EO, for both sensitive and resistant strains. Erythromycin had bacteriostatic activity when using alone, but when it was combined with the EO it behaved as a bactericidal antibiotic. If we consider that erythromycin can accumulate intracellularly, the bactericidal effect achieved with the EO combination would be considered as a promising alternative for the treatment of staphylococcal infections in bovine mastitis in a future, taking this work as starting point. The analysis of biological systems using mathematical models allows to obtain more information that simplifies collecting data from the observation of the results of an *in vitro* test. We will continue investigating about the intracellular efficacy of the combination between this macrolide and the EO.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

NM: conceived, supervised, and designed the experiments. DB: performed all the experimental assay, statistical analysis, and written the manuscript. LGC, AVB, and LM: contributed with experimental assays. AB: performed the EO quality assay. All authors contributed to the review, revision, and approved the final manuscript.

Funding

This work is partially financed by the Laboratory of Pharmacological and Toxicological Studies (LEFyT) and the National Agency for Scientific and Technical Promotion (ANPCyT) (PICT 2018-00932 and PICT 2020-01429).

Acknowledgments

The authors thank to National Scientific and Technical Research Council (CONICET) for their collaboration in granting Ph.D. Scholarships.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Ruegg PL. A 100-year review: mastitis detection, management, and prevention. *J Dairy Sci.* (2017) 100:10381–97. doi: 10.3168/jds.2017-13023

2. Krömker V, Leimbach S. Mastitis treatment- Reduction in antibiotic usage in dairy cows. *Reprod Dom Anim.* (2017) 52:21–9. doi: 10.1111/rda.13032

3. Gomes F, Henriques M. Control of bovine mastitis: Old and recent therapeutic approaches. *Curr Microbiol.* (2016) 72:377–82. doi: 10.1007/s00284-015-0958-8

4. Leitner G, Krifucks O, Kiran MD, Balaban N. Vaccine development for the prevention of staphylococcal mastitis in dairy cows. *Vet Immunol Immunopathol.* (2011) 142:25–35. doi: 10.1016/j.vetimm.2011.03.023

5. Oliveira M, Nunes SF, Carneiro C, Bexiga R, Bernardo F, Vilela CL. Time course biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. *Vet Microbiol.* (2007) 124:187–91. doi: 10.1016/j.vetmic.2007.04.016

6. Monistero V, Graber HU, Pollera C, Cremonesi P, Castiglioni B, Bottini E, et al. *Staphylococcus aureus* isolates from bovine mastitis in eight countries: genotypes, detection of genes encoding different toxins and other virulence genes. *Toxins.* (2018) 10:247. doi: 10.3390/toxins10060247

7. Mensa J, Soriano A, Llinares P, Barberán J, Montejo M, Salavert M et al. Guía de tratamiento antimicrobiano de la infección por *Staphylococcus aureus. Rev Esp Quimioter.* (2013) 26:1–84.

8. Algharib SA, Dawood A, Xie S. Nanoparticles for treatment of bovine *Staphylococcus aureus* mastitis. *Drug Deliv.* (2020) 27:292–308. doi: 10.1080/10717544.2020.1724209

9. de los Santos R, Zunino P, Gil A, Laport A, Hirigoyen D. Resistencia a los antibióticos de *Staphylococcus aureus* asociada con mastitis subclínica y clínica en Uruguay durante un período de ocho años. *Austral J Vet Sci.* (2017) 49:191–94. doi: 10.4067/S0719-81322017000300191

10. Prescott JF. Lincosamidas, macrólidos y pleuromutilinas. In: Prescott JF, Baggot JD, Walter RD (eds). *Terapéutica Antimicrobiana en Medicina Veterinaria, 3rd ed*. Buenos Aires, Argentina: Editorial Intermédica (2002). p. 204-32.

11. Lucas MF, Mestorino N, Errecalde J. Macrólidos: novedades de un clásico grupo de antimicrobianos. *Analecta Vet.* (2007) 27:36–45.

12. Sádaba B and Azanza JR. Antibióticos macrólidos y otros antibióticos. In: Leza Cerro, J.C., Lizasoaín Hernández I, Lorenzo Fernández P, Moreno González A, Moro Sánchez MA (eds). *Velásquez Farmacología básica y clínica, 20th ed.* Buenos Aires, Argentina: Editorial Panamericana (2005). p. 825–39.

13. Moncada Cárdenas A. *Farmacocinética y Farmacodinamia celular de antimicrobianos utilizados en la terapia de la mastitis subclínica bovina* [Doctoral thesis]. La Plata, Argentina: Universidad Nacional de La Plata.

14. Van Bambeke F. Macrolides and Ketolides. In: Vinks AA, Derendorf H, Mouton JW (eds). *Fundamentals of Antimicrobial Pharmacokinetics and Pharmacodynamics*. (2014). New York, United States: Editorial Springer. p. 257–79.

15. Pemán J, Cantón E, Ramón MS, Jiménez T, Gobernado M. Penetración y actividad de los antimicrobianos en el interior de los fagocitos. *Rev Esp Quimioterap.* (1991) 4:27–36.

16. Shryock TR, Mortensen JE, Baumholtz M. The effects of macrolides on the expression of bactericidal virulence mechanisms. *J Antimicrob Chemother.* (1998) 41:505–12. doi: 10.1093/jac/41.5.505

17. Ziv G. Practical pharmacokinetic aspects of mastitis therapy 2. Practical and therapeutic applications. *Vet Med Small Anim Clin.* (1980) 75:469–74.

18. World Health Organization. *Ranking of Medically Important Antimicrobials for Risk Management of Antimicrobial Resistance due to Non-Human Use. Critically Important Antimicrobials for Human Medicine, 6th revision*. (2018). Geneva, Switzerland: WHO.

19. European Medicine Agency. *Answers to the Requests for Scientific Advice on the Impact on Public Health and Animal Health of the Use of Antibiotics in Animals, Document of 18 December 2014 EMA/381884/2014, Veterinary Medicines Division/CVMP/CHMP*. (2014) London, England: EMA.

20. Mushtaq S, Shah AM, Shah A, Lone SA, Hussain A, Hassan QP et al. Bovine mastitis: an appraisal of its alternative herbal cure. *Microb Pathog.* (2018) 114:357–61. doi: 10.1016/j.micpath.2017.12.024

21. Nazzaro F, Fratianni F, De Martino L, Coppola R, De Feo V. Effect of essential oils on pathogenic bacteria. *Pharmaceuticals (Basel).* (2013) 6:1451–74. doi: 10.3390/ph6121451

22. Rodrigues FF, Costa JG, Coutinho HD. Synergy effects of the antibiotic gentamicin and the essential oil of *Croton zehntneri*. *Phytomedicine.* (2009) 16:1052–55. doi: 10.1016/j.phymed.2009.04.004

23. Rosato A, Vitali C, De Laurentis N, Armenise D, Milillo AM. Antibacterial effect of some essential oils administered alone or in combination with Norfloxacin. *Phytomedicine.* (2007) 14:727–32. doi: 10.1016/j.phymed.2007.01.005

24. Si H, Hu J, Liu Z, Zeng ZL. Antibacterial effect of oregano essential oil alone and in combination with antibiotics against extended-spectrum β-lactamase- producing *Escherichia coli*. *FEMS Immunol Med Microbiol.* (2008) 53:190–4. doi: 10.1111/j.1574-695X.2008.00414.x

25. Yap PS, Yiap BC, Ping HC, Lim SH. Essential oils, a new horizon in combating bacterial antibiotic resistance. *Open Microbiol J.* (2014) 8:6–14. doi: 10.2174/1874285801408010006

26. Amri I, Mancini E, De Martino L, Marandino A, Hamrouni L, Mohsen H, et al. Chemical composition and biological activities of the essential oils from three *Melaleuca* species grown in Tunisia. *Int J Mol Sci.* (2012) 13:16580–91. doi: 10.3390/ijms131216580

27. Buldain D, Gortari Castillo L, Marchetti L, Julca Lozano K, Bandoni A, Mestorino N. Modelling the growth and death of *Staphylococcus aureus* against *Melaleuca armillaris* essential oil at different pH conditions. *Antibiotics.* (2021) 10:222. doi: 10.3390/antibiotics10020222

28. Buldain D, Buchamer AV, Marchetti L, Aliverti F, Bandoni A, Mestorino N. Combination of cloxacillin and essential oil of *Melaleuca armillaris* as an alternative against *Staphylococcus aureus*. *Front Vet Sci.* (2018) 5:177. doi: 10.3389/fvets.2018.00177

29. Buldain D, Gortari Castillo L, Buchamer AV, Aliverti F, Bandoni A, Marchetti L, et al. *Melaleuca armillaris* essential oil in combination with rifaximin against *Staphylococcus aureus* isolated of dairy cows. *Front Vet Sci.* (2020) 7:344. doi: 10.3389/fvets.2020.00344

30. Holmgren PK, Holmgren NH, Barnett LC. *Index Herbariorum. The Herbaria of the World Part I, 8th ed*. (1990). New York Botanical Gardens: New York, NY.

31. National Mastitis Council. *Laboratory Handbook on Bovine Mastitis*. Madison, WI: National Mastitis Council Inc. (1999).

32. Federation of Animal Science Societies. *Guide for the Care and Use of Agricultural Animals in Teaching and Research, 3th ed.* (2010). Champaign, IL, USA: Federation of Animal Science Societies.

33. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. Second Informational Supplement, CLSI document VET01- S2*. Wayne, PA: Clinical and Laboratory Standards Institute (2013).

34. Eliopoulos GM, Moellering RC. Antimicrobial combinations. In: Lorian V, editor. *Antibiotics in Laboratory Medicine*. (1996). Baltimore, MD: The Williams &Wilkins Co. p. 330–96.

35. European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases, ESCMID, 2000. EUCAST Definitive Document E. Def 1.2, May 2000: Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. *Clin Microbiol Infect*. (2000) 6:503–08.

36. Sidhu PK, Landoni MF, Aliabadi FS, Lees P, PK-PD. integration and modeling of marbofloxacin in sheep. *Res Vet Sci.* (2011) 88:134–41. doi: 10.1016/j.rvsc.2009.05.013

37. Weidmann C, Dell'Elce A. Evaluación de la Actividad antibacteriana *in vitro* de marbofloxacina sobre *Escherichia coli*; Entre la biología descriptiva y el modelo matemático. In: *Proceedings of the XXI Encuentro de Jóvenes Investigadores de la Universidad Nacional del Litoral, Santa Fe, Argentina*. (2017) (accessed on October3–4, 2017).

38. Yu Y, Rüppel D, Weber W, Derendorf H. PK/PD Approaches. In: Hock F, Gralinski M (eds). *Drug discovery and evaluation: Methods in clinical pharmacology*. Cham, Switzerland: Springer International Publishing (2018). p. 1–23.

39. Pankey GA, Sabath D. Clinical relevance of bacteriostatic vs. bactericidal mechanisms of action in the treatment of gram-positive bacterial infections. *Clin Infect Dis.* (2004) 38:864–70. doi: 10.1086/381972

40. Veras HN, Campos AR, Rodrigues FF, Botelho MA, Coutinho HD, Menezes IR. et al. Enhancement of the antibiotic activity of erythromycin by volatile compounds of Lippia alba (Mill) NE Brown against *Staphylococcus aureus*. *Pharmacogn Mag.* (2011) 7:334–7. doi: 10.4103/0973-1296.90415

41. Bezerra Dos Santos AT, Araújo TF, Nascimento da. Silva LC, da Silva CB, de Oliveira AF, Araújo JM et al. Organic extracts from Indigofera suffruticosa leaves have antimicrobial and synergic actions with erythromycin against *Staphylococcus aureus*. *Front Microbiol.* (2015) 6:13. doi: 10.3389/fmicb.2015.00013

42. Magi G, Marini E, Facinelli B. Antimicrobial activity of essential oils and carvacrol, and synergy of carvacrol and erythromycin, against clinical, erythromycin-resistant Group A Streptococci. *Front Microbiol.* (2015) 6:165. doi: 10.3389/fmicb.2015.00165

43. Piatkowska E, Piatkowski J, Przondo-Mordarska A. The strongest resistance of *Staphylococcus aureus* to erythromycin is caused by decreasing uptake of the antibiotic into the cells. *Cell Mol Biol Lett.* (2012) 17:633–45. doi: 10.2478/s11658-012-0034-3

44. Leclercq R. Mechanisms of resistance to macrolides and lincosamides: Nature of the resistance elements and their clinical implications. *Clin Infect Dis.* (2002) 34:482–92. doi: 10.1086/324626

45. Li L, Li ZW, Yin ZQ, Wei Q, Jia RY, Zhou LJ et al. Antibacterial activity of leaf essential oil and its constituents from *Cinnamomum longepaniculatum*. *Int J Clin Exp Med*. (2014) 7:1721–27.

46. Hayouni EA, Bouix M, Abedrabba M, Leveau JY, Hamdi M. Mechanism of action of *Melaleuca armillaris* (Sol. ex Gaertu) Sm essential oil on six LAB strains as assessed by multiparametric flow cytometry and automated microtiter-based assay. *Food Chem.* (2008) 111:707–18. doi: 10.1016/j.foodchem.2008.04.044

47. Gómez-Lus ML, Calvo A, Bouza E, Prieto J. Quimioterapia antiinfecciosa y antitumoral: Antibióticos y quimioterápicos. Generalidades. In: Leza Cerro JC, Lizasoaín Hernández I, Lorenzo Fernández P, Moreno González A, Moro Sánchez MA. (eds). *Velásquez. Farmacología básica y clínica, 20th ed*. (2005). Buenos Aires, Argentina: Editorial Panamericana. p. 825–39.

48. Bosnar M, Kelnerić Z, Munić V, Eraković V, Parnham MJ. Cellular uptake and efflux of azithromycin, erythromycin, clarithromycin, telithromycin, and cethromycin. *Antimicrob Agents Chemother.* (2005) 49:2372–7. doi: 10.1128/AAC.49.6.2372-2377.2005

49. Villa P, Sassella D, Corada M, Bartosek I. Toxicity, uptake, and subcellular distribution in rat hepatocytes of roxithromycin, a new semisynthetic macrolide, and erythromycin base. *Antimicrob Agents Chemother.* (1988) 32:1541–6. doi: 10.1128/AAC.32.10.1541

50. Byung-Wook C, Chun-Nam C, Soo-Mi L, Mee-Jeong K, Ju-Yeon P, Chang-Yeol Y et al. Therapeutic effect of oregano essential oil on subclinical bovine mastitis caused by *Staphylococcus aureus* and *Escherichia coli*. *KSVS*. (2015) 55:253–7. doi: 10.14405/kjvr.2015.55.4.253

51. Mullen KA, Lee AR, Lyman RL, Mason SE, Washburn SP, Anderson KL. An *in-vitro* assessment of the antibacterial activity of plant derived oils. *Int J Dairy Sci.* (2014) 97:5587–91. doi: 10.3168/jds.2013-7806

52. McPhee CS, Anderson KL, Yeatts JL, Mason SE, Barlow BM, Baynes RE. Milk and plasma disposition of thymol following intramammary administration of a phytoceutical mastitis treatment. *Int J Dairy Sci.* (2011) 94:1738–43. doi: 10.3168/jds.2010-3988

53. Armorini S, Yeatts JE, Mullen KA, Mason SE, Mehmeti E, Anderson KL et al. Development of a HS-SPME-GC-MS/MS method for the quantitation of thymol and carvacrol in bovine matrices and to determine residue depletion in milk and tissues. *J Agric Food Chem.* (2016) 64:7856–5. doi: 10.1021/acs.jafc.6b02899

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 27 January 2023
DOI 10.3389/fvets.2023.980187

OPEN ACCESS

EDITED BY
Dayou Shi,
South China Agricultural University, China

REVIEWED BY
Hongbin Si,
Guangxi University, China
Ahmed Nisar,
Lasbela University of Agriculture, Water and Marine Sciences, Pakistan
Yi Wu,
Nanjing Agricultural University, China

*CORRESPONDENCE
Youjun Hu
✉ 748810399@qq.com
Zhiwen Xu
✉ abtcxzw@126.com

†These authors share first authorship

SPECIALTY SECTION
This article was submitted to Veterinary Pharmacology and Toxicology, a section of the journal Frontiers in Veterinary Science

RECEIVED 28 June 2022
ACCEPTED 10 January 2023
PUBLISHED 27 January 2023

CITATION
Su B, Wang Y, Jian S, Tang H, Deng H, Zhu L, Zhao X, Liu J, Cheng H, Zhang L, Hu Y and Xu Z (2023) *In vitro* and *in vivo* antiviral activity of monolaurin against *Seneca Valley virus*. *Front. Vet. Sci.* 10:980187. doi: 10.3389/fvets.2023.980187

In vitro and *in vivo* antiviral activity of monolaurin against *Seneca Valley virus*

Bo Su[1†], Yingjie Wang[1†], Shanqiu Jian[1†], Huaqiao Tang[1], Huidan Deng[1], Ling Zhu[1], Xiaonan Zhao[2], Jian Liu[2], Huangzuo Cheng[2], Lina Zhang[3], Youjun Hu[2*] and Zhiwen Xu[1*]

[1]College of Veterinary Medicine, Sichaun Agricultural University, Chengdu, China, [2]Innovation Center of Guangdong Nuacid Biotechnology Co., Ltd., Qingyuan, China, [3]College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang, China

Introduction: Surveillance of the *Seneca Valley virus* (SVV) shows a disproportionately higher incidence on Chinese pig farms. Currently, there are no vaccines or drugs to treat SVV infection effectively and effective treatment options are urgently needed.

Methods: In this study, we evaluated the antiviral activity of the following medium-chain fatty acids (MCFAs) or triglycerides (MCTs) against SVV: caprylic acid, caprylic monoglyceride, capric monoglyceride, and monolaurin.

Results: *In vitro* experiments showed that monolaurin inhibited viral replication by up to 80%, while *in vivo* studies showed that monolaurin reduced clinical manifestations, viral load, and organ damage in SVV-infected piglets. Monolaurin significantly reduced the release of inflammatory cytokines and promoted the release of interferon-γ, which enhanced the viral clearance activity of this type of MCFA.

Discussion: Therefore, monolaurin is a potentially effective candidate for the treatment of SVV infection in pigs.

KEYWORDS

MCFA, antiviral agent, monolaurin, SVV, inflammatory response

Introduction

Seneca Valley virus (SVV) belongs to the genus *Senecavirus* in the family *Picornaviridae*. Phylogenetic analysis of the whole-genome sequence of *Senecavirus* A shows that it is closely related to members of the genus *Cardiovirus* (1). In 2015, Brazilian scientists isolated the complete genome of SVV from vesicular fluid and serum of pigs with vesicular disease and elucidated that SVV infection was associated with idiopathic vesicular disease in pigs (2). Subsequently, many other countries have also reported cases of pigs infected with SVV, where newborn piglets are more vulnerable to SVV infection. The main clinical manifestations of SVV infection in pigs are blisters and ulcers on the hoofs and snout (3, 4). Clinical symptoms can be similar to foot-and-mouth disease, swine vesicular disease, and vesicular stomatitis, with a potential impact on the immune system of pigs (5). The virus is shed through the oral cavity, nasal secretions, and feces with a viral shedding duration of ~28 days after infection (3). Presently, SVV is sporadically and locally prevalent, but its transmission mechanism is not completely clear. Currently, there is no vaccine or specific drug available for the prevention and treatment of SVV infection in pigs. Therefore, the control of SVV in pigs depends on the hygiene measures implemented on pig farms.

Replacing antibiotics in animal feed with biologically active substances has become a hot topic in China. Medium-chain fatty acids (MCFAs) are a class of saturated fatty acids containing 6–12 carbon atoms. Even-numbered carbon MCFAs, such as caproic acid (C6), caprylic acid (C8), capric acid (C10), and lauric acid (C12), are found in natural foods, such as coconut oil, palm kernel oil, and milk. MCFAs undergo esterification with glycerol to form triglycerides,

known as medium-chain fatty acid triglycerides (MCTs). In addition to being a source of energy, MCFAs can also improve intestinal morphological structure and growth, prevent infection, regulate immunity, and act as an alternative to antibiotics (6, 7). Both MCFAs and MCTs exhibit strong bacteriostatic activity against a variety of pathogens, including gram-positive and gram-negative bacteria, viruses, fungi, algae, and protozoa (8, 9).

The antimicrobial properties of fatty acids have been reported extensively in the literature (10). Previously, studies have confirmed the antiviral activity of free MCFAs such as capric, lauric, myristic, and long-chain unsaturated oleic, linoleic and linolenic acids against vesicular stomatitis virus (VSV), herpes simplex virus (HSV) and visna virus (11). Other studies reported similar antiviral activity of MCFAs, together with their alcohol and monoglyceride derivatives, against HSV 1 and 2 (12). Research also showed that MCFAs (caprylic, capric, and lauric acids) and monolaurin can inhibit ASFV in liquid conditions and reduce Asfivirus (ASFV) infectivity, which may help to prevent disease progression and virus transmission (13). On the one hand, SVV is a small, non-enveloped picornavirus, unknown until 2002 when it was discovered incidentally as a cell culture contaminant, and the family Picornaviridae also contains foot-and-mouth disease virus (FMDV) and swine vesicular disease virus (SVDV). On the other hand, since the vesicular lesions caused by SVV infection are clinically similar from those caused by other vesicular disease viruses, such as FMDV, SVDV, VSV and vesicular exanthema of swine virus (VESV). Thus, we evaluated the antiviral activity of MCFAs or MCTs against SVV. In this study, the anti-SVV activity of selected MCFAs and MCTs was evaluated *in vitro*, and the most effective compound was selected and tested *in vivo*. The clinical symptoms, viral load, and proinflammatory cytokines were recorded and analyzed to evaluate the anti-SVV activity of monolaurin. Our results provide a reliable basis for the potential clinical use of monolaurin for the treatment of SVV infection in pigs.

Materials and methods

Samples and reagents

BHK-21 cells and the Seneca virus A strain SVV-SC-MS (complete genome GenBank: MN700930.1) were obtained from the Animal Biotechnology Center (ABTC) at Sichuan Agricultural University School of Veterinary Medicine. Fetal bovine serum, cell culture medium (DMEM), trypsin, and PBS buffer were purchased from Solarbio (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China); DMSO was purchased from Sigma (USA); the CCK8 kit (code: Beyotime. C0038) used in this study was purchased from Beyotime Biotechnology Co., Ltd. Caprylic, caprylic monoglyceride, capric monoglyceride, and monolaurin were prepared by Guangdong Nuacid Biotechnology Co., Ltd. The PrimeScript™ RT reagent Kit (Perfect Real Time), DNA/RNA extraction kit, and TB Green® Premix Ex Taq™ (Tli RNaseH Plus) were purchased from Takara (Dalian) Engineering Co., Ltd.

Maximum nontoxic dose

BHK-21 cells were cultured in a 96-well plate at 37°C under 5% CO_2 for 24–36 h until the cells grew into a monolayer. Caprylic, caprylic monoglyceride, capric monoglyceride, and monolaurin were dissolved in DMSO independently to prepare a 10 mg/mL stock solution. A cell maintenance solution of 100 μg/mL was prepared from the stock solution for these four test MCFA, whereafter a total of 11 concentration gradients were prepared from the cell maintenance solution following 2-fold gradient dilution. The concentrations of the cell maintenance solution for these four test MCFA were 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195, 0.098, and 0.049 μg/mL. Supernatant from all wells of a 96-well-plate with monolayer BHK-21 cells was discarded, and the MCFA sample solution (100 μL/well) was added. Two percent DMEM and one precebt DMSO controls were also set at the same time. The cells were cultured in an incubator for 48 h at 37°C and 5% CO_2. A cytotoxicity assay was performed according to the instructions of the CCK8 kit, and the cell viability was calculated. The concentration corresponding to a cell viability >90% was recorded as the maximum non-toxic dose (MNTD), which was used as the working dose for subsequent experiments.

In vitro calculation of viral inhibition rate

A mixture of 1 MNTD and 100 $TCID_{50}$ viral suspension was prepared by mixing the virus solution with the MCFA solution. BHK-21 cells were seeded in a 96-well-plate and grown to monolayers at 37°C in a 5% CO_2 incubator. The supernatants were discarded, and an equal volume of the viral suspension was added to the wells (100 μL/well) of the experimental group. The 2% DMEM (A) and 1% DMSO (B) controls were also set. The plates were incubated for 1 h at 37°C with 5% CO_2 in a cell incubator. After incubation, the supernatant was discarded, and 100 μL of the sample solution with a concentration of 1 MNTD or 100 μL of the maintenance solution was added to the corresponding wells and then incubated at 37°C in a 5% CO_2 incubator. Cell infection was stopped when complete CPE was developed in virus-only control wells (for ∼36–48 h post-infection). The supernatants were collected and measured with the CCK8 method, and the virus inhibition rate was calculated.

In vivo evaluation of anti-SVV activity

A total of 25 weaned piglets at 21 days old were obtained from a pig farm (Sichuan gistar group) in Sichuan Province, China. All the piglets were SVV negative for the antigen and antibody test by PCR or ELISA kits (detection methods were established by ATBC). Before the experiment, the animal lab was sterilized with formaldehyde and pasteurizer. All the pigs were cared for according to original farm procedures to prevent stress and bacterial infection in the pigs. The piglets were fed common complete feed. Piglets were first observed for 3 days and then subjected to viral challenge and drug administration. The 25 weaned piglets were divided into 5 groups ($n = 5$), as shown in Table 1.

Clinical symptoms

The clinical symptoms of piglets in each group were observed daily, and scores were assigned as follows: fever at 2 points; lethargy at 2 points; decreased feed intake at 1 point; anorexia at 2 points;

TABLE 1 Piglet grouping, viral challenge, and drug administration information.

Group	*n*	SVV	Monolaurin	Treatment time
Control	5	Cell supernatants	Saline	Days 1, 2, and 3
Model	5	10^5TCID$_{50}$/mL	Saline	
Low	5	10^5TCID$_{50}$/mL	0.5 g	
Medium	5	10^5TCID$_{50}$/mL	1 g	
High	5	10^5TCID$_{50}$/mL	2 g	

TABLE 2 The PCR master mix used for quantitative SVV detection from piglet stool and blood.

Primer F: AGGTACTGGAGAAGGACGCT	0.5 μL
Primer R: GGTTGACGTACAGGCCGAAA	0.5 μL
ddH2O	3 μL
SYBR Green Premix Ex TaqII	5 μL
cDNA	1 μL
Total	10 μL

blisters or ulcers at 2 points; and death as 5 points. Additionally, for every piglet in each group, morning feces and 0.5 mL jugular blood were collected daily for the determination of SVV load. Whenever blisters and crusts appeared or the piglets were on the verge of death, they were immediately sacrificed and necropsied. On day 14, all the remaining piglets were sacrificed, and the lungs, spleens, kidneys, and livers were aseptically collected and fixed in 4% paraformaldehyde.

Quantitative detection of SVV load in stool and blood by RT–qPCR

The feces and blood samples were thoroughly mixed with 3.0 mL of PBS and then centrifuged at 12,000 r/min for 3 min. Supernatants were collected for RNA extraction. Extracted RNA was reverse transcribed to obtain cDNA, which was added to the PCR master mix as detailed in Table 2, and loaded into a fluorescent quantitative PCR machine to detect the SVV viral load under the following thermocycling conditions: 40 cycles of denaturation at 95°C for 30 s, annealing at 95°C for 5 s, and elongation at 58°C for 30 s. At the end of the amplification, melting curve analysis was performed from 65 to 95°C with 0.5°C per second.

Histopathology

Briefly, the Lung, spleen, liver, and kidney tissues were fixed in 4% paraformaldehyde for 36 h and then embedded in paraffin. According to standard method, tissue sections (4 μm), were stained with Hematoxylin and Eosin (H&E) for histopathological examination. Finally, histological lesions were recorded with a light microscope (OLYMPUS, Japan) at 400× magnifications.

In vivo detection of inflammatory cytokines

Piglet venous blood was collected at 0 and 3 dpi. The expression of IL-6, IL-8, IL-10, IL-1β, IFN-γ, and TNF-α in the supernatant was detected using ELISA kits according to the manufacturer's instructions (Multisciences (Lianke) Biotech Co., Ltd., Hangzhou, China). The absorbance was measured using a microplate reader at 450 nm. In brief, the samples were added to the wells, followed by the antigen in the samples bounding to the capture antibody, the microplate was washed, the detection antibody was added, and the microplate was washed again. After the substrate was added, the microplate reader detected the colored reaction products and calculated light density (OD) values, which were used to calculate and analyze the amount of antigen in each sample.

Statistical analysis

Statistical results were expressed as means and standard deviation (SD). Significant differences were determined with one-way analysis of variance (ANOVA), followed by Duncan's multiple range test in SPSS 20.0 (IBM Corp., Armonk, NY, USA). Significance was set at $P < 0.05$.

Results

Maximum non-toxic concentration determination

After adding different concentrations of drugs and culturing for 96 h, the OD_{450} value or cell viability was determined using a CCK-8 kit following the manufacturer's instructions. If the cell viability was >90%, the dose was recorded as MBTD. BHK-21 cells showed good tolerance to glycerol caprylate and monoglycery laurate with an MNTD of 50 μg/mL. Caprylic and capric monoglycerides showed little toxicity to these cells with an MNTD of 25 μg/mL (Figure 1).

Monolaurin inhibits virus proliferation

Although all four treatments had an inhibitory effect on SVV, the inhibitory rate of monolaurin was the highest, with values up to 80% (Figure 2). The anti-SVV activity of caprylic monoglycerides was better than that of capric monoglycerides. Monolaurin was selected for the subsequent *in vivo* tests.

Clinical symptoms and scores

After the piglets were challenged with the virus and treated with monolaurin, the development and progression of their clinical symptoms were observed and monitored continuously for 14 days (Figure 3). One piglet from the low-dose group died at 2 dpi. There were no other piglet deaths recorded in this group on the subsequent days. One piglet from the model group died at 3 dpi. No piglets died in the middle-dose, high-dose, or control groups. The piglets in the low-dose and model groups showed decreased feed intake and symptoms such as anorexia, lethargy, and fever after the virus

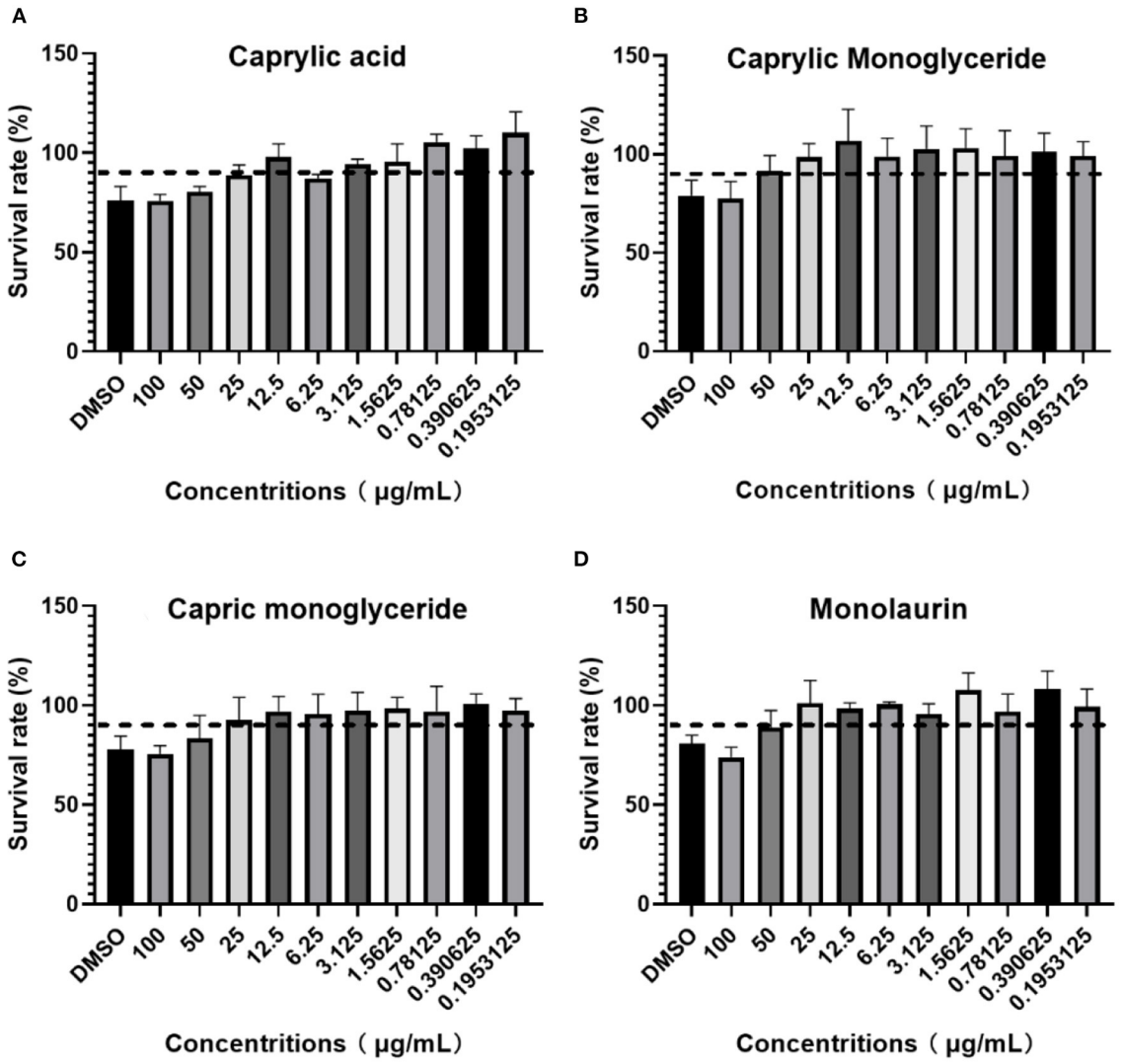


FIGURE 1
BHK-21 cell survival rate in: **(A)** Caprylic acid; **(B)** Caprylic monoglyceride; **(C)** Carpric monoglyceride; **(D)** Monolaurin.

challenge. These piglets had blisters and ulcers on their snouts and hooves at 7 dpi. Except that erosions, ulcerations, and vesicular lesions of the snout, oral mucosa, and distal limbs, especially around the coronary band, as well as more general symptoms of illness such as fever, lethargy, and anorexia, may be observed from the model group. Hoof sloughing and lameness can also occur. By contrast the clinical symptoms, mental status, and feed intake of the monolaurin-treated piglets were better than those of piglets in the model group. The piglets in the middle-dose and high-dose groups had similar clinical symptoms, with a lower post-challenge score than that of the model group.

Post-challenge effects of monolaurin on SVV load

Piglet viral load peaked at 3 dpi and then continued to decline (Figure 3). Monolaurin reduced the viral load in the feces and blood of piglets infected with SVV in a dose-dependent manner (Figure 4). The treatment with high-dose Monolaurin was the most effective for viral load in fecal and blood of SVV-infected piglets. Compared with fecal viral load of SVV infected piglets, blood viral load decreased more significantly after 3 dpi (Figure 4B).

Histopathological examination

Blisters and ulcers manifested on the snout and hoofs at 7 dpi. Pigs in the model and low-dose groups exhibited the following clinical manifestations: parts of the lung were atrophied, the alveolar septum was thickened, and the rest of the lung tissues had compensatory emphysema. In the model group, spleens showed diffuse hemorrhage, severe swelling of hepatocytes, partial cell necrosis, glomerular atrophy, and partial shedding of the renal tubular epithelium. There were no significant changes in the spleens, livers, and kidneys of pigs in the low-dose group. The pigs in the middle-dose and high-dose groups had alveoli without obvious

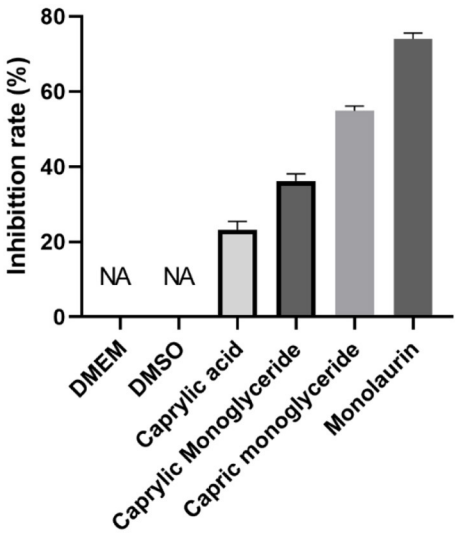


FIGURE 2
In vitro evaluation of the anti-SVV activity of MCFAs.

lesions, while their spleens, kidneys, and livers had clear structures but no obvious lesions (Figure 5).

In vivo detection of inflammatory cytokines

Proinflammatory cytokines were significantly increased by SVV infection, and treatment with monolaurin showed some degree of anti-inflammatory activity (Figure 6). High doses of monolaurin significantly decreased the levels of IL-1β, IL-10, and TNF-α to levels ($p < 0.05$), without a significant difference compared with the corresponding control groups ($p > 0.05$). However, with the increase of the monolaurin dose, the trend of the increase of IFN-γ level is more obvious. So, high doses of monolaurin significantly increased the amount of IFN-γ in a dose-dependent manner.

Discussion

Monolaurin, a monoglyceride formed from 12-carbon saturated fatty acids and glycerol, is naturally found in coconut oil, palm oil, and breast milk, and is a safe and highly effective monoglyceride with bacteriostatic activity (14). Monolaurin has broad antibacterial activities, including inhibiting bacterial growth, reducing the production of exotoxin, and forming biofilms (15). As a lipid, monolaurin can bind to the phospholipid bilayer of bacteria and disrupt the normal physiological processes of the bacteria, thereby inducing a bacteriostatic effect. Monolaurin is also reported to have a strong inhibitory effect on the growth and reproduction of gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Helicobacter pylori*, *Bacillus*, and *Campylobacter jejuni*, among others (16). Furthermore, monolaurin can block the release of gram-positive bacterial exotoxins (such as enterotoxins and streptococcal pyrogenic exotoxins, etc.) (17). Monolaurin can also bind to the lipid bilayer membrane enveloped viruses and inhibit viral activity by compromising viral integrity and infectivity. Monolaurin has shown a good inhibitory effect on some enveloped viruses, such as the HSV, influenza virus, PRRS virus, and porcine epidemic diarrhea virus (18, 19). In this study, we show that monolaurin has a strong inhibitory effect on SVV even though it is a non-enveloped virus. However, the anti-SVV mechanism of monolaurin needs further research.

Even though SVV causes blisters and ulcers on the snout and hoofs of pigs, there are only a few reports on autopsy symptoms and microscopic pathogenesis of this viral infection. Pathological experiments in this study showed lesions in the lungs, livers, spleens, and kidneys of infected piglets. Preventing SVV from destroying the integrity of the intestinal barrier helps to reduce the damage of the virus. Monolaurin has great potential for application in animal health, as it promotes growth and gut health. For example, some studies have found that monolaurin can significantly improve the growth performance of weaned piglets (20, 21). Additionally, dose-related monolaurin has been found to improve body weight, regulation of gut microbiota, and systemic inflammation in mice fed a low-fat diet (22). These studies show a significant positive correlation between monolaurin and the increased abundance of probiotics, such as *Lactobacillus reuteri* and *Ruminococcus gnavus* (22). Normal intestinal flora is necessary for the integrity of the tight junctions of the intestinal tract. Here, monolaurin significantly improves the health of the intestinal tract, which reduces the chance of viruses invading the intestinal epithelial cells and the bloodstream. Our findings corroborate the findings of these previous studies, as we found that monolaurin-treated pigs had significantly reduced viral loads in their blood and feces as well as reduced clinical symptoms associated with SVV infection. During the trial, one piglet from the low-dose group died at 2 dpi, one piglet from the model group died at 3 dpi. No piglets died in the middle-dose, high-dose.

Many viruses can induce inflammatory responses and even cause an inflammatory factor storm (23). The mechanism is the excessive activation of immune cells by increasing intracellular inflammatory factors, including interleukin, TNF-α, and complement protein molecules (24). The storm-like suicide attack induced by pathogenic microorganisms in infected cells can cause bystander damage to other tissues by increasing vascular permeability and circulatory disorders, which can even result in multiple organ functional failure (MOF) (25). Usually, inflammation is a protective immune response that is conducive to clearing pathogenic microorganisms. However, uncontrollable excessive inflammation can cause autoimmune damage (26). In this study, we observed that SVV infection induced the release of many inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-10, and TNF-α, triggering an inflammatory cytokine storm. Previous studies have found that monolaurin affects the lipid dynamics of human T cells and regulates T-cell signaling and the release of functional factors. It also inhibits the immune response that is overactivated by the virus, thereby reducing the amount of SVV-induced inflammatory cells (27). *Seneca Valley* virus infection can reduce the level of IFN-γ in the serum, hence reducing the antiviral activity of the body. On the other hand, monolaurin can increase the level of IFN-γ, possibly explaining one of the anti-viral mechanisms of MCFAs.

The results from this study support the efficacy of Monolaurin against SVV. Data suggest that monolaurin block virus proliferation

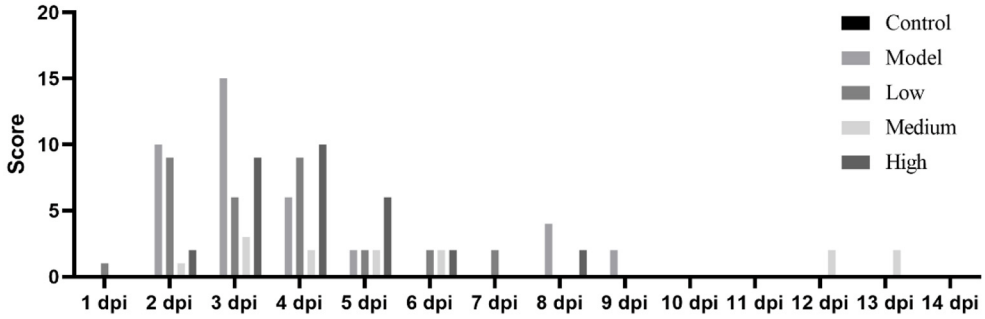


FIGURE 3
Clinical symptom scores for piglets infected with SVV.

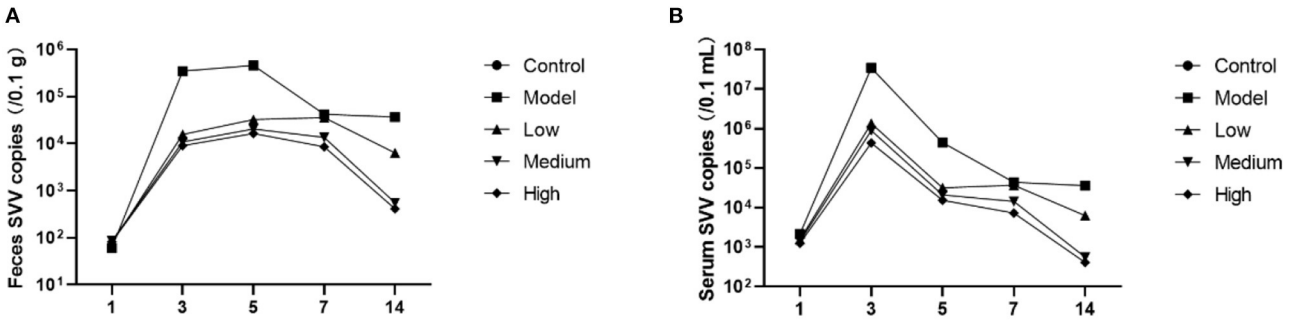


FIGURE 4
Viral load in the feces and blood of piglets infected with SVV. **(A)** Viral load in feces; **(B)** Viral load in blood.

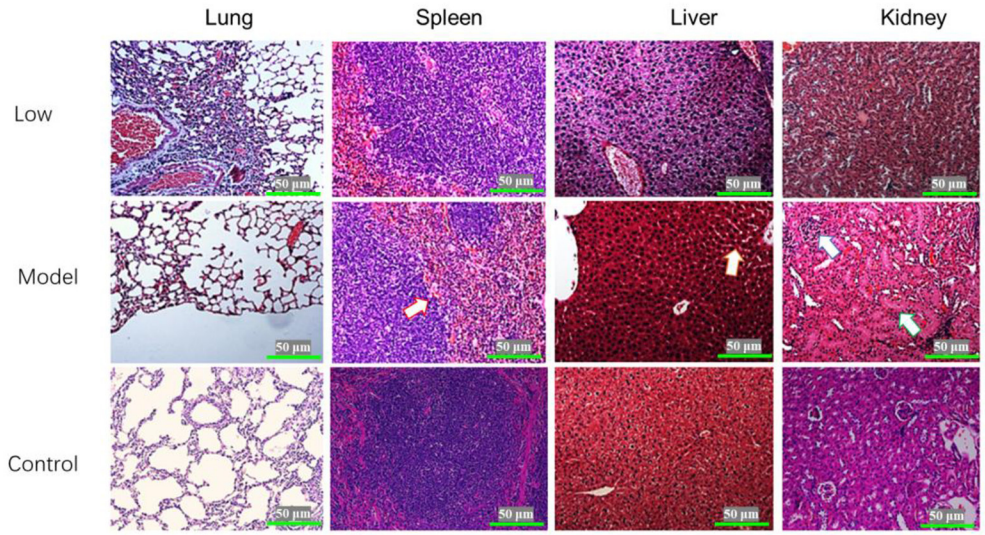


FIGURE 5
Histopathological examination of the lungs, spleen, liver, and kidneys of piglets infected with SVV (HE×400). The arrow on Model-spleen indicated the hemorrhage diffuse; the arrow on Model-liver indicated the cell swelling; and the arrows on Model-kidney indicated the glomerular atrophy and tubular swelling.

and systemic inflammation response. Despite the promising results, our sample sizes were small and it is not clear whether other mechanisms are involved in the regulation of the efficacy of antiviral therapies. A large-scale evaluation of the anti-viral potential and mechanism of monolaurin should be the subject of future studies.

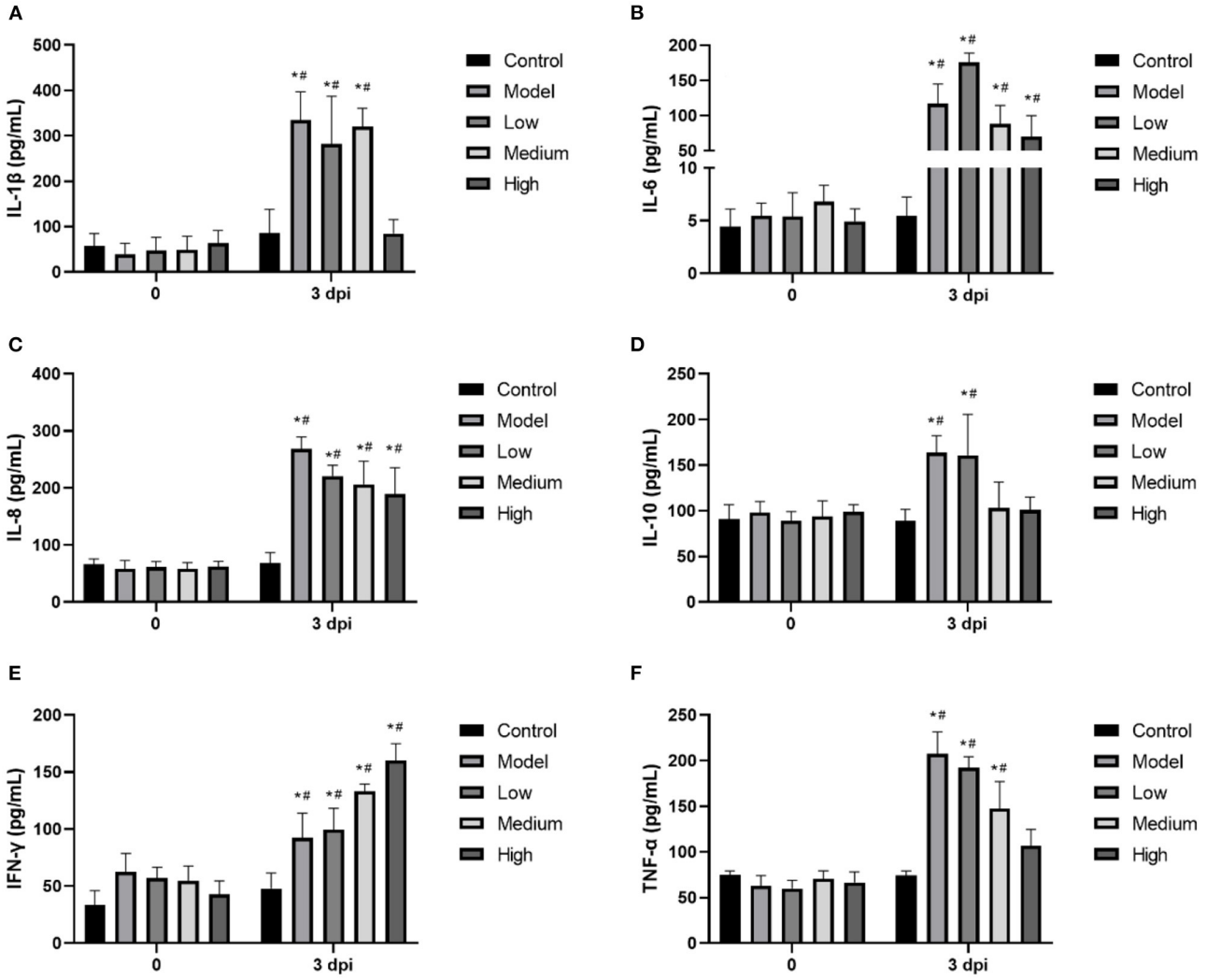


FIGURE 6
(A–F) The relative mRNA expressions of cellular inflammatory factors IL-1β, IL-6, IL-8, IL-10, IFN-γ. and TNF-α in piglet blood. Effects of monolaurin on cytokine release in piglet blood infected with SVV: $^{*}p < 0.05$ *vs.* 0 dpi, $^{\#}p < 0.05$ *vs.* control.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the Animal Ethical Committee of Sichuan Agricultural University.

Author contributions

BS, YW, and SJ conceived of the presented idea. HT and HD developed the theory and performed the computations. All authors contributed to the article and approved the submitted version.

Conflict of interest

XZ, JL, HC, and YH were employed by Innovation Center of Guangdong Nuacid Biotechnology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Venkataraman S, Reddy SP, Loo J, Idamakanti N, Hallenbeck PL, Reddy VS. Structure of *Seneca Valley* virus-001: an oncolytic picornavirus representing a new genus. *Structure.* (2008) 16:1555–61. doi: 10.1016/j.str.2008.07.013

2. Vannucci FA, Linhares D, Barcellos D, Lam H, Collins J, Marthaler D. Identification and complete genome of *Seneca Valley* virus in vesicular fluid and sera of pigs affected with idiopathic vesicular disease, Brazil. *Transbound Emerg Dis.* (2015) 62:589–93. doi: 10.1111/tbed.12410

3. Joshi LR, Fernandes MH, Clement T, Lawson S, Pillatzki A, Resende TP, et al. Pathogenesis of Senecavirus A infection in finishing pigs. *J Gen Virol.* (2016) 97:3267–79. doi: 10.1099/jgv.0.000631

4. Montiel N, Buckley A, Guo B, Kulshreshtha V, VanGeelen A, Hoang H, et al. Vesicular disease in 9-week-old pigs experimentally infected with Senecavirus A. *Emerg Infect Dis.* (2016) 22:1246. doi: 10.3201/eid2207.151863

5. Xue Q, Liu H, Zhu Z, Yang F, Ma L, Cai X, et al. *Seneca Valley* virus 3Cpro abrogates the IRF3-and IRF7-mediated innate immune response by degrading IRF3 and IRF7. *Virology.* (2018) 518:1–7. doi: 10.1016/j.virol.2018.01.028

6. Dahmer PL, Leubcke GE, Lerner AB, Jones CK. Effects of medium-chain fatty acids as alternatives to ZnO or antibiotics in nursery pig diets. *Transl Anim Sci.* (2020) 4:txaa151. doi: 10.1093/tas/txaa151

7. Lawrence JM, Lerner AB, Jones CK. *The Effect of Feeding Medium Chain Fatty Acids (MCFA) on Nursery Pig Growth in an Attempt to Replace ZnO or Carbadox, Animal Sciences and Industry Undergraduate Research Symposium*. Spring (2019).

8. Hanczakowska E, Swiatkiewicz M, Natonek-Wiśniewska M, Okoń K. Medium chain fatty acids (MCFA) and/or probiotic *Enterococcus faecium* as a feed supplement for piglets. *Livest Sci.* (2016) 192:1–7. doi: 10.1016/j.livsci.2016.08.002

9. Huang CB, Alimova Y, Myers TM, Ebersole JL. Short-and medium-chain fatty acids exhibit antimicrobial activity for oral microorganisms. *Arch Oral Biol.* (2011) 56:650–4. doi: 10.1016/j.archoralbio.2011.01.011

10. Desbois AP. Potential applications of antimicrobial fatty acids in medicine, agriculture and other industries. *Recent Pat Antiinfect Drug Discov.* (2012) 7:111–22. doi: 10.2174/157489112801619728

11. Thormar H, Isaacs CE, Brown HR, Barshatzky MR, Pessolano T. Inactivation of enveloped viruses and killing of cells by fatty acids and monoglycerides. *Antimicrob Agents Chemother.* (1987) 31:27–31. doi: 10.1128/AAC.31.1.27

12. Hilmarsson H, Kristmundsdottir T, Thormar H. Virucidal activities of medium- and long-chain fatty alcohols, fatty acids and monoglycerides against herpes simplex virus types 1 and 2: comparison at different pH levels. *APMI.* (2005) 113:58–65. doi: 10.1111/j.1600-0463.2005.apm1130109.x

13. Jackman JA, Hakobyan A, Zakaryan H, Elrod CC. Inhibition of African swine fever virus in liquid and feed by medium-chain fatty acids and glycerol monolaurate. *J Anim Sci Biotechnol.* (2020) 11:1–10. doi: 10.1186/s40104-020-00517-3

14. Jackman JA, Boyd RD, Elrod CC. Medium-chain fatty acids and monoglycerides as feed additives for pig production: towards gut health improvement and feed pathogen mitigation. *J Anim Sci Biotechnol.* (2020) 11:1–15. doi: 10.1186/s40104-020-00446-1

15. Rozenbaum RT, Su L, Umerska A, Eveillard M, Håkansson J, Mahlapuu M, et al. Antimicrobial synergy of monolaurin lipid nanocapsules with adsorbed antimicrobial peptides against *Staphylococcus aureus* biofilms *in vitro* is absent *in vivo*. *J Control Release.* (2019) 293:73–83. doi: 10.1016/j.jconrel.2018.11.018

16. Yoon BK, Jackman JA, Valle-González ER, Cho NJ. Antibacterial free fatty acids and monoglycerides: biological activities, experimental testing, and therapeutic applications. *Int J Mol Sci.* (2018) 19:1114. doi: 10.3390/ijms19041114

17. Preuss HG, Echard B, Dadgar A, Talpur N, Manohar V, Enig M, et al. Effects of essential oils and monolaurin on *Staphylococcus aureus*: *in vitro* and *in vivo* studies. *Toxicol Mech Methods.* (2005) 15:279–85. doi: 10.1080/15376520590968833

18. Hierholzer JC, Kabara JJ. *In vitro* effects of monolaurin compounds on enveloped RNA and DNA viruses 1. *J Food Saf.* (1982) 4:1–12. doi: 10.1111/j.1745-4565.1982.tb00429.x

19. Lieberman S, Enig MG, Preuss HG. A review of monolaurin and lauric acid: natural virucidal and bactericidal agents. *Altern Complemen Ther.* (2006) 12:310–4. doi: 10.1089/act.2006.12.310

20. Ren C, Wang Y, Lin X, Song H, Zhou Q, Xu W, et al. A combination of formic acid and monolaurin attenuates enterotoxigenic *Escherichia coli* induced intestinal inflammation in piglets by inhibiting the NF-κB/MAPK pathways with modulation of gut microbiota. *J Agric Food Chem.* (2020) 68:4155–65. doi: 10.1021/acs.jafc.0c01414

21. Thomas LL, Woodworth JC, Tokach MD, Dritz SS, DeRouchey JM, Goodband RD, et al. Evaluation of different blends of medium-chain fatty acids, lactic acid, and monolaurin on nursery pig growth performance. *Transl Anim Sci.* (2020) 4:548–57. doi: 10.1093/tas/txaa024

22. Li Y, Liu T, Zhang X, Zhao M, Zhang H, Feng F. Lactobacillus plantarum helps to suppress body weight gain, improve serum lipid profile and ameliorate low-grade inflammation in mice administered with glycerol monolaurate. *J Funct Foods.* (2019) 53:54–61. doi: 10.1016/j.jff.2018.12.015

23. Gupta KK, Khan MA, Singh SK. Constitutive inflammatory cytokine storm: a major threat to human health. *J Interferon Cytokine Res.* (2020) 40:19–23. doi: 10.1089/jir.2019.0085

24. Pan P, Shen M, Yu Z, Ge W, Chen K, Tian M, et al. SARS-CoV-2 N protein promotes NLRP3 inflammasome activation to induce hyperinflammation. *Nat Commun.* (2021) 12:4664. doi: 10.1038/s41467-021-25015-6

25. Iwasaki M, Saito J, Zhao HL, Sakamoto A, Hirota K, Ma DQ. Inflammation triggered by SARS-CoV-2 and ACE2 augment drives multiple organ failure of severe COVID-19: molecular mechanisms and implications. *Inflammation.* (2021) 44:13–34. doi: 10.1007/s10753-020-01337-3

26. Deretic V. Autophagy in inflammation, infection, and immunometabolism. *Immunity.* (2021) 54:437–53. doi: 10.1016/j.immuni.2021.01.018

27. Fosdick MG, Chheda PR, Tran PM, Wolff A, Peralta R, Zhang MY, et al. Suppression of human T cell activation by derivatives of glycerol monolaurate. *Scientific report.* (2021) 11:8943. doi: 10.1038/s41598-021-88584-y

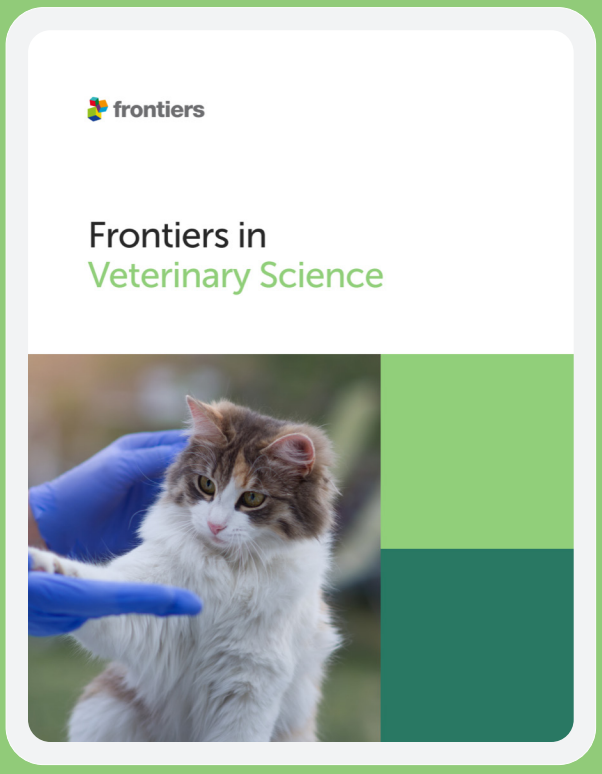
Frontiers in Veterinary Science

Transforms how we investigate and improve animal health

The third most-cited veterinary science journal, bridging animal and human health with a comparative approach to medical challenges. It explores innovative biotechnology and therapy for improved health outcomes.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact