Journal Pre-proofs

Effects of ursolic acid on growth performance, serum biochemistry, antioxidant capacity, and intestinal health of broilers

X.W. Zhang, X. Li, Y. Yin, M. Wang, Y.F. Wang, J.Y. Chen, Y.R. Zhao

PII: S1751-7311(24)00322-7
DOI: https://doi.org/10.1016/j.animal.2024.101385
Reference: ANIMAL 101385

To appear in: *Animal*

Received Date: 25 March 2024
Revised Date: 20 November 2024
Accepted Date: 21 November 2024

Please cite this article as: X.W. Zhang, X. Li, Y. Yin, M. Wang, Y.F. Wang, J.Y. Chen, Y.R. Zhao, Effects of ursolic acid on growth performance, serum biochemistry, antioxidant capacity, and intestinal health of broilers, *Animal* (2024), doi: https://doi.org/10.1016/j.animal.2024.101385

Effects of ursolic acid on growth performance, serum biochemistry, antioxidant capacity, and intestinal health of broilers

X. W. Zhang [a], X. Li [a], Y. Yin [a], M. Wang [a], Y. F. Wang [a], J. Y. Chen [b] Y. R. Zhao [a 1]

[a]College of Animal Science and Technology, Hunan Agricultural University, Changsha 410128, China

[b]Academician Workstation, Changsha Medical University, Changsha 410219, China

[1]Correspondence author: Y. R. Zhao; E-mail address: zyr@hunau.edu.cn

Highlights

1) Exploring the growth promotion reasons of ursolic acid in broilers.

2) Ursolic acid improved the morphological structure of the jejunum in broilers.

3) Ursolic acid improved physical barrier function of the jejunum and ileum.

4) Ursolic acid regulated structural composition of cecal microbiota in broilers.

5) Ursolic acid promotes broiler growth by maintaining intestinal health.

Abstract

Previous studies have shown that adding 450 mg/kg ursolic acid (**UA**) can improve the growth performance of broilers. However, the specific mechanism is still unclear.

Therefore, the purpose of this study was to further explore whether UA promotes the growth of broilers by affecting the intestinal environment of broilers. We randomly divided 120 broilers with similar body weight (46.53 ± 0.05 g) into 2 groups. Each group had six replicates, with 10 broilers per replicate. The broilers were fed either the corn-soybean meal basal diet (**CON group**) or the corn-soybean meal basal diet supplemented with 450 mg/kg UA (**UA group**). This study lasted 42 days. Adding UA increased the daily weight gain and feed conversion ratio of broilers ($P < 0.05$). The UA group exhibited reduced aspartate aminotransferase, total cholesterol, interleukin 6 and interleukin 1, and triacylglycerol levels, with increased interleukin 10 and high-density lipoprotein cholesterol in serum ($P < 0.05$). The UA supplementation improved total antioxidant capacity, total superoxide dismutase, and glutathione peroxidase activity in serum ($P < 0.05$), and increased these levels in the jejunum ($P < 0.05$). It reduced malondialdehyde concentration in the jejunum and ileum ($P < 0.05$), improved jejunal morphology by increasing villus height and villus-to-crypt ratio, and decreased crypt depth ($P < 0.05$). Gene expression of *zona occludens 1* and *Claudin-1* was higher, while *interleukin 6* was lower in the UA group ($P < 0.05$). Additionally, *interleukin 10* gene expression in jejunal mucosa was higher ($P < 0.05$). Significant differences were observed in the abundance of Bacteroides, proteobacteria, and desulfurization bacteria ($P < 0.05$), with higher *Barnesiella* and *Clostridia_UCG-014*, and *lower Romboutsia* in the UA group ($P < 0.05$). *Barnesiella* negatively correlated with interleukin 6, interleukin 1, and triacylglycerol, but positively correlated with interleukin 10 ($P < 0.05$). In conclusion, adding 450 mg/kg UA to broiler feed can improve serum and jejunal antioxidant capacity, reduce jejunal and ileal inflammation, improve jejunal morphology, and regulate cecal microbiota structure composition, promoting broiler growth.

Keywords: **ursolic acid, oxidative stress, inflammation, intestinal morphology, intestinal microorganism**

Implications

Prior research indicated that adding 450 mg/kg of ursolic acid in broiler feed boosts growth, yet the mechanism was unknown. We explore the reasons for its growth promotion. Our study found that adding 450 mg/kg ursolic acid to broiler feed improves serum and jejunal antioxidant levels, lessens inflammation in the jejunum and ileum, improves jejunal structure, and adjusts the composition of cecal microbiota, thereby promoting growth. These results clarify the primary cause of ursolic acid's growth-promoting effect on broilers. However, the key regulatory targets of ursolic acid in promoting broiler growth still need further study.

Introduction

Commercial broiler production has developed rapidly in the poultry industry due to its advantages including fast slaughter, fresh meat, and a high feed conversion ratio (Deng et al., 2023). However, adverse stimulation of microbial infection, high-density-feeding, and heat stress lead to poor growth and intestinal health of broilers (Liu et al., 2023). Guo et al. (2023) reported that substances with antioxidant, anti-inflammatory, and bacteriostatic properties can effectively improve the growth performance and intestinal health of broilers. With the prohibition of antibiotics, plant extracts with antioxidant, anti-inflammatory, and bacteriostatic effects are accepted by farmers and consumers because, in addition to promoting animal growth, they have non-toxic side effects, no residues, and do not cause pollution (Latek et al., 2022).

Ursolic acid (**UA**) a pentacyclic triterpenoid compound, is insoluble in water and soluble in ethanol and has a special smell. It naturally occurs in food products such as apples, flowering quince, as well as in more than 120 plant species such as *Rosemary herb* and *Hedyotis diffusa* (Karthik et al., 2023; Wang et al., 2018). Zhao et al. (2019) found that administration of 100 mg/Kg UA to acute kidney injury model mice by tube feeding can significantly reduce the secretion of tumor necrosis factor α, interleukin 6 and interleukin 1β, block TLR4/MyD88 signaling pathway in LPS-stimulated macrophage model, enhance macrophage autophagy, and exert anti-inflammatory properties. Fu et al. (2023) found that intragastric administration of 100 mg/kg UA in an experimental autoimmune myocarditis mouse model can reduce oxidative stress level (lower ROS level) and alleviate oxidative damage in experimental autoimmune myocarditis by up-regulating Nrf2/HO-1 signaling pathway. It can be seen that UA has significant anti-oxidant and anti-inflammatory properties. Kang et al. (2022) study has shown that adding 450 mg/kg UA can improve the production performance of broilers. However, the specific mechanism is still unclear.

Zhang et al. (2019) found that UA could reduce intestinal inflammation, relieve intestinal oxidative stress, and reduce intestinal permeability in mouse models. Tian et al. (2023) showed that UA could regulate the intestinal microbiota structure of mice induced by high-fat, mainly manifested as the increase in the genera of *Lactobacillus* and *Akkermansia* after UA intervention. In modern poultry breeding enterprises, commercial broilers can easily suffer from intestinal oxidative stress, intestinal inflammation, and intestinal microbiota imbalance under the adverse stimulation (microbial infection, high-density-feeding, heat stress) of the external environment, which has adverse effects on the growth of broilers (Ducatelle., 2023). Based on previous studies, the purpose of this experiment was to add 450 mg/kg UA to the corn-soybean meal basal diet of broilers and to further explore how UA can promote the growth of broilers by affecting the intestinal environment of broilers, to provide some reference for the application of UA in broiler production.

Material and methods

Experimental design, animals and diets

A total of 120 Arbor Acres broilers (half male and half female) were randomly divided into 2 groups: control group (fed with corn-soybean meal basal diet; **CON group**) and experimental group (fed with corn-soybean meal basal diet supplemented with 450 mg/kg UA; UA purity ≥ 99%; **UA group**) with 6 replicates in each group and 10 chickens in each replicate. The experimental broilers were raised in three layers of cages. Before the experiment, formalin and potassium permanganate were used to clean and disinfect the broiler houses and cages, and then fumigation and ventilation were carried out. The henhouse was heated to 32-35°C before brooding, and then the temperature was reduced by 2-3°C every week until it was maintained at 20-23°C. The relative humidity was adjusted by 65-70% with seasonal changes and growth characteristics of broilers (Wang et al., 2022). Feeding and drinking water were free, and routine immunization was performed. The diet formula was formulated regarding NRC 1994 (Table 1).

Preparation and sample collection

Feed intake was recorded per replicate per day. Broilers were weighed at the beginning of each stage (day 1) and at the end of each stage (days 21 and 42). The death of chickens was checked every day, and the dead broilers were recorded and weighed to adjust the feeding frequency and feed amount as appropriate. The average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion ratio (**FCR**) were calculated. After fasting for 12 hours, broilers were weighed at 42 days of age. A 42-day-old cock broiler with an average body weight close to the average weight of the group was selected from each replicate. Blood was collected 5 mL from the wing vein, centrifuged at 3000 × g for 10 min to prepare serum, and stored in a refrigerator at -20°C for testing (Zhang et al., 2021). After slaughtering, approximately 1 cm of the mid-jejunum and mid-ileum were taken and fixed with 4% paraformaldehyde (0.44 mol/L) to make tissue sections (De et al., 2020). The jejunal and ileal mucosa and cecal contents were collected for post-test analysis (De et al., 2020).

Serum biochemistry analysis

Using a spectrophotometer (UH5700, Shimadzu Instrument Co., Ltd, Suzhou, China), we measured triacylglycerol, high-density lipoprotein cholesterol, total

cholesterol, low-density lipoprotein cholesterol, as well as the activities of alanine aminotransferase and aspartate aminotransferase in serum, the kits were provided by Nanjing Jiancheng Bioengineering Institute, China. Enzyme-linked immunosorbent assay was used to measure the concentrations of inflammatory markers interleukin 1, interleukin 6, interleukin 10, and tumor necrosis factor α in the serum. The kits for this assay were provided by the Jiangsu Meimian Industrial Co., Ltd. The measurements were performed using a microplate reader (Infinite M PLEX, Swiss Kentucky Company, Austria).

Antioxidant index analysis

Malondialdehyde, total antioxidant capacity, total superoxide dismutase, and glutathione peroxidase activities were assessed in both the serum and intestinal mucosa using the kit from Nanjing Jiancheng Bioengineering Institute, China. The measurements were performed using a microplate reader (Infinite M PLEX, Swiss Kentucky Company, Austria). All operations are strictly in accordance with the kit instructions.

Intestinal tissue slice

The collected jejunal and ileal samples were fixed with xylene for 48 hours, embedded in paraffin, sliced using a 4 μm blade of a microtome (Biocut, Leica, Germany, Weztlar, Germany), and mounted on a slide in a 37°C thermostatic water bath. The hematoxylin-eosin staining kit of China Nanjing Jiancheng Institute of Bioengineering was used for staining. The steps are briefly described as follows. The slides were transparent with xylene twice, soaked in different concentrations of alcohol three times, washed with tap water, then stained with hematoxylin and eosin, washed with water, dehydrated with alcohol five times, fixed with xylene once, dried in a fume hood, sealed with neutral resin, and stored in a cool and dry place for subsequent observation. Finally, an optical microscope and Image J (https://imagej.net/Fiji) were used to analyze the intestinal villus height, crypt depth, and villus-to-crypt ratio.

Real-time fluorescence quantitative expression

The relative expression of jejunum and ileum mucosal genes was detected by Real-time fluorescence quantitative expression. Total RNA was extracted from the

tissues using the RNA extraction kit (SteadyPure, Ecoray Biology, Changsha, Hunan, China). The reverse transcription kit (Evo M-MLVA, Ecoray Biology, Changsha, Hunan, China) was utilized to obtain cDNA, and the quantitative kit (AG11701, Ecoray Biology, Changsha, Hunan, China) was used to detect the relative expression of genes. Primers were synthesized in Beijing Qingke Biotechnology Co., Ltd., and their sequences can be found in Table 2. The gene expression level was analyzed using the $2^{-\Delta\Delta Ct}$ method after normalizing the internal reference gene *β-actin*.

Gut microbiome

Refer to the collection method of Zou et al., (2019). There were 6 samples in each group, totaling 12 samples for microbial sequencing analysis. The sequencing analysis steps are summarized as follows. The PCR products were obtained by amplifying the V3-V4 region of the bacterial 16s rDNA gene after extracting bacterial DNA from the samples for testing. These were analyzed using the NEB Next Ultra II DNA Library Prep Kit (Biolabs, New England, USA). The PCR products were sequenced using the Illumina Miseq/Novaseq 6000 platform from Illumina (United States). Afterward, the data were split, spliced, and filtered in a short sequence before the filter was removed. Uparse algorithm of Vsearch (v2.7.1) software was utilized to cluster qualified sequences into operational taxonomic units with a similarity threshold of 97%. The indices of abundance and variety using operational taxonomic units information were computed. R (v3.6.0) was used for plotting. Spearman analysis was used to analyze the correlation between different bacteria and inflammation and lipid metabolism indicators.

Statistical analysis

SPSS 26 F-test was employed to analyze whether the data of each group (6 samples in each group) conformed to the normal distribution. The differences in serum biochemical, oxidative stress, and inflammation data between the two groups were analyzed by SPSS 26 independent sample T-test. The initial body weight block was included as a random effect and the UA treatment as a fixed effect. The cage was the experimental unit for growth performance, while the selected broiler from each pen was used as an experimental unit for analysis of serum index, antioxidant index, intestinal morphology, and cecal microbiota. The results are expressed as mean and pooled standard error of the means ($\bar{X}$ + SEM). Microsoft Office Excel 2023 was used for data tables. Origin 2022 and SigmaPlot 12.5 were used for drawing gene expression. A significant difference was defined as $P < 0.05$.

Results

Growth performance and serum biochemistry

As shown in Table 3, the UA group had higher ADG in broilers aged 1-21 days ($P < 0.05$), and the UA improved FCR ($P < 0.05$), but had no impact on ADFI ($P > 0.05$). At the age of 1-42 days, the UA improved FCR ($P < 0.05$), but the ADG and ADFI had no effect between the two groups ($P > 0.05$). In the serum, adding UA decreased the activity of aspartate aminotransferase and the concentrations of triacylglycerol and total cholesterol, as well as increased high-density lipoprotein cholesterol ($P < 0.05$; Table 4). However, there was no significant import on alanine aminotransferase activity and the concentration of low-density lipoprotein cholesterol between the two groups ($P > 0.05$; Table 4). As shown in Table 4, the UA group had lower interleukin 1 and interleukin 6 concentrations, but the interleukin 10 concentration was higher ($P < 0.05$).

Antioxidant capacity and intestinal morphology

As shown in Table 5, the UA group had higher activity of total superoxide dismutase and the level of total antioxidant capacity in the serum ($P < 0.05$). In the jejunum, the UA group of total antioxidant capacity level and glutathione peroxidase activity was higher and had lower malondialdehyde content ($P < 0.05$). In the ileum, adding UA increased glutathione peroxidase activity but the content of malondialdehyde was lower ($P < 0.05$). As shown in Table 6, in the jejunum, adding UA could increase villus height and villus-to-crypt ratio, as well as decrease crypt depth ($P < 0.05$). But the UA did not affect villus height, crypt depth, and villus-to-crypt ratio of the ileum ($P > 0.05$).

Intestinal barrier function gene expression

The UA group had higher gene expression of *ZO-1* and *Claudin-1* in the jejunal mucosa ($P < 0.05$; Fig. 1A). Adding UA up-regulated *interleukin 10* gene expression and down-regulated *interleukin 6* gene expression in the jejunal mucosa ($P < 0.05$; Fig. 1B). Adding UA up-regulated gene expression of *ZO-1* and *Claudin-1* in the ileal mucosa ($P < 0.05$; Fig. 1C). Otherwise, The UA group had lower *interleukin 6* gene expression in the ileal mucosa ($P < 0.05$; Fig. 1D).

Intestinal microbiota structure composition and correlation analysis

As shown in Fig. 2. The UA group had higher values of Chao1 index, Shannon and Simpson indices ($P < 0.05$). However, there was no significant effect on the PD index ($P > 0.05$). Both the UA and CON groups exhibited Firmicutes and Bacteroidetes as the predominant phyla at the phylum level (Fig. 3A). The UA group had higher Bacteroidetes and had lower Proteobacteria ($P < 0.05$; Table 7). As shown in Fig. 3B, the prevailing bacteria in the UA group were *Barnesiella*, *Clostridia_UCG-014*, and *Romboutsia*. Adding UA increased relative abundance of *Barnesiella* and *Clostridia_UCG-014,* as well as decreased *Romboutsia* ($P < 0.05$; Table 8). As shown in Fig. 3C, spearman correlation analysis found that *Barnesiella* exhibited a negative

correlation with serum levels of interleukin 1, interleukin 6, and triacylglycerol, whereas showing a positive correlation with interleukin 10 ($P < 0.05$). *Romboutsia* exhibited a positive correlation with serum levels of interleukin 6 ($P < 0.05$). However, *Clostridia_UCG-014* does not correlate with serum indicators of inflammation and lipid metabolism ($P > 0.05$).

Discussion

The health of the liver and intestinal environment is a prerequisite for the healthy growth of broilers. This study focuses on the effect of UA on the intestinal environment of broilers and also pays attention to the effect of UA on the liver function of broilers. This study found that UA had the effect of reducing aspartate aminotransferase. In medicine, the reduction of aspartate aminotransferase activity usually reflects the enhancement of liver function (Wan et al., 2020). Therefore, the UA enhances the liver function of broilers, which will be beneficial to the healthy growth of broilers. In addition, quantitative analysis of intestinal histological sections and villus structure (villus height, crypt depth, villus-to-crypt ratio) can be used to evaluate the integrity of intestinal morphology and reflect the absorption capacity of intestinal nutrients (Rysman et al., 2023). This experiment shows that the UA group had a higher villus height and villus-to-crypt ratio, and a lower crypt depth. Prakatur et al. (2019) reported that as villus height increases, so does the intestinal tract's capacity to absorb nutrients. Deeper crypts indicate a faster tissue metabolism. A higher villus-to-crypt ratio signifies improved intestinal development, which facilitates nutrient absorption. Therefore, this may be an important reason for UA to increase the ADG and reduce the FCR of broilers. However, the changes in the intestinal morphology of broilers may be related to the antioxidant function of UA (Bao et al., 2022).

Intestinal oxidative stress produces a large amount of ROS, which damages the integrity of intestinal morphology. The enhancement of intestinal antioxidant capacity is conducive to maintaining the integrity of intestinal morphology and improving the intestinal morphological structure (Gevawy et al., 2004). The UA has been proved to be a plant extract with high antioxidant properties, which could increase the activities of superoxide dismutase and glutathione peroxidase and reduced the concentration of malondialdehyde (Habtemariam et al., 2019; Kamzi et al., 2022). In this experiment, the UA increased the glutathione peroxidase activity in the jejunum and ileum, reduced the malondialdehyde content in the jejunum and ileum, and increased the activities of serum total superoxide dismutase and glutathione peroxidase. The changes in these results suggest that UA may maintain the integrity of intestinal morphology by increasing the activity of antioxidant enzymes and reducing the production of ROS. In addition, excessive ROS can act as an upstream stimulus to cause abnormal activation of the intestinal immune system, causing damage to the intestinal mucosal barrier, destroying the tight junctions between intestinal epithelial cells, and leading to intestinal inflammation (Xie et al., 2022). Importantly, free radical immune cells activate multiple inflammatory signaling pathways, promote the release of pro-inflammatory cytokines (tumor necrosis factor α, interleukin 6, and interleukin 1β), and further increase the

oxidative stress level of the intestinal tract (Li et al., 2023). Therefore, the UA may reduce the stimulation of ROS on intestinal microbacteria and immune cells by increasing the activities of antioxidant enzymes (total superoxide dismutase, glutathione peroxidase), thereby reducing the damage to the intestinal mucosal barrier and avoiding the occurrence of intestinal inflammation.

Therefore, this experiment further evaluated the status of intestinal barrier function. The connection between intestinal epithelial cells plays an important role in the function of the whole intestinal barrier (Citi., 2018; Wang et al., 2024). Zhang et al. (2022) reported that tight junction proteins mainly include occludin, claudin, and ZO-1, which are important in maintaining intestinal epithelial barrier function. In this study, the UA significantly increased the relative gene expression of *ZO-1* and *Claudin-1*, suggesting decreased intestinal permeability. Ibrahim et al. (2021) reported that blocking the expression of PIK3R3 protein could inhibit the transcription of NF-κB upregulate the expression of TJ (ZO-1, Claudin-1, occludin, etc.) proteins, and reduce the expression of inflammatory factors interleukin 1β, interleukin 6, and tumor necrosis factor α, thereby reducing the damage caused by inflammation to the body. Sheng et al. (2021) found that UA decreased serum and colonic interleukin 6 levels, and downregulated three classical inflammatory pathways (interleukin 6/STAT3, MAPK, and PI3K). In this study, the UA could reduce the gene expression of *interleukin 6* in the Jejunal and ileal mucosa and also reduce the concentrations of serum interleukin 6 and interleukin 1. The changes in these indicators further confirmed that UA can reduce intestinal permeability, reduce intestinal inflammation, and improve intestinal barrier function. The mechanism may be achieved by inhibiting the expression of PIK3R3 protein in the PI3K pathway.

The host's gastrointestinal function, immune function, and healthy intestinal homeostasis benefit from the complex ecosystem of microbiota (Sanders et al., 2021; Abd El-Hack et al., 2020). In this experiment, the sequencing results of cecal contents of broilers showed that UA could increase α diversity, which was manifested by the increase of Chao1 and Shannon index. Ciocan et al. (2018) reported that the increase of α diversity was considered to be beneficial to improve intestinal microbial diversity and maintain intestinal immune homeostasis. Therefore, we further analyzed the differences in microbial diversity at the phylum level and found that UA could reduce the proportion of Bacteroidetes, which may be an important reason for its maintenance of intestinal immune homeostasis because the reduction of Bacteroidetes is conducive to alleviating the process of intestinal inflammation. It is worth noting that this study found that the serum triglycerol and total cholesterol concentrations in the UA group were significantly reduced. Importantly, lipid metabolism in the body has a significant correlation with the changes in Firmicutes. Stojanov et al. (2020) reported that Firmicutes played a key role in host nutrition and metabolism through short-chain fatty acid synthesis. However, our results showed that UA had no significant effect on the relative abundance of Firmicutes. Therefore, we further analyzed the differences of microorganisms at the genus level and found an interesting phenomenon. The relative abundance of *Barnesiella* in the Bacteroidetes increased significantly in the UA group.

Karlsson et al. (2013) and Song et al. (2023) reported that *Barnesiella* was related to inflammation and lipid metabolism, and the relative abundance of *Barnesiella* was significantly reduced in people with obesity and inflammation. Therefore, through correlation analysis, it was found that *Barnesiella* was negatively correlated with triglycerol, interleukin 1, and interleukin 6, and positively correlated with interleukin 10. In addition, the UA also significantly increased the relative abundance of *Romboutsia* and significantly reduced the relative abundance of *Clostridia_UCG-014*. Similarly, correlation analysis showed that the effects of these two genera on inflammation and lipid metabolism under the conditions of this experiment were not as significant as those of *Barnesiella*, although studies have reported the effects of these two genera on inflammation and lipid metabolism. Therefore, we speculate that the significant increase in the abundance of *Barnesiella* may be an important reason for the significant decrease in the concentration of total cholesterol and triacylglycerol in serum, but its specific action bacteria and related mechanisms still need further study. In addition, the abundance of the microbiota depends on sex, which means that if more male or female broilers were slaughtered in one of the treatments, there is a bias (Feye et al., 2020). In this study, the slaughter of male broilers found that UA had a significant effect on cecal microorganisms, but whether it could have the same effect on the cecal microorganisms of female broilers still needed further study.

Conclusion

In summary, the inclusion of UA not only improved the early growth performance of broilers but also improved the FCR throughout the entire growth period. Furthermore, the UA contributed to a reduction in serum levels of aspartate aminotransferase, total cholesterol, and triacylglycerol, while simultaneously increasing levels of high-density lipoprotein cholesterol. Concurrently, the additive bolstered antioxidant and antiinflammatory capabilities, and improved intestinal barrier function, microbial diversity, and structural composition of broilers. Consequently, incorporating 450 mg/kg of UA into a broiler diet is anticipated to serve as a high-quality, plant-derived feed supplement.

Ethics approval

The Institutional Animal Care and Use Committee of Hunan Agricultural University approved all the procedures (approval number HUNAU2022003).

Data and model availability statement

None of the data were deposited in an official repository. Information can be made available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

Author ORCIDs

X. W. Zhang: https://orcid.org/0009-0004-7915-085X.

Declaration of interest

The writers participating in this project affirm that they do not have any competing interests to disclose.

Acknowledgements

None.

Financial support statement

The National Key R&D Program of China (2023YFD1301200) supports this study.

References

Abd El-Hack, M. E., El-Saadony, M. T., Shafi, M. E., Qattan, S. Y. A., Batiha, G. E., Khafaga, A. F., Abdel-Moneim, A. E., Alagawany, M., 2020. Probiotics in poultry feed: A comprehensive review. Journal of animal physiology and animal nutrition 104, 1835–1850.

Bao, C. L., Zhang, W. X., Wang, J., Liu, Y. J., Cao, H., Li, F. Y. Liu, S. Z., Shang, Z. D., Cao. Y. H., Dong, B., 2022. The effects of dietary bacillus amyloliquefaciens TL106 supplementation, as an alternative to antibiotics, on growth performance, intestinal immunity, epithelial Barrier Integrity, and intestinal microbiota in broilers. Animals 12, 3085.

Citi, S., 2018. Intestinal barriers protect against disease. Science 359, 1097-1098.

Ciocan, D. V., Rebours, C. S., Voican, L., Wrzosek, V., Puchois, A., Cassard, M., Perlemuter G., 2018. Characterization of intestinal microbiota in alcoholic patients with and without alcoholic hepatitis or chronic alcoholic pancreatitis. Scientific reports 8, 4822.

De, G. A., Leleu, S., Delezie, E., Rapp, C., De Smet, S., Goossens, E., Haesebrouck, F., Van F., Immerseel, R., Ducatelle, R., 2020. Dietary zinc source impacts intestinal morphology and oxidative stress in young broilers. Poultry science 99, 441-453.

Deng, F., Tang, S., Zhao, H., Zhong, R., Liu, L., Meng, Q., Zhang, H., Chen, L., 2023. Combined effects of sodium butyrate and xylo-oligosaccharide on growth performance, anti-

inflammatory and antioxidant capacity, intestinal morphology and microbiota of broilers at early stage. Poultry science 102, 102585.

Ducatelle, R., Goossens, E., Eeckhaut, V., Van-I, F., 2023. Poultry gut health and beyond. Animal nutrition 13, 240-248.

Feye, K. M., Baxter, M. F. A., Tellez-Isaias, G., Kogut, M. H., Ricke, S. C., 2020. Influential factors on the composition of the conventionally raised broiler gastrointestinal microbiomes. Poultry science 99, 653-659.

Fu, Y. N., Liu, T. L., He, S. K., Zhang, Y. C., Tan, Y. T., Bai, Y., Shi, J. W., Deng, W. H., Qiu, J. N., Wang, Z., Chen, Y. H., Jin, Q. F., Xie, M. X., Wang, J., 2023. Ursolic acid reduces oxidative stress injury to ameliorate experimental autoimmune myocarditis by activating Nrf2/HO-1 signaling pathway. Frontiers in pharmacology 14, 1189372.

Gaweł, S., Wardas, M., Niedworok, E., Wardas. P., 2004. Dialdehyd malonowy (MDA) jako wskaźnik procesów peroksydacji lipidów w organizmie [Malondialdehyde (MDA) as a lipid peroxidation marker]. Wiadomosci lekarskie 57, 453-455.

Guo, S. W., Ma, X. J., Xing, Y. Y., Xu, Y. Q., Jin, X., Yan, S. M., Shi, L. L., Zhang, L. H., Shi. B. L., 2023. Effects of Artemisia Annua L. Water extract on growth performance and intestinal related indicators in broilers. Poultry Science 60, 2023024.

Habtemariam, S., 2019. Antioxidant and anti-inflammatory mechanisms of neuroprotection by ursolic acid: addressing brain injury, cerebral ischemia, cognition deficit, anxiety, and depression. Oxidative medicine and cellular longevity 2019, 8512048.

Ibrahim, S., Zhu, X., Luo, X., Feng, Y., Wang. J., 2020. PIK3R3 regulates ZO-1 expression through the NF-kB pathway in inflammatory bowel disease. International immunopharmacology 85, 106610.

Karlsson, F. H., Tremaroli, V., Nookaew, I., Bergström, G., Behre, C. J., Fagerberg, B., Nielsen, J., Bäckhed, F., 2013. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature 498, 99-103.

Kazemi Pordanjani, M., Banitalebi, E., Roghani, M., Hemmati, R., 2022. Ursolic acid enhances the effect of exercise training on vascular aging by reducing oxidative stress in aged type 2 diabetic rats. Food science & nutrition 11, 696-708.

Kang, K. L., 2022. Single-cell sequencing and multi-omic analysis reveals pathogenesis in wooden breast in broilers and nutritional regulation. PH.D. thesis, Hunan Agricultural University, Hunan, China.

Karthik, M., Manoharan, S., Muralinaidu, R., 2023. Ursolic acid-loaded chitosan nanoparticles suppress 7,12-dimethylbenz (a) anthracene-induced oral tumor formation through their antilipid peroxidative potential in golden syrian hamsters. Naunyn-Schmiedeberg's archives of pharmacology 396, 3061-3074.

Latek, U., Chłopecka, M., Karlik, W., Mendel, M., 2022. Phytogenic compounds for enhancing intestinal barrier function in poultry-a review. Planta medica 88, 218-236.

Liu, L., Li, C. L., T., Li, H., Wang, H. Y., Zhang, X. F., Ren, Q. D., Zhang, H. P., Jin, N. Y., Li, C., Zhao, C. Q., 2023. Effects of lactiplantibacillus plantarum LPJZ-658 supplementation on the production, meat quality, intestinal morphology, and cecal microbiota of broilers chickens. Microorganisms 11, 1549.

Li, L., Peng, P. L., Ding, N., Jia, W. H., Huang, C. H., Tang, Y., 2023. Oxidative stress, inflammation, gut dysbiosis: What can polyphenols do in inflammatory bowel disease? Antioxidants (Basel) 12, 967.

Prakatur, I., Miskulin, M., Pavic, M., Marjanovic, K., Blazicevic, V., Miskulin, I., Domacinovic. M., 2019. Intestinal morphology in broiler chickens supplemented with propolis and bee pollen. Animals 9, 301.

Rysman, K., Eeckhaut, V., Ducatelle, R., Goossens, E., Van-I, F., 2023. Broiler performance correlates with gut morphology and intestinal inflammation under field conditions. Avian pathology 52, 232-241.

Stojanov, S., Berlec, A., Š trukelj, B., 2020. The influence of probiotics on the firmicutes/bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease Microorganisms 8, 1715.

Sheng, Q. S., Li, F., Chen, G. Q., Li, J. C., Li, J., Wang, Y. F., Lu, Y. Y., Li, Q., Li, M. Q., Chai, K. Q., 2021. Ursolic acid regulates intestinal microbiota and inflammatory cell infiltration to prevent ulcerative colitis. Journal of immunology research 2021, 6679316.

Sanders, D. J., Inniss, S., Sebepos-R, G., Rahman, F. Z., Smith, A. M., 2021. The role of the microbiome in gastrointestinal inflammation. Bioscience reports 41, BSR20203850.

Song, B. C., He, J., Pan, X., Kong, L. L., Xiao, C. P., Keerqin, C., Song, Z. G., 2023. Dietary macleaya cordata extract supplementation improves the growth performance and gut health of broiler chickens with necrotic enteritis. Journal of animal science and biotechnology 14, 113.

Tian, C. F., J. Li, Y. Bao, L. Gao, L. X. Song, K. Li, and M. Sun. 2023. Ursolic acid ameliorates obesity of mice fed with high-fat diet via alteration of gut microbiota and amino acid metabolism. Frontiers in microbiology 14, 1183598.

Wan, S. Z., Luo, F. Y., Huang, C. K., Liu, C., Luo, Q. T., Zhu, X., 2020. Ursolic acid reverses liver fibrosis by inhibiting interactive NOX4/ROS and RhoA/ROCK1 signalling pathways. Aging 12, 10614-10632.

Wang, S. B., Wu, H. Z., Zhu, Y. H., Cui, H. X., Yang, J., Lu, M. Y., Cheng, H. Z., Gu, L. H., Xu, T. S., Xu, L., 2022. Effect of lycopene on the growth performance, antioxidant enzyme

activity, and expression of gene in the Keap1-Nrf2 signaling pathway of arbor acres broilers. Frontiers in veterinary science 9, 833346.

Wang, M., Fu, R. J., Xu, D. Q., Chen, Y. Y., Yue, S. J., Zhang, S., Tang, Y. Q., 2024. Traditional chinese medicine: a promising strategy to regulate the imbalance of bacterial microbiota, impaired intestinal barrier and immune function attributed to ulcerative colitis through intestinal microecology. Journal of ethnopharmacology 318, 116879.

Xie, S., Zhang, R., Li, Z. Y., Liu, C. R., Xiang, W. W., Lu, Q. Q., Chen, Y. Y., Yu, Q. H., 2022. Indispensable role of melatonin, a scavenger of reactive oxygen species (ROS), in the protective effect of Akkermansia muciniphila in cadmium-induced intestinal mucosal damage. Free radical biology & medicine 193, 447-458.

Zhao, J., Zheng, H., Sui, Z., Jing, F., Quan, X., Zhao, W., Liu, G., 2019. Ursolic acid exhibits anti-inflammatory effects through blocking TLR4-MyD88 pathway mediated by autophagy. Cytokine 123, 154726.

Zhang, W., D., Gan, Jian, J., Huang, C., Luo, F., Wan, S., Jiang, M., Wan, Y., Wang, A., Li, B., Zhu, X., 2019. Protective effect of ursolic acid on the intestinal mucosal barrier in a rat model of liver fibrosis. Frontiers in physiology 10, 956.

Zhang, C., Li, C. X., Shao, Q., Chen, W. B., Ma, L., Xu, W. H., Y. Li, X., Huang S. C., Ma, Y. B., 2021. Effects of glycyrrhiza polysaccharide in diet on growth performance, serum antioxidant capacity, and biochemistry of broilers. Poultry science 100, 100927.

Zou, X., Ji, J., Qu, H., Wang, J., Shu, D. M., Wang, Y., Liu, T. F., Li, Y., Luo, C. L., 2019. Effects of sodium butyrate on intestinal health and gut microbiota composition during intestinal inflammation progression in broilers. Poultry science 98, 4449-4456.

Zhang, B. L., Liu, N., Hao, M. L., Xie, Y. X., Song, P. Y., 2022. Effects of substitution of soybean meal with rapeseed meal and glutamine supplementation on growth performance, intestinal morphology, and intestinal mucosa barrier of Qian dong nan xiao xiang Chicken. Animal bioscience 35, 1711-1724.

Table 1

Basal diet composition and nutrient levels in broilers (as-fed basis, %)

	Phase 1	Phase 2		Phase 1	Phase 2

Ingredients	(d 1 to 21)	(d 22 to 42)	Chemical composition [2]	(d 1 to 21)	(d 22 to 42)
Corn	**54.32**	**57.28**	Metabolizable energy, **MJ/kg**	**12.54**	**12.96**
Soybean meal	**37.35**	**33.68**	**CP**	**21.50**	**20.00**
Soybean oil	**3.99**	**5.15**	**Lys**	**1.15**	**1.00**
L-Lys·Hcl (79%)	**0.17**	**0.08**	**Met**	**0.50**	**0.40**
DL-Met (98%)	**0.18**	**0.10**	**Met+Cys**	**0.91**	**0.76**
CaHPO4	**1.59**	**1.39**	**Val**	**0.85**	**0.74**
Limestone	**1.10**	**1.02**	**Trp**	**0.21**	**0.18**
NaCl	**0.30**	**0.30**	**Thr**	**0.81**	**0.72**
Premix [1]	**1.00**	**1.00**	**Ca**	**1.00**	**0.95**
			DM	**90.00**	**89.00**
			Available phosphorus	**0.45**	**0.40**

[1]Nutrient levels of premix (per kg diet): vitamin A, 8 000 IU; vitamin D3, 2000 IU; vitamin E, 20 IU; vitamin K3, 15 mg; vitamin B1, 2.25 mg; vitamin B2, 8.20 mg; vitamin B6, 2.80 mg; vitamin B12, 0.015 mg; folic acid, 0.95 mg; nicotinic acid, 35 mg; pantothenic acid, 10 mg; biotin, 0.18 mg; choline chloride, 700 mg; manganese (manganese sulfate), 80 mg; iron (ferrous sulfate), 80 mg; zinc (zinc sulfate), 100 mg; copper (copper sulfate), 9 mg; selenium (sodium selenite), 0.30 mg; iodine (potassium iodide), 0.40 mg.

[2]Chemical composition: metabolizable energy, amino acids, and available phosphorus were calculated values, while the others were measured values.

Table 2

Oligo nucleotide primers in broilers

Genes	Sequences (5´→3´)	Product size (bp)	Accession No.
Interleukin 6	F:GAGGCGAATGTTGGTGGAA R:CAGCAGGTGTTGATGAATGTC	205	NM_204628.2
Interleukin 1β	F:CAGAGATGGCGTTCGTTCC R:CTGTGGTGTGCTCAGAATCC	207	XM_015297469.3
Interleukin 10	F:CGCTGTCACCGCTTCTTCA R:GGCTCACTTCCTCCTCCTCAT	168	NM_001004414.4
Tumor necrosis factor α	F:GGACAGCCTATGCCAACAAGT R:CACCACACGACAGCCAAGT	172	XM_046927262.1
ZO-1	F:GCCTACTGCTGCTCCTTACAACTC R:GCTGGATCTATATGCGGCGGTAAG	129	XM_040680630.1
Claudin-1	F:ACACCCGTTAACACCAGATTT R:GCATTTTTGGGGTAGCCTCG	152	NM_001013611.2

Occludin	F:GATGGACAGCATCAACGACC R:CTCTTCTGCACGGCCATCTT	142	NM_205128.1
β-actin	F:TGCGTGACATCAAGGAGAAG R:TGCCAGGGTACATTGTGGTA	199	L08165

Abbreviation: ZO-1 = zona occludens-1.

Table 3

Effect of ursolic acid on growth performance in broilers

Items	CON group	UA group	SEM	*P*-value
BW, g				
on 1 d	46.58	46.53	0.121	0.564
on 21 d	788.66	842.25	2.312	0.001
on 42 d	2671.28	2703.70	1.553	0.691
1 to 21 d of age				

ADG, g/d	**35.34**	**37.88**	**1.241**	**0.001**
ADFI, g/d	**49.25**	**49.95**	**0.922**	**0.403**
FCR	**1.40**	**1.34**	**0.031**	**0.041**
1 to 42 d of age				
ADG, g/d	**63.28**	**65.19**	**2.751**	**0.148**
ADFI, g/d	**104.61**	**104.18**	**1.884**	**0.582**
FCR	**1.65**	**1.60**	**0.021**	**0.042**

Abbreviations: CON = control; UA = ursolic acid; ADFI = average daily feed intake; ADG = average daily gain; FCR = feed conversion ratio; SEM = pooled SEM; n = 6 / group.

Table 4

Effect of ursolic acid on serum biochemical in broilers

Items	CON group	UA group	SEM	*P*-value
Alanine aminotransferase, U/L	**4.41**	**4.10**	**0.458**	**0.555**
Aspartate aminotransferase, U/L	**43.44**	**34.81**	**2.964**	**0.016**
Total cholesterol, mmol/L	**3.37**	**3.10**	**0.357**	**0.027**

Triacylglycerol, mmol/L	0.63	0.52	0.110	0.017
High-density lipoprotein cholesterol, mmol/L	3.01	3.46	0.182	0.033
Low-density lipoprotein cholesterol, mmol/L	0.75	0.78	0.041	0.509
Interleukin 1, ng/L	249.18	232.22	6.038	0.019
Interleukin 6, ng/L	46.08	42.96	0.160	0.011
Tumor necrosis factor α, ng/L	87.28	87.91	1.848	0.522
Interleukin 10, ng/L	56.29	59.00	0.824	0.032

Abbreviations: CON = control; UA = ursolic acid; SEM = pooled SEM; n = 6 / group.

Table 5

Effects of ursolic acid on serum and intestinal antioxidation in broilers

Items	CON group	UA group	SEM	*P*-value
Serum				
Total antioxidant capacity, mmol/L	0.51	0.63	0.050	0.042
Total superoxide dismutase, U/ml	140.73	180.95	14.988	0.022
Glutathione peroxidase, U/ml	345.33	417.19	15.451	0.001
Malondialdehyde, nmol/mg prot	3.66	3.78	0.781	0.876

Jejunum				
Total antioxidant capacity, mmol/g prot	**0.27**	**0.30**	**0.009**	**0.012**
Total superoxide dismutase, U/mg	**68.29**	**69.28**	**1.180**	**0.441**
Glutathione peroxidase, U/mg	**38.32**	**46.09**	**0.562**	**0.001**
Malondialdehyde, nmol/mg prot	**1.09**	**0.58**	**0.194**	**0.025**
Ileum				
Total antioxidant capacity, mmol/g prot	**0.31**	**0.32**	**0.009**	**0.917**
Total superoxide dismutase, U/mg	**68.65**	**65.61**	**3.780**	**0.425**
Glutathione peroxidase, U/mg	**28.06**	**34.82**	**2.327**	**0.016**
Malondialdehyde, nmol/mg prot	**0.96**	**0.73**	**0.071**	**0.012**

Abbreviations: CON = control; UA = ursolic acid; SEM = pooled SEM; prot = total protein; n = 6 / group.

Table 6

Effect of ursolic acid on intestinal morphology in broilers

Items	CON group	UA group	SEM	*P*-value

Jejunum				
Villus height, μm	**354.27**	**471.31**	**28.292**	**0.012**
Crypt depth, μm	**193.15**	**165.28**	**11.502**	**0.039**
Villus-to-crypt ratio	**2.15**	**2.84**	**0.122**	**0.001**
Ileum				
Villus height, μm	**321.19**	**320.09**	**15.074**	**0.944**
Crypt depth, μm	**179.83**	**182.92**	**6.831**	**0.661**
Villus-to-crypt ratio	**1.79**	**1.75**	**0.086**	**0.569**

Abbreviations: CON = control; UA = ursolic acid; SEM = pooled SEM; n = 6 / group.

Table 7

Effect of ursolic acid on the abundance of cecal microbial phylum level in broilers (%)

Items	CON group	UA group	SEM	*P*-value
Firmicutes	**50.53**	**50.68**	**1.655**	**0.951**
Bacteroidetes	**12.09**	**9.77**	**0.175**	**0.001**
Proteobacteria	**2.44**	**0.82**	**0.248**	**0.033**
Desulfobacterota	**1.22**	**0.77**	**0.654**	**0.582**
Actinobacteriota	**0.28**	**0.12**	**0.079**	**0.072**

Abbreviations: CON = control; UA = ursolic acid; SEM = pooled SEM; n = 6 / group.

Table 8

Effect of ursolic acid on the abundance of cecal microbial genus level in broilers (%)

Items	CON group	UA group	SEM	*P*-value
Barnesiella	1.30	31.67	3.821	0.001
Alistipes	7.38	7.60	1.388	0.878
Lactobacillus	3.91	3.41	1.254	0.699
Faecalibacterium	6.52	8.17	1.336	0.254
Bacteroides	5.71	7.57	2.533	0.481
Ruminococcus	5.71	4.99	0.370	0.078
Clostridia_UCG-014	2.81	3.97	0.464	0.032
Romboutsia	4.08	1.82	0.426	0.001
Christensenellaceae_R-7	3.06	2.34	0.413	0.113
Negativibacillus	2.66	2.63	0.717	0.971
Blautia	1.87	1.65	0.307	0.495

Abbreviations: CON = control; UA = ursolic acid; SEM = pooled SEM; n = 6 / group.

Fig.1. Effects of ursolic acid on gene expression of Jejunal and ileal barrier function in broilers. (A) Jejunal tight junction protein genes expression level; (B) Jejunal inflammatory factor genes expression level; (C) Ileal tight junction protein genes expression level; (D) Ileal inflammatory factor genes expression level. Asterisks indicate statistical significance: * *P* < 0.05; ** *P* < 0.01. Abbreviations: CON = control; UA = ursolic acid; ZO-1 = zona occludens-1. n = 6 / group.

Fig.2. Difference in intestinal microbiota diversity of broilers by adding ursolic acid to the diet. Asterisks indicate statistical significance: * *P* < 0.05; ** *P* < 0.01; PD: pedigree diversity is a diversity index that takes into account species abundance and evolutionary distance. The larger the value, the higher the community diversity. Abbreviations: CON = control; UA = ursolic acid. n = 6 / group.

Fig.3. Structural composition diagram of the effects of ursolic acid on cecal microbiota of broilers, and correlation analysis. (A) Phylum level; (B) Genus level; (C) Correlation analysis between microbial differential bacteria and lipid metabolism and inflammatory factors. Red and blue represent positive and negative correlations respectively. Asterisks indicate statistical significance: * *P* < 0.05; ** *P* < 0.01. Abbreviations: CON = control; UA = ursolic acid. n = 6 / group.