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Molecular Characteristics and Effects of *Ascaridia galli* Infection on Intestinal Microbiota Isolates in Wenzhou, China

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ABSTRACT

Background: *Ascaridia galli* (*A. galli*) is a parasitic nematode that infects the small intestine of chickens, causing widespread parasitic infections in poultry. Infected chicks exhibit growth retardation, digestive disorders, alternating diarrhea and constipation and ultimately succumb to emaciation.

Methods: In this study, *A. galli* samples were isolated from chickens in Wenzhou City, Zhejiang Province, China and analyzed using molecular biological techniques for identification. Phylogenetic analysis of the COX1 genetic sequence revealed slight variations compared to *A. galli* isolates from other regions. The impact of *A. galli* infection on chicken intestinal microbiota was evaluated by 16S rRNA sequencing.

Result: The results indicated that the abundance of *Desulfovibrio*, a pathogen known to potentially harm both human and animal health, was significantly increased in *A. galli* infection group. This study not only provides valuable insights into the healthy breeding of chickens but also offers critical scientific data for the prevention and control of opportunistic pathogens associated with parasitic infections, which has significant public health implications.

Key words: 16S rRNA, *Ascaridia galli*, Chickens, COX1, *Desulfovibrio.*

INTRODUCTION

Ascaridia galli (*A. galli*) is prevalent in various poultry species, including chickens, geese, pigeons, turkeys and several wild bird species (Tu *et al.*, 2019; Manjunatha *et al.*, 2023). *A. galli* resides in the small intestine of chickens, where large numbers of parasites can lead to intestinal obstruction, perforation, diarrhea, inflammation and, in severe cases, host mortality, particularly affecting chicks (Dänicke *et al.*, 2009; Moudgil *et al.*, 2018; Abdel *et al.*, 2019). *A. galli* absorbs nutrients from the host, thereby negatively impacting the health and productivity of chickens. This can result in anemia, reduced egg production rates and weight loss (Sharma *et al.*, 2018; Al-Musawi *et al.*, 2020). The parasite is globally distributed across numerous countries and regions (Li *et al.*, 2013; Yazwinski *et al.*, 2013; Thapa *et al.*, 2015; Sharma *et al.*, 2018), causing significant mortality in chickens and substantial economic losses in the poultry farming industry. Identification of *A. galli* in chickens primarily relies on molecular biology techniques, complemented by morphological characteristics. Several studies have demonstrated that the COX1 gene in mitochondrial DNA serves as a reliable genetic marker for species identification of *A. galli* (Mlondo *et al.*, 2022; Kusnoto *et al.*, 2024; Ritu *et al.*, 2024; Hu *et al.*, 2025).

The gut microbiota plays a critical role in maintaining the proper function of the scavenger system and significantly influences both systemic immune response and overall health. It facilitates the degradation of dietary fiber, vitamins and complex sugars, producing metabolites such as short-chain fatty acids serving as an energy source to the host (Ramsteijn *et al.*, 2024). Gut microbiota stimulates the proliferation and repair of intestinal epithelial cells, enhancing intestinal barrier function and mitigating adverse effects of substance transmission while inhibiting the colonization and proliferation of pathogenic microorganisms. Parasite infections may disrupt the balance of gut microbiota (Cohen and Denkers, 2015; Stensvold *et al.*, 2018; Shimokawa, 2025), facilitating secondary infections. Restoring and maintaining the balance of intestinal flora is essential for the prevention and treatment of parasite-related diseases.

This study aimed to amplify the COX1 gene of *A. galli via* PCR and construct a phylogenetic tree to investigate genetic differences between *A. galli* and other parasitic species. Additionally, the impact of *A. galli* infection to

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chicken intestinal microbiota was analyze by 16S rRNA sequencing of intestinal contents.

MATERIALS AND METHODS

Parasites collected and DNA extraction

From November to December in 2020, Worms were collected from the intestines of free-range chickens at multiple locations in Wenzhou, Zhejiang Province, China and preserved in 70% ethanol for subsequent analysis. TIANamp Genomic DNA Kit (DP304-02; TIANGEN, Beijing; China) was used to extract the total genomic DNA of collected worms in Wenzhou Vocational College of Science and Technology (Wenzhou, Zhejiang, China) according to theprotocol given by manufacturer and the total genomic DNA was stored at -40°C.

PCR reactions, sequence and phylogenetic analysis

The COX1 genes of worms were amplified by the primers as follow: the forward primers is 5′-ATTATTACTG CTCATGCTATTTTGATG-3′ and reverse primers is 5′-CAA AACAAATGTTGAAAATCAAAGG-3′. The PCR reaction protocol included an initial activation of Taq polymerase at 95°C for 10 minutes, followed by 35 cycles comprising denaturation at 95°C for 30 seconds, annealing at 55°C for 40 seconds, extension at 72°C for 30 seconds, and with a final extension at 72°C for 10 minutes. Thereafter, the PCR products were subjected to sequencing analysis at Sangon Biotech (Shanghai) Co., Ltd. The sequences of COX1 genes of isolated worms were compared with sequences form GenBank database. The homology of COX1 genes between isolated worms and others was analyzed by constructing a phylogenetic tree with MEGA 5.05 software.

Histopathology

Intestinal tissue samples were collected from both infected and uninfected chickens via surgical dissection. The samples were fixed in a 4% buffered neutral formaldehyde solution and Hematoxylin-eosin (HE) staining was conducted on sections with thickness of about 3.0 μm. The deparaffinization of sections was performed in xylene and the rehydration of sections was conducted through 100%, 95%, 85% and 75% alcohol respectively. Following this, Hematoxylin was used to stain the cell nuclei of sections and eosin was used to stain the cytoplasm. Subsequently, the sections were dehydrated with 75%, 85%, 95% and 100% alcohol respectively and rendered transparent using xylene. Finally, the slides were sealed for microscopic examination.

Intestinal contents collection, DNA extraction and PCR amplification

Samples from chickens infected with worms (group A) and uninfected chickens (group B) were collected in free-range farm. Intestinal contents were collected from five chickens in each group, transferred to cryogenic tubes and immediately immersed in liquid nitrogen for subsequent analysis. The QIAamp Fast DNA Stool Mini Kit (Qiagen, Catalog #51604) was used to extract bacterial DNA of chicken cecal contents. Following this, The sequences of V3-V4 hypervariable region in 16S rRNA gene were amplified by PCR reaction with barcoded primers.

Library construction, sequencing and data preprocessing

Sequencing libraries were prepared following standard protocols at Biomarker Technologies Co., Ltd. (Beijing, China) and paired-end sequencing was performed using the Illumina HiSeq 2500 platform. The raw image data files produced by high-throughput sequencing technologies, including Illumina HiSeq, underwent base calling analysis to generate sequencing reads. These reads were saved in FASTQ format files, which contain both sequence data and associated quality scores. FLASH v1.2.7 software was used to merge paired-end readsbased on whose overlapping regions were used to produce raw tags. High-quality clean tags were yielded by filtering raw tags with Trimmomatic v0.33 software. Effective tags were generated by removing chimeric sequences with UCHIME v4.2 software.

Bioinformatics and statistical analysis

Taxonomic annotation of operational taxonomic units (OTUs) was performed using Usearch software, referencing the Silva bacterial taxonomic database. OTU numbers per sample were determined at a 97% similarity threshold. Low-abundance OTUs were filtered out. Representative sequences were aligned against microbial reference databases to derive taxonomic classification information for each OTU. Community composition was analyzed at various taxonomic levels (phylum, class, order, family, genus, species) using the Qiime v2.0 package. Alpha diversity indices were evaluated with Mothur software (version 1.30). Shannon diversity rarefaction curves were generated using Mothur and the DESeq2 package in RStudio (version 4.0.0), which were also used to calculate Shannon indices at various sequencing depths. The Shannon index reflects microbial diversity within the samples; higher Shannon indices indicate greater OTU richness and species diversity. Two-dimensional Principal Component Analysis (PCA) plots were created in RStudio (version 4.0.0) using the Unweighted UniFrac distance matrix obtained from QIIME. Lnear discriminant analysis effect size algorithm (LEfSe) was utilized to identify biomarkers. Additionally, Metastats software was employed to perform T-tests on species richness data between the groups.

RESULTS AND DISCUSSION

PCR amplification, sequence alignment and phylogenetic analysis

Specific PCR amplification yielded fragments of approximately 533 bp, the expected length of the target fragment, with no non-specific bands observed. The blank control was negative. Homologous sequence comparison in the GenBank database confirmed that these worms were *Ascaridia galli*. The phylogenetic tree (Fig 1) constructed in this study revealed that all *A. galli* sequences exhibited a

close phylogenetic relationship with *A. galli* isolates collected South Africa (KT388440.1, KT388438.1), Italy (FM178545.1), Ghana (MW243594.1) and China (KT613902.1). However, they did not cluster within the same clade. Furthermore, these sequences exhibited a distant phylogenetic relationship with pigeon-derived sequences (JX624729.1, NC_021643.1) and did not cluster within the same clade.

Histopathology

Paraffin sections were prepared from the posterior duodenal tissues of both uninfected and worm-infected chickens and stained with hematoxylin and eosin (HE) (Fig 2). Microscopic analysis revealed that intestinal villi in uninfected chickens remained intact and well-formed. In contrast, villi in chickens infected with worms displayed underdevelopment, fragmentation and desquamation. These findings suggest that worm infection causes significant damage to the intestinal mucosal villi in chickens.

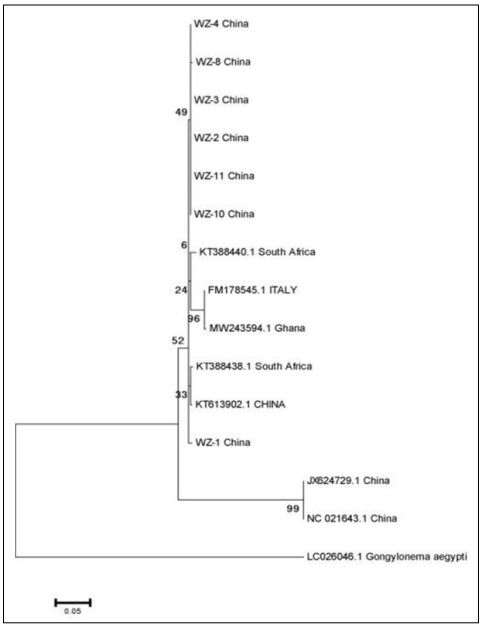


Fig 1: Phylogenetic tree of COX1 gene.

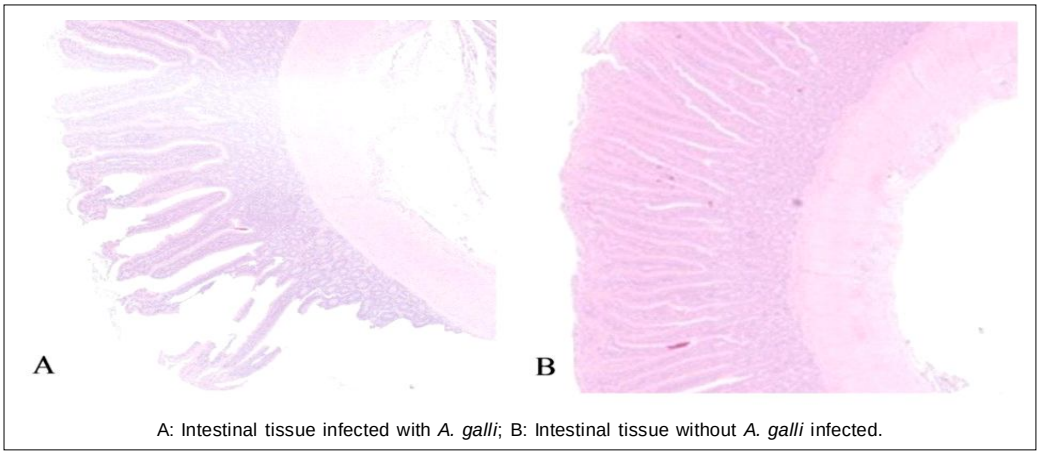
Analysis of composition differences in gut microbiota

The Shannon diversity rarefaction curve demonstrated that the number of operational taxonomic units (OTUs) in Group A was significantly higher than in Group B (Fig 3A), indicating greater species richness in the microflora of Group A compared to Group B. Principal Component Analysis (PCA) was utilized to visualize compositional differences among samples. Samples clustering closely together suggested a high degree of similarity in their species composition. PCA revealed a distinct separation in bacterial community composition between Group A and Group B, reflecting significant differences in abundance. The principal components, namely PC1 and PC2, accounted for 41.93% and 21.14% of the total variance, respectively (Fig 3B).

Gut microbiota analysis

Using the LEfSe algorithm (Fig 4), we identified the dominant genera in Groups A and B. T-tests on species richness data was performed using Metastats software and compositional differences between infected and uninfected groups were screened based on the q-value (q <0.05, obtained after p-value correction). Synthesis of results from the evolutionary branch graph of LEfSe analysis and t-tests indicated that Group A exhibited significantly higher abundances of *Desulfovibrionales*, *Desulfovibrionaceae*, *Desulfovibrio* and uncultured_ bacterium_ g_Desulfovibrio species at the order, family, genus and species levels, respectively, compared to Group B.

*A. galli*_is a severe intestinal helminth disease causing substantial economic losses to the chicken breeding industry annually. Although the mortality rate of chickens infected with roundworms did not significantly increased, the flock showed a decrease in feed intake, weight loss, pale cockscomb and a significant decrease in body weight. Due to changes in animal welfare regulations and people's consumption habits of liking high-quality chicken, chickens are increasingly being raised free-range



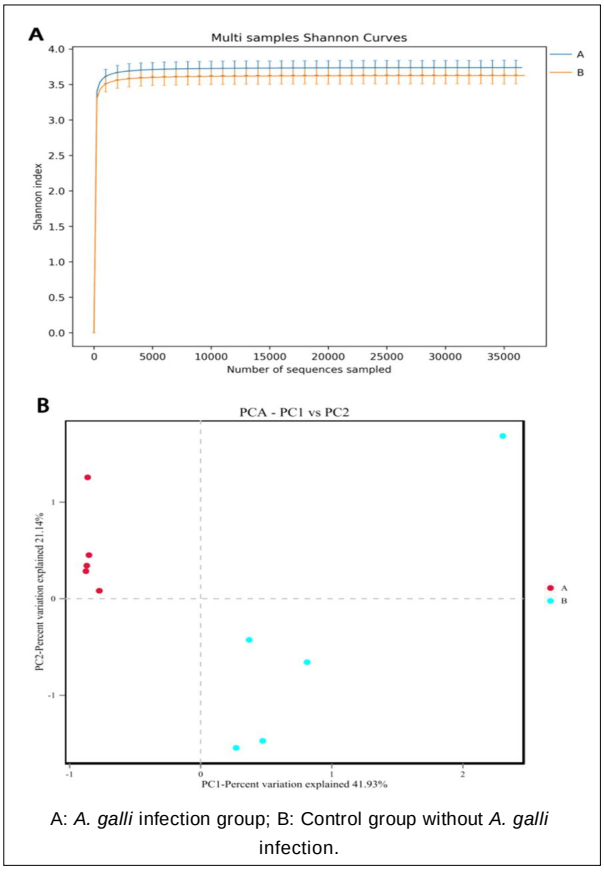
A: Intestinal tissue infected with *A. galli*; B: Intestinal tissue without *A. galli* infected.

Fig 2: HE staining of intestinal tissue (10×40).

rather than in cages. As this parasite is transmitted directly via the fecal-oral route through eggs containing infective larvae, this shift increases the risk of parasite infection (Höglund *et al.*, 2023).

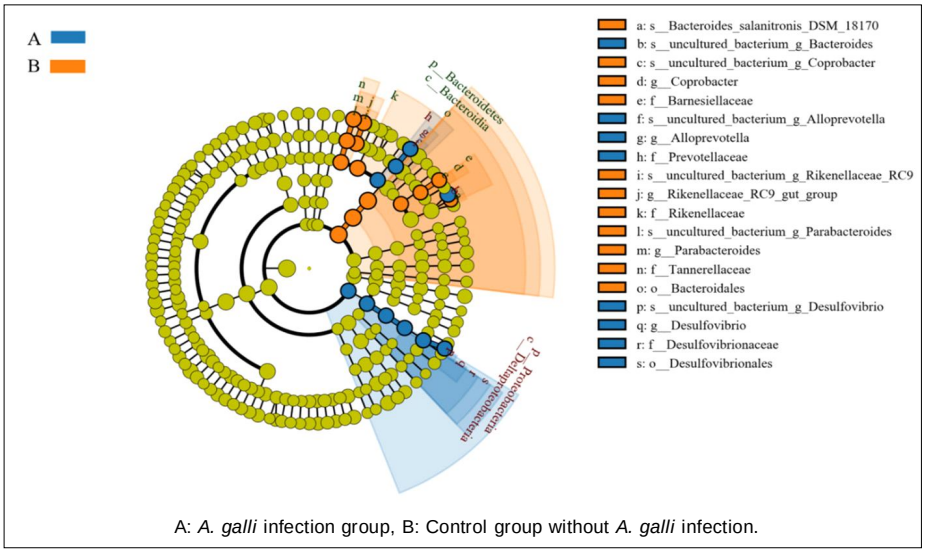
To effectively diagnose and control parasite infections, the isolated helminths were classified and identified using molecular biology techniques. In this study, the phylogenetic tree revealed genetic variation between the *A. galli* isolates from this study and those from other countries, such as South Africa, Ghana, China and Italy. These findings contribute to enriching the genetic information database for chicken *A. galli.* Contrary to previous reports (Okulewicz *et al.*, 1985), which found lower bacterial abundance in infected content compared to uninfected content, our study demonstrated that *A. galli* infection increased gut microbiota diversity, as measured by the Shannon index. This finding aligns with observations of other helminth infections in humans (Lee *et al.*, 2014) or animals (Zhou *et al.*, 2020; Song *et al.*, 2023). Histological analysis confirmed that *A. galli* infection resulted in significant intestinal damage in chickens.

In this study, we identified significant differences in the fecal microbiota composition of *Desulfovibrio* between Groups A and B using LEfSe analysis, which were confirmed by t-tests. Several studies suggest that *Desulfovibrio* species, by increasing the microbial sulfate reduction rate, are primary contributors to hydrogen sulfide (H, S) production in feces (Huang *et al.*, 2019; Karnachuk *et al.*, 2021). H, S is toxic and can cause severe poisoning in animals by inhibiting cytochrome oxidase (Firer *et al.*, 2008). As a metabolic byproduct of *Desulfovibrio*, H, S adversely affects the comfort, health and production efficiency of both animals and humans (Huang *et al.*, 2024). Certain species of the *Desulfovibrio* genus have been revealed to induce gut inflammation by disrupting the gut barrier, damaging the intestinal lining and increasing the secretion of inflammatory cytokines such as IL-1β, iNOS and TNF-α. Additionally, those bacteria reduce the levels of anti-inflammatory short-chain fatty acids (Parada *et al.*, 2019). Therefore, we propose that the increased abundance of *Desulfovibrio* bacteria caused by *A. galli* infection contributes to pathological losses in chickens.



A: *A. galli* infection group; B: Control group without *A. galli* infection.

Fig 3: Sample shannon index curve and PCA analysis graph.



A: *A. galli* infection group, B: Control group without *A. galli* infection.

Fig 4: LEfSe cladogram of the 16S rRNA sequence analysis.

Researches has established a significant positive correlation between *Desulfovibrio* overgrowth and various human diseases, including Parkinson's disease, inflammatory bowel disease, colitis, colorectal cancer, atherosclerosis and obesity (Singh *et al.*, 2023). In this study, we found that *A. galli* infection increases the abundance of *Desulfovibrio*, potentially facilitating the transmission of this opportunistic pathogen to humans. Thus, addressing *A. galli* is critical not only for poultry health but also for safeguarding human health.

Fecal egg examination and parasite autopsy have been employed to diagnose chicken roundworm disease. However, it takes approximately 30 days for visible worms or eggs to develop in chickens infected with roundworm eggs after the larvae return to the intestinal tract. During this period, significant economic losses have already occurred. Therefore, the development of an early diagnostic method for chicken roundworm infection holds great significance.

The prevention and control of *A. galli* require a multifaceted approach, encompassing vaccination, routine environmental sanitation and disinfection, enhanced breeding management practices, regular health examinations and appropriate pharmacological interventions (Sharma *et al.*, 2019). These measures can effectively mitigate the spread of *A. galli*.

CONCLUSION

Based on molecular biology analysis, we identified the worms from the chicken as *A. galli* which have some genetic differences with other places. 16s rRNA analysis showed that *Desulfovibrio* is of great importance in *A. galli* infection. This study is crucial for the healthy breeding of chickens and has important public health implication.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the Wenzhou Vocational College of Science and Technology of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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