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Parasitism: The good, the bad and the ugly

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Parasitism: The good, the bad and the ugly

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Editorial: Parasitism: the good, the bad and the ugly

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Editorial on the Research Topic
Parasitism: the good, the bad and the ugly

Parasitism is a tight association between species in which one organism, the parasite, lives on or inside the host, causing it harm, and is structurally adapted to this way of life (1). Until the twenty-first century, parasitism was studied by parasitologists, rather than ecologists or evolutionary biologists. Today, parasitism is a major element of evolutionary ecology, as nearly all free-living animals, are hosts to at least one parasite species (2, 3). Since it is in the parasite's evolutionary interest for its host to flourish, long-term coevolution can lead to a stable relationship bordering on mutualism (1, 4). According to Lynn Margulis, when resources are scarce, natural selection moves relationships from parasitism to mutualism, as was brilliantly illustrated in Margulis' endosymbiosis theory, where eukaryotic mitochondria and chloroplasts descended from formerly free-living prokaryotes (5).

While worm parasitism clearly affects animals, it may also reduce the prevalence and severity of autoimmune diseases in animal hosts, including humans (6). Helminth infections might safeguard hosts from immune-mediated illnesses like allergies and autoimmune disease, as well as suppress detrimental inflammatory host's reaction. In this symbiotic relationship, the helminth, and the host benefit from each other's presence (7–9). The boundary between mutualism, symbiosis, and pathological parasitism is narrow and is frequently overlapping without a theory to clearly explain this relationship between the parasite, host, and ecological niche (10–12). Parasites are frequently seen as dangerous and primarily viewed as harmful to the host (3, 13). However, removing them entirely would be detrimental to the host, the environment, and the parasites themselves (3). Complex natural ecosystems with diverse species and their interactions are the result of a long evolutionary history (12, 14). Parasites make up half of life's diversity and play vital ecological roles (3, 15). Parasites allow for inter-species DNA exchange, allowing for evolutionary change (16). To complete their life cycles, many parasites rely on predator-prey or other stable ecological interactions (12, 17). At individual and species levels, hosts have developed adaptations to their coevolving parasites (10, 11, 15, 18). Often, the expression of normal behavior or the development of a healthy immune system depends on parasite infections (8). In instances where parasites are not present, hosts may occasionally exhibit phenotypes that are less than ideal (14). Conserving parasite species is supported by their evolutionary heritage and potential, their role in ecosystem services, and their value as indicators of ecosystem quality (3, 19). Parasites imply a healthy ecology (20). Researchers have highlighted the functional significance of parasites in preserving the health of individual hosts, host populations,

and entire ecosystems in order to raise awareness of parasite conservation (3, 14). For individual hosts, parasites impose energetic demands that reduce growth, fecundity, and survival (21, 22), but these apparent negative effects on host fitness may be compensated by positive consequences, such as improving normal host immune system function (8, 9, 23), regulating their host's microbiome (7, 8, 24–26), inducing specific interaction between gut microbiota and host immune responses (27–29), and providing protection against pollutants such as heavy metals (20, 30, 31). Even though parasites decrease host fitness, they could potentially maintain host population viability by controlling host population size (18, 32, 33), and increasing genetic variety while avoiding the detrimental effects of inbreeding (21). Parasites can act as keystone species, allowing competing species to coexist while lowering the dominance of superior competitors (17). Parasites may mediate predatory and competitive interactions among free-living species at higher levels of organization, adjusting community structure and diversity, food web complexity, and energy flow throughout the entire ecosystem (20, 34–36). While parasites typically remain absent from food web representations, they consistently hold the top position (15).

With 220 million people affected annually by malaria, measures such as prophylaxis, eradicating mosquito vectors using pesticides, and developing a malaria vaccine, have been attempted to stop malaria transmission (37). Nonetheless, medication resistance, mosquito pesticide resistance, and vaccination failures due to parasite mutations or parasite immune manipulation have all been issues (38–40). Similar problems have been observed with other economically significant groups of parasites that cause disease in livestock, poultry, and domestic animals, such as protozoa, helminths, and ectoparasites (41, 42).

Due to their potential benefits in numerous ecosystems, parasites should not be eliminated from Earth's environments (3). Nonetheless, some parasites do represent a serious threat to human and animal health; therefore, we must find an appropriate equilibrium between parasite burden, human and animal fitness, and ecosystem quality, all while adhering to the current one health concept (14, 27, 33, 43–46). To achieve this equilibrium, parasite managements must rely on suitable treatment, prevention and control strategies, and their appropriate implementation at field (3, 29, 38, 47). Studies on parasites management can serve as a link between health and environmental sciences, enabling accomplish the objective of the one health concept through an interdisciplinary and holistic approach (14, 44, 48).

Several issues regarding holistic approaches to parasite management have aroused the interest of scientific community, with resulting in several investigations and publications to answer if parasites are good, bad, or ugly for human's and animal's health. This Research Topic contains all various initiatives to harness knowledge, such as the following:

1. Genetic studies for both ecto- and endo parasites. Generated mitochondrial genome data provided further flea taxonomic and epidemiological studies (Liu et al.). Genetic analysis of *Fasciola hepatica*, demonstrated population structure in South America not previously realized using nuclear and mitochondrial markers (Garcia-Corredor et al.).

2. Innovative lab procedures. Currently, it is feasible to use harmless methods for preserving avian *Eimeria* sp. sporulated oocysts for challenge testing or live coccidiosis vaccines (Laverty et al.). Isolates of live-attenuated *Histomonas meleagridis* were successfully obtained by serial passaging in a lab setting (Beer et al.). These attenuated vaccine strains proved to be immunoprotective against challenge with virulent *H. meleagridis*. However, commercial application of these attenuated vaccines is not currently feasible from a production, labor, and economic standpoint. Nowdays, the identification of *E. tenella* antigens through novel immunological approaches is crucial for the development of successful genetically engineered vaccines (Juárez-Estrada et al.). Lab procedures indicates that Ferritin 2 has emerged as a promising antigen for a universal vaccination against avian mites (Win et al.).

3. Bibliometric analysis for screening assistance. Bibliometric data provides insight for the selection and evolution of anticoccidial drugs and, contributes to understand anticoccidial medication utilization trends (Kandeel et al.).

4. Experimental infection models are necessary to mimic complex multi-factorial interactions. An experimental mixed avian *Eimeria* sp. challenge model that particularly evaluates performance, intestinal permeability, and dysbiosis is suitable to analyse different strategies for avian coccidiosis control (Graham et al.). An experimental model revealed that horizontal transmission of *H. meleagridis* could vary depending on the pathogenicity of the challenge strain and Turkey diet composition (Barros et al.). Turkeys experimentally vaccinated with a candidate *E. meleagrimitis* strain with or without amprolium intervention showed altered ileal and cecal microbiome (Trujillo-Peralta et al.). In an experimental model of priming/challenge, an *E. meleagrimitis* strain candidate vaccine caused a moderate infection that induced protective immunity (Trujillo-Peralta et al.). Coccidiosis infection in lambs impacts rumen fermentation in a way that is independent of plane nutrition, but this effect does not translate to performance response (Sujani et al.).

5. Alternatives to antibiotics. As demand rises to decrease (eliminate) antibiotic use in poultry; natural resources such as *Yucca schidigera* and *Trigonella foenum* are promising coccidiosis control options (Benarbia et al.). Soybean lecithin has also been linked to increased resilience in several animal species to physical and chemical stressors (Wee et al.).

Novel research on parasitology contributes to identify new prevention and control measures that we need now more than ever front to current climate changing (43, 49, 50).

Author contributions

MJ-E: Conceptualization, Data curation, Writing—original draft, Writing—review and editing. GT-I: Conceptualization, Data

curation, Funding acquisition, Writing—original draft, Writing—review and editing. DG: Conceptualization, Data curation, Writing—original draft, Writing—review and editing. XH-V: Data curation, Visualization, Writing—review and editing.

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References

1. Rózsa L, Garay J. Definitions of parasitism, considering its potentially opposing effects at different levels of hierarchical organization. *Parasitol.* (2023) 150:761–68. doi: 10.1017/S0031182023000598

2. Santiago MFM, King KC, Drew GC. Interactions between insect vectors and plant pathogens span the parasitism–mutualism continuum. *Biol Lett.* (2023) 19:20220453. doi: 10.1098/rsbl.2022.0453

3. Lymbery AJ, Smit NJ. Conservation of parasites: a primer. *Int J Parasitol Parasites Wildl.* (2023) 21:255–63. doi: 10.1016/j.ijppaw.2023.07.001

4. Hasik AZ, de Angeli Dutra D, Doherty JF, Duffy MA, Poulin, R, Siepielski AM. Resetting our expectations for parasites and their effects on species interactions: a meta-analysis. *Ecol Lett.* (2023) 26:184–99. doi: 10.1111/ele.14139

5. López-García P, Moreira D. The symbiotic origin of the eukaryotic cell. *C R Biol.* (2023) 346:55–73. doi: 10.5802/crbiol.118

6. Abu-Shakra M, Shoenfeld Y. For debate parasitic infection and autoimmunity. *Autoimmunity.* (1991) 9:337–44. doi: 10.3109/08916939108997136

7. Loke P, Lim YAL. Helminths and the microbiota: parts of the hygiene hypothesis. *Parasite Immunol.* (2015) 37:314–23. doi: 10.1111/pim.12193

8. Ramanan D, Bowcutt R, Lee SC, Tang MS, Kurtz ZD, Ding Y, et al. Helminth infection promotes colonization resistance via type 2 immunity. *Science.* (2016) 352:608–12. doi: 10.1126/science.aaf3229

9. Atagozli T, Elliott DE, Ince MN. Helminth lessons in inflammatory bowel diseases (IBD). *Biomedicine.* (2023) 11:1200. doi: 10.3390/biomedicines11041200

10. Taraschewski H. Hosts and parasites as aliens. *J Helminthol.* (2006) 80:99–128. doi: 10.1079/JOH2006364

11. Lymbery AJ, Morine M, Kanani HG, Beatty SJ, Morgan DL. Co-invaders: the effects of alien parasites on native hosts. *Int J Parasitol Parasites Wildl.* (2014) 3:171–77. doi: 10.1016/j.ijppaw.2014.04.002

12. Best A, Ashby B. How do fluctuating ecological dynamics impact the evolution of hosts and parasites? *Phil Trans R Soc B.* (2023) 378:20220006. doi: 10.1098/rstb.2022.0006

13. Vilcinskas A. Pathogens as biological weapons of invasive species. *PLoS Pathog.* (2015) 11:e1004714. doi: 10.1371/journal.ppat.1004714

14. Selbach C, Mouritsen KN, Poulin R, Sures B, Smit NJ. Bridging the gap: aquatic parasites in the One Health concept. *Trends Parasitol.* (2022) 38:109–11. doi: 10.1016/j.pt.2021.10.007

15. Jephcott TG, Sime-Ngando T, Gleason FH, Macarthur DJ. Host–parasite interactions in food webs: Diversity, stability, and coevolution. *Food Webs.* (2016) 6:1–8. doi: 10.1016/j.fooweb.2015.12.001

16. Combes C. *Parasitism: The Ecology and Evolution of Intimate Interactions*. Chicago: University of Chicago Press Chicago, USA. (2001).

17. Ramesh A, Hall SR. Niche theory for within-host parasite dynamics: Analogies to food web modules via feedback loops. *Ecol Lett.* (2023) 26:351–68. doi: 10.1111/ele.14142

18. Papkou A, Gokhale CS, Traulsen A, Schulenburg H. Host–parasite coevolution: why changing population size matters. *Zoology.* (2016) 119:330–38. doi: 10.1016/j.zool.2016.02.001

19. Almeida D, Cruz A, Llinares C, Torralva M, Lantero E, Fletcher DH, et al. Fish morphological and parasitological traits as ecological indicators of habitat quality in a Mediterranean coastal lagoon. *Aquatic Conserv: Mar Freshw Ecosyst.* (2023) 2023:1–16. doi: 10.1002/aqc.3996

20. Marcogliese DJ. Parasites of the superorganism: are they indicators of ecosystem health? *Inter J Parasitol.* (2005) 35:705–16. doi: 10.1016/j.ijpara.2005.01.015

21. Coltman DW, Pilkington JG, Smith JA, Pemberton JM. Parasite-mediated selection against inbred soay sheep in a free-living, island population. *Evol.* (1999) 53:1259–67. doi: 10.2307/2640828

22. Marcogliese DJ. Parasites: Small players with crucial roles in the ecological theater. *Ecohealth.* (2004) 1:151–64. doi: 10.1007/s10393-004-0028-3

23. Sorci G, Guivier E, Lippens C, Faivre B. Microbes, Parasites and Immune Diseases. In: Alvergne A, Jenkinson C, Faurie C, editors. *Evolutionary Thinking in Medicine: From Research to Policy and Practice.* Cham: Springer International Publishing. (2016) p. 211–23.

24. Giacomin P, Agha Z, Loukas A. Helminths and intestinal flora team up to improve gut health. *Trends Parasitol.* (2016) 32:664–66. doi: 10.1016/j.pt.2016.05.006

25. Partida-Rodríguez O, Serrano-Vázquez A, Nieves-Ramírez ME, Moran P, Rojas L, Portillo T, et al. Human intestinal microbiota: Interaction between parasites and the host immune response. *Arch Med Res.* (2017) 48:690–700. doi: 10.1016/j.arcmed.2017.11.015

26. Llewellyn MS, Leadbeater S, Garcia C, Sylvain FE, Custodio M, Ang KP, et al. Parasitism perturbs the mucosal microbiome of Atlantic Salmon. *Sci Rep.* (2017) 7:43465. doi: 10.1038/srep43465

27. Mutapi F. The gut microbiome in the helminth infected host. *Trends Parasitol.* (2015) 31:405–06. doi: 10.1016/j.pt.2015.06.003

28. Li C, Liu Y, Liu X, Bai X, Jin X, Xu F, et al. The gut microbiota contributes to changes in the host immune response induced by *Trichinella spiralis*. *PLoS Negl Trop Dis.* (2023) 17:e0011479. doi: 10.1371/journal.pntd.0011479

29. Boisseau M, Dhorne-Pollet S, Bars-Cortina D, Courtot É, Serreau D, Annonay G, et al. Species interactions, stability, and resilience of the gut microbiota - Helminth assemblage in horses. *iScience.* (2023) 26:106044. doi: 10.1016/j.isci.2023.106044

30. Sánchez MI, Pons I, Martínez-Haro M, Taggart MA, Lenormand T, Green AJ. When parasites are good for health: Cestode parasitism increases resistance to arsenic in brine shrimps. *PLoS Pathog.* (2016) 12:e1005459. doi: 10.1371/journal.ppat.1005459

31. Keke UN, Mgbemena AS, Arimoro FO, Omalu ICJ. Biomonitoring of effects and accumulations of heavy metals insults using some helminth parasites

of fish as bio-indicators in an Afrotropical stream. *Front Environ Sci.* (2020) 2:576080. doi: 10.3389/fenvs.2020.576080

32. Hudson PJ, Dobson AP, Newborn D. Prevention of population cycles by parasite removal. *Science.* (1998) 282:2256–58. doi: 10.1126/science.282.5397.2256

33. Scott ME. Helminth-host-environment interactions: Looking down from the tip of the iceberg. *J Helminthol.* (2023) 97:e59. doi: 10.1017/S0022149X23000433

34. Hudson PJ, Dobson AP, Lafferty KD. Is a healthy ecosystem one that is rich in parasites? *Trends Ecol Evol.* (2006) 21:381–85. doi: 10.1016/j.tree.2006.04.007

35. Dunn AM, Hatcher MJ. Parasites and biological invasions: parallels, interactions, and control. *Trends Parasitol.* (2015) 31:189–99. doi: 10.1016/j.pt.2014.12.003

36. Vannatta JT, Minchella DJ. Parasites and their impact on ecosystem nutrient cycling. *Trend Parasitol.* (2018) 34:452–55. doi: 10.1016/j.pt.2018.02.007

37. El Moustapha I, Ouldabdallahi Moukah M, Ould Ahmedou Salem MS, Brahim K, Briolant S, Basco L, et al. Malaria prevalence in Mauritania: a systematic review and meta-analysis. *Malar. J.* (2023) 22:146. doi: 10.1186/s12936-023-04569-4

38. Orwa T, Mbogo R, Luboobi L. Mathematical model for the in-host malaria dynamics subject to malaria vaccines. *Lett Biomath.* (2018) 5:22–251. doi: 10.30707/LiB5.1Orwa

39. Aidoo EK, Aboagye FT, Botchway FA, Osei-Adjei G, Appiah M, Duku-Takyi R, et al. Reactive case detection strategy for malaria control and elimination: a 12 year systematic review and meta-analysis from 25 malaria-endemic countries. *Trop Med Inf Dis.* (2023) 8:180. doi: 10.3390/tropicalmed8030180

40. Miyauchi E, Shimokawa C, Steimle A, Desai MS, Ohno H. The impact of the gut microbiome on extra-intestinal autoimmune diseases. *Nat Rev Immunol.* (2023) 23:9–23. doi: 10.1038/s41577-022-00727-y

41. Blake DP, Tomley FM. Securing poultry production from the ever-present *Eimeria* challenge. *Trends Parasitol.* (2014) 30:12–9. doi: 10.1016/j.pt.2013.10.003

42. Naz S, Abbasi SW, Cavezza S, Jaiswal AK, Azevedo V. Parasite Control Strategies: Immunoprophylaxis. In: Rizwan HM, Sajid MS, editors. *Parasitism and Parasitic Control in Animals.* Oxfordshire: CABI. (2023) 231–47. doi: 10.1079/9781800621893.0015

43. Iacovino A, Randazzo L. Climate change and food security in the One Health perspective. *BioLaw J.* (2023) 2:293–314.

44. Díaz AV, Walker M, Webster JP. Reaching the World Health Organization elimination targets for schistosomiasis: the importance of a one health perspective. *Phil Trans R Soc B.* (2023) 378:20220274. doi: 10.1098/rstb.2022.0274

45. Douchet P, Gourbal B, Loker ES, Rey O. Schistosoma transmission: scaling-up competence from hosts to ecosystems. *Trends Parasitol.* (2023) 39:563–74. doi: 10.1016/j.pt.2023.04.001

46. Vicente-Santos A, Willink B, Nowak K, Civitello DJ, Gillespie TR. Host-pathogen interactions under pressure: a review and meta-analysis of stress-mediated effects on disease dynamics. *Ecol Lett.* (2023) 1–18. doi: 10.1111/ele.14319

47. Coates A. Research pre-empting parasite adaptation is key to sustainable disease management in aquaculture. *Aquacult Environ Interact.* (2023) 15:35–43. doi: 10.3354/aei00451

48. Hodžić A, Dheilly NM, Cabezas-Cruz A, Berry D. The helminth holobiont: a multidimensional host–parasite–microbiota interaction. *Trends Parasitol.* (2023) 39:91–100. doi: 10.1016/j.pt.2022.11.012

49. Hector TE, Gehman ALM, King KC. Infection burdens and virulence under heat stress: ecological and evolutionary considerations. *Phil Trans R Soc B.* (2023) 378:20220018. doi: 10.1098/rstb.2022.0018

50. Poglayen G, Gelati A, Scala A, Naitana S, Musella V, Nocerino M, et al. Do natural catastrophic events and exceptional climatic conditions also affect parasites? *Parasitol.* (2023) 15:1–9. doi: 10.1017/S0031182023000471

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REVIEW
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Histomonosis in Poultry: A Comprehensive Review

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Histomonas meleagridis, the etiological agent of histomonosis, is a poultry parasite primarily detrimental to turkeys. Characteristic lesions occur in the liver and ceca, with mortalities in turkey flocks often reaching 80–100%. Chickens and other gallinaceous birds can be susceptible but the disease was primarily considered sub-clinical until recent years. Treating and preventing *H. meleagridis* infection have become more difficult since 2015, when nitarsone was voluntarily removed from the market, leaving the poultry industry with no approved prophylactics, therapeutics, or vaccines to combat histomonosis. Phytogenic compounds evaluated for chemoprophylaxis of histomonosis have varied results with *in vitro* and *in vivo* experiments. Some recent research successes are encouraging for the pursuit of antihistomonal compounds derived from plants. Turkeys and chickens exhibit a level of resistance to re-infection when recovered from *H. meleagridis* infection, but no commercial vaccines are yet available, despite experimental successes. Safety and stability of live-attenuated isolates have been demonstrated; furthermore, highly efficacious protection has been conferred in experimental settings with administration of these isolates without harming performance. Taken together, these research advancements are encouraging for vaccine development, but further investigation is necessary to evaluate proper administration age, dose, and route. A summary of the published research is provided in this review.

Keywords: *Histomonas meleagridis*, protozoa, parasite, turkey, histomonosis

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INTRODUCTION

The first known histomonosis outbreak was described by Cushman (1) and occurred in a Rhode Island turkey flock. Smith (2) further characterized histomonosis and attributed it to the protozoan *Amoeba meleagridis* obtained from liver lesions. Shortly thereafter, Tyzzer (3) more appropriately renamed this protozoon as *Histomonas meleagridis*. Further studies confirmed *H. meleagridis* as the etiological agent, although the mode of cecal invasion was still uncertain (4). Common synonyms for the disease have included blackhead disease, infectious enterohepatitis, histomoniasis, and typhlohepatitis (5–7). Blackhead disease is an unfortunate misnomer as a cyanotic head is neither pathognomonic nor common (8, 9); therefore, histomonosis will be the preferred terminology used throughout this review based on the Standardized Nomenclature of Animal Parasitic Diseases (10). Turkeys are especially susceptible to *H. meleagridis* infection, although other gallinaceous birds such as chickens, pheasants, and peafowls can be affected (7, 11, 12). Annual economic losses to the turkey industry have been estimated to exceed 2 million USD, and a 2020 survey listed histomonosis in position #11 of current issues facing the industry (9, 13).

Graybill and Smith (14) implicated *Heterakis* spp. in the role of transmitting *H. meleagridis* as they were unable to initiate the disease in absence of cecal worms. Further research showed that unprotected histomonads did not survive long periods outside the host, although duration in the environment when protected by feces or other materials was not well-characterized (15, 16). The separate rearing of poultry species is critical as chickens are considered partially resistant to histomonosis, frequently serving as asymptomatic carriers and reservoirs of *H. meleagridis*-infected heterakid eggs [**Figure 1**; (11, 17–21)]. Direct transmission within a flock is considered to occur through cloacal drinking which transfers materials from the vent region into the ceca through waves of reverse peristalsis (22–25). Horizontal transmission of *H. meleagridis* has occurred by comingling and contact of infected with uninfected turkeys, regardless of floor type and in absence of *H. gallinarum* (26, 27). The breed of turkeys or chickens may affect susceptibility to *H. meleagridis* infection, although male and female turkeys appear to be similarly susceptible; however, research is limited on the possible influence on disease development (28–31).

BIOLOGY OF *H. meleagridis*

H. meleagridis is a unicellular parasite belonging to the phylum Parabasalia, class Tritrichomonadea, order Tritrichomonadida, and family *Dientamoebidae* (32, 33). Interestingly, the morphology can change between flagellated and amoeboid forms depending on location within the ceca or liver, respectively, with an average histomonad size of 10–14 μm [**Figure 2**; (3, 5, 9, 34–36)]. The cell morphology and associated phenotypic changes have been mimicked experimentally *in vitro* (37). *H. meleagridis* typically exhibits a single-flagellated form within the cecal lumen, but this flagellum is lost upon mucosal invasion with the development of pseudopods (38). *H. wenrichi* (alternatively *Parahistomonas wenrichi*), a non-pathogenic but separate species, appears as 4-flagellated or amoeboid in form with a larger size of 20–30 μm (16, 39–41). *H. meleagridis* reproduce via binary fission; lacking mitochondria, these protozoa rely on hydrogenosomes as modified organelles for energy metabolism (3, 42–44).

Early *in vitro* work indicated that histomonads can be grown at temperatures of 36.5–37°C but not when reduced to 18–22°C for 48 h or 5°C for 24 h, suggesting that environmental survival of protozoa shed from infected birds is not likely to be culpable in mass infectivity (35). Currently, *in vitro* propagation occurs anaerobically at 40–41°C with a Medium 199-based cell culture and bacterial co-culture to simulate the body temperature and environment of a healthy turkey (44, 45). Dwyer's medium comprised of Medium 199, chick embryo extract, horse sera, and rice powder has been utilized, although other cell culture media such as L-15, MEM, or RPMI have been substituted effectively for Medium 199 (46). Modified Dwyer's medium, which removes

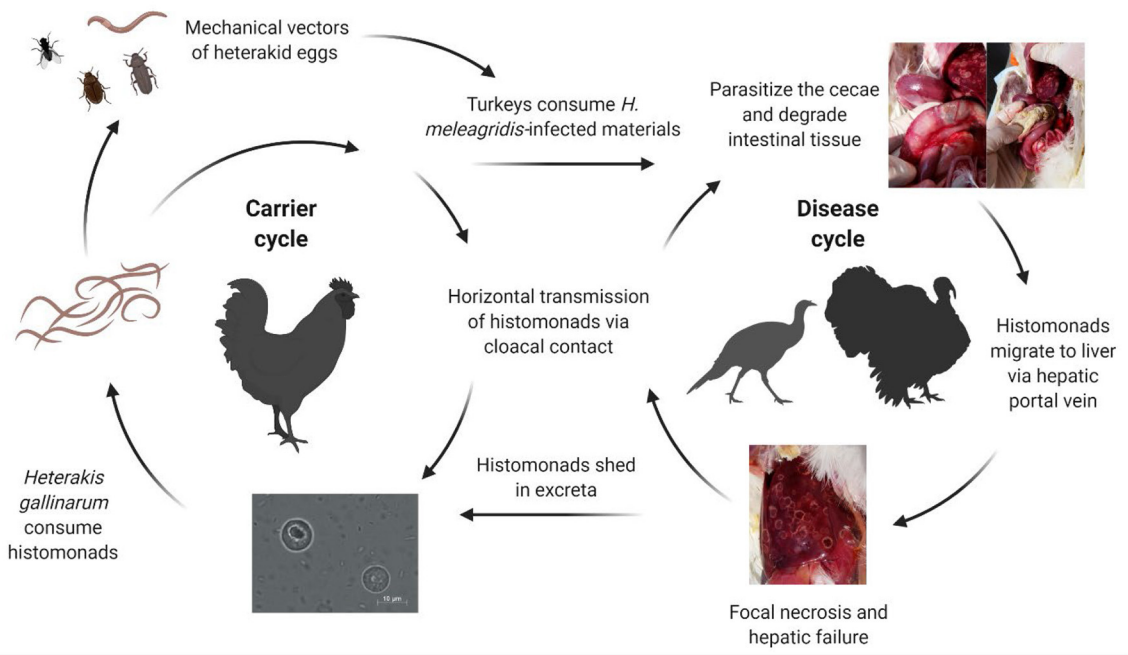

FIGURE 1 | Complex transmission of *Histomonas meleagridis*. Histomonads can be consumed by *Heterakis gallinarum* and can be subsequently incorporated into the nematode ova. Carrier birds such as chickens can harbor the cecal worms and shed infected heterakid eggs into the environment. Earthworms, flies, and other invertebrates can serve as intermediate mechanical vectors of infected heterakid ova. Turkeys may ingest infected materials such as excreta or invertebrates contaminated with the protozoa. Once inside the intestine, the histomonads will migrate to the ceca, replicating and degrading the cecal lining. Direct transmission can occur rapidly from turkey-to-turkey due to cloacal drinking and reverse peristalsis movement of materials into the vent region (Created with BioRender.com).

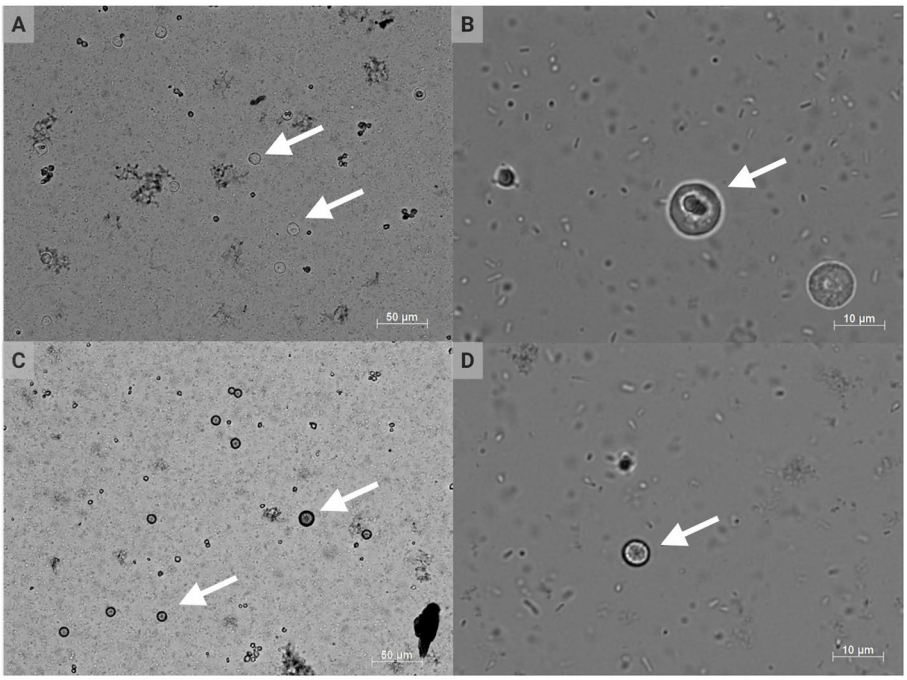

FIGURE 2 | Conventional DIC photomicrographs of *Histomonas meleagridis*. Amoeboid or irregular morphology of histomonads at 200× **(A)** and 1,000× (**B**) total magnification; spherical morphology of histomonads at 200× **(C)** and 1,000× (**D**) total magnification. The arrows indicate selected histomonads (Created with BioRender.com).

the chick embryo extract and increases rice powder from 0.096 (w/v) to 0.8% (w/v), improved histomonad growth following revival of aliquots from liquid nitrogen and serial passage (47). Further increasing rice powder from 0.8 (w/v) to 4–8% (w/v) resulted in a nearly 10-fold growth increase, but this was not sustained longer than 2 days as the remaining nutrients became exhausted (48). Cholesterol supplementation has improved *H. meleagridis* growth *in vitro,* even in the absence of serum, which is typically required for adequate growth (49). Chute and Chute (50) cryogenically preserved *H. meleagridis* isolates in combination with 8% dimethylsulfoxide for up to 345 days and demonstrated viability of infection to birds following thaw. Honigberg and Dwyer (51) demonstrated that either 5 or 10% dimethylsulfoxide effectively preserved the protozoa in cryogenic storage as observed after 7 weeks; therefore, isolates could be maintained for future studies.

Field isolates of *H. meleagridis* can be cultivated from infected carcasses, particularly cecal samples, if shipment to a laboratory occurs soon after bird mortality with greater recovery potential if temperatures are maintained above 30°C (52). *In vitro* growth from cecal samples can usually be confirmed between 1 and 4 days after inoculation into culture media; intracloacal inoculation back into live birds can be used to further diagnose *H. meleagridis* as the original cause of infection in field outbreaks (53). Histomonads have also been isolated effectively from liver lesions, but *in vitro* propagation attempts in absence of bacteria have been unsuccessful (16, 34, 53, 54). Attempts to culture the protozoa in absence of live bacteria and serum were achieved with difficulty, but supplementation of palmitic acid or cholesterol was required along with antibiotic-killed bacteria and hamster liver extract; however, these results have not been easily replicated (45, 55–57). *In vitro* growth of *H. meleagridis* was better sustained with undefined populations of turkey cecal bacteria than with mixed chicken cecal bacteria (58). Moreover, histomonads have been grown with supplementation of single species of bacteria and monoxenic cultures have been established (59, 60).

PATHOGENESIS

After parasitizing and degrading the cecal tissue, histomonads migrate to the liver via the hepatic portal blood; the resulting pathognomonic lesions are exhibited as target-like liver lesions and caseous cecal cores [**Figures 3**, **4**; (44, 61, 62)]. Histomonads have been observed in the bursa of Fabricius of 6-week-old commercial chickens diagnosed with histomonosis, further implicating the intracloacal route for natural infection (63). Although less common, *H. meleagridis* has also been shown to infect areas including the brain, pancreas, heart, lungs, kidneys, and spleen (64–68). Turkeys are especially vulnerable to histomonosis, and chickens (**Figure 5**) are less susceptible but function to serve as reservoirs and can develop the disease (17). Cloacal transmission seems less important to chickens than turkeys for transfer of histomonosis, as horizontal transmission did not occur in the absence of vectors and was not exacerbated with *Eimeria adenoeides* challenge, which is not surprising as this *Eimeria* spp. is turkey-specific (69). While cloacal drinking

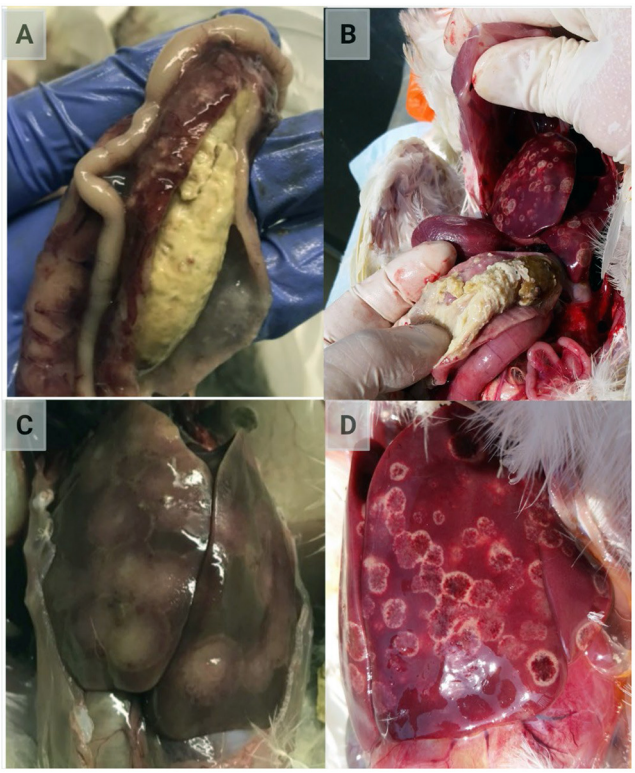

FIGURE 3 | Classic lesions resulting from *Histomonas meleagridis* infection. **(A,B)** Caseous cheese-like cecal core; **(C,D)** focal necrosis resulting in target-like liver lesions (Created with BioRender.com).

is a well-known occurrence in chickens and turkeys, species differences in horizontal transmission could result from higher litter moisture and huddling behavior in turkeys than chickens, allowing greater survival and subsequent transmission of *H. meleagridis* in the absence of vectors (22, 69). Mortalities in turkey flocks can reach 80–100%; organic farms co-rearing turkeys and broilers have struggled with series of outbreaks with broiler and turkey mortalities reaching 100 and 67.2%, respectively, possibly due to co-infection with *Eimeria* spp. (19, 44, 70). Susceptibility of different poultry species and genetic lines has only been evaluated briefly, but infection incidence and severity do appear different (28, 29, 68, 71, 72). In chickens, sex-related variations and environmental differences have influenced intestinal structure and function; therefore, it seems reasonable that these differences could factor into the incidence and severity of histomonosis (73). In addition to age and genetic line of poultry, variations in mortality rate and lesion severity could result from strain-specific differences in virulence of *H. meleagridis* or exposure dose (23, 74, 75). Although chickens were previously regarded as sub-clinically affected by histomonosis, outbreaks have occurred recently in broiler breeder and free-range flocks (76, 77). Interestingly, recent research has indicated that *H. meleagridis* infection and replication are similar regardless of chicken genetic line, further suggesting that chickens may be asymptomatic or sub-clinically infected but not actually resistant to infection (78).

A virulent clonal strain of *H. meleagridis* induced similar mortality and pathology in turkeys regardless of age, sex, or dose (31). A low dose of 3,162 histomonads induced 100% mortality in British United turkeys (BUT-Big6) by 2-weeks post-infection (30). Three different genetic lines of turkeys showed similar susceptibility to histomonosis, although wild Canadian turkeys exhibited higher mortality rates and lower liver lesions than BUT-Big6 or Kelly-Bronze lines (72). Concurrent infection with *E. tenella* can aggravate the development of histomonosis in broiler chickens, specifically increasing liver lesions (79). Conversely, turkeys co-infected with *E. adenoeides* and *H. meleagridis* resulted in significantly reduced cases of histomonosis (24). The dosage and timing of *Eimeria* vaccination of chickens will influence the severity of aggravation due to histomonosis, although further co-infection studies are necessary to conclude effects of combined pathogens to severity in chickens and turkeys (80).

Bradley and Reid (81) inoculated gnotobiotic (bacteria-free) turkeys with *H. meleagridis* in combination with either *Escherichia coli*, *Bacillus subtilis*, or *Clostridium perfringens* and suggested that a combination of the protozoa and bacteria populations was required to initiate histomonosis. Incidence of *H. meleagridis* infection in gnotobiotic chickens and turkeys increased when concurrently challenged with a mixture of *E. coli* and *C. perfringens*, whereas histomonosis was lessened with administration of a single bacteria species (82). Healthy turkey ceca contain predominantly (>50%) anaerobic *Lactobacillus* spp. and relatively low (<1%) coliforms and *Enterococcus* spp. (61). *Salmonella typhimurium*, *E. coli*, and *H. meleagridis* infections have been found concurrently in broiler chicken flocks (83). Cultures of *H. meleagridis* were identified to favor obligate anaerobes of the *Clostridiaceae* family, aerotolerant anaerobes of the *Bacteriodaceae* family, or facultative to obligate anaerobes of the *Baccillaceae* family (84). The Proteobacteria phylum increased in relative abundance in birds with severe histomonosis, but *E. coli* populations were maintained at the same level in turkeys regardless of the level of gut inflammation (85). *E. coli* mutually benefited histomonad growth *in vitro* and increased cecal involvement *in vivo* (60, 86). Co-infection of laying chickens with *H. meleagridis* and *E. coli* produced severe dysbiosis, increased microscopic lesions, and enhanced colonization of the cecal tissue (86). Recently however, the gastrointestinal pathology and *E. coli* load were not associated with severity of histomonosis, while microbiota composition and dysbiosis were directly attributed to the severity of inflammation (85). In addition to providing direct nutrients, bacteria appear to serve a mutualistic role with the protozoa by supplying essential proteins and metabolites during replication, as well as regulating *in vitro* environmental conditions (87).

Histomonosis has been produced in experimental settings with the intracloacal inoculation of infected liver, cecal tissues, or with a suspension of *in vitro* cultivated *H. meleagridis* (15, 62). Variations in host resistance, challenge dose, pathogen virulence, and frequency of exposure are some factors influencing disease severity (88). A case reproductive rate of 8.4 was estimated in a horizontal transmission study and turkeys recovered from histomonosis were shown to remain infectious for 5.7 days

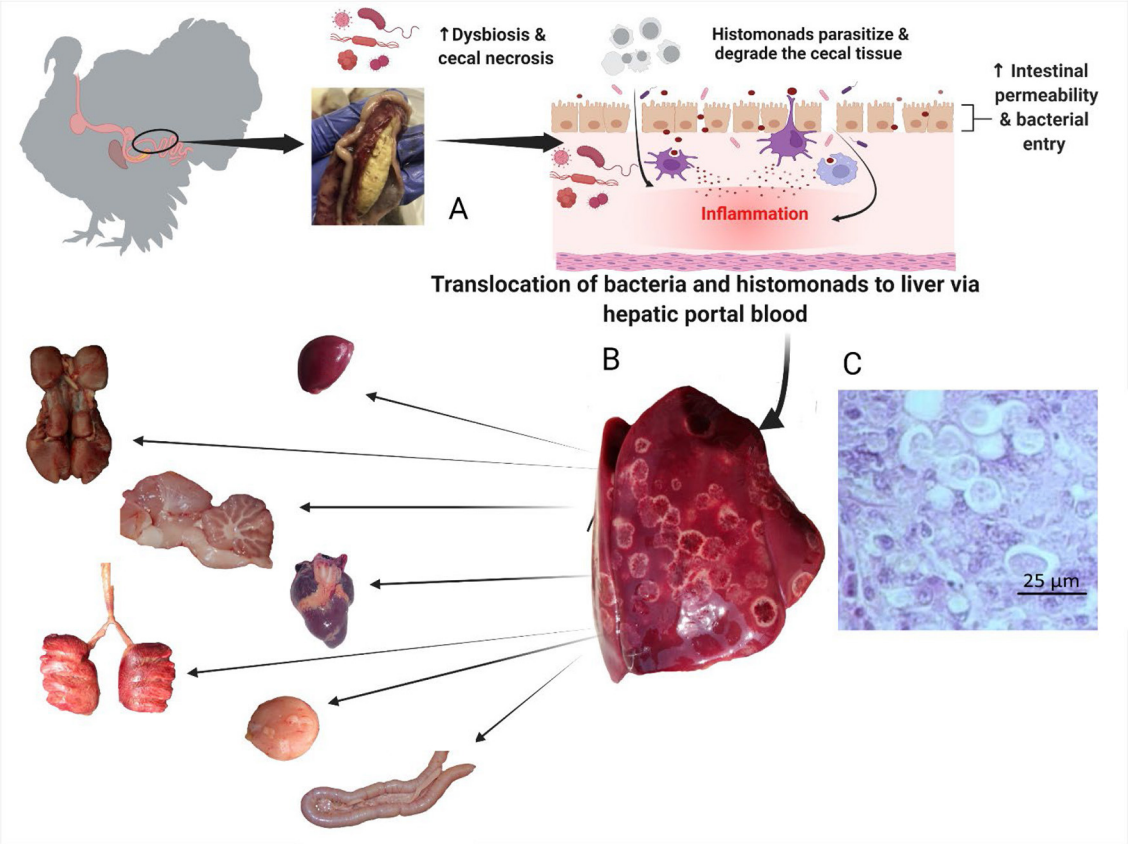

FIGURE 4 | Pathogenesis of histomonosis. The parasite induces a severe inflammatory reaction in the ceca. The inflammatory reaction is followed by necrosis, with dysbiosis causing increased permeability in the ceca **(B)**. This allows bacterial and parasitic translocation to the liver via hepatic portal blood; the resulting pathognomonic lesions are exhibited as target-like liver lesions and caseous cecal cores **(A)**. *Histomonas meleagridis* in the liver of a turkey, Periodic acid–Schiff (PAS), 40× **(C)**. From the liver, bacteria and histomonads migrate to other parenchymal organs (spleen, heart, kidneys, pancreas, lungs, brain, bursa of Fabricius) causing chronic systemic inflammation and multiple organ failure (Created with BioRender.com).

after recovery (89). A retrospective data analysis implicated an increased relative risk of male commercial turkey grow-out flocks to contracting histomonosis when located within 1 mile of a broiler breeder flock (90). Lund (74) reported a positive correlation between infective dose (10^2-10^5 histomonads/birds) and mortality; conversely, a low dose of 10 histomonads induced 100% mortality in turkeys (91). Liebhart and Hess (92) administered a virulent isolate via oral administration to 1-day-old turkeys with successful initiation of histomonosis, but the oral route of infection remains controversial. Presumably, histomonads cannot survive the low pH in the ventriculus unless protected by a vector such as *Heterakis* spp. or with a neutral to alkaline pH in the gastrointestinal tract to allow survival of the protozoa (67, 93). *H. meleagridis* has been shown to persist up to 9 h in non-chlorinated water and fecal droppings and up to 6 h on materials such as feathers and feed (94). Histomonads are fragile when shed unprotected into the environment, but not much is known about the methods for disinfection (95). Consequently, the importance of *H. meleagridis*-infected water as a possible source of involvement for cloacal transmission has been suggested as an important risk factor (94, 96). Although previously disregarded to form resistant structures, cyst-like forms have recently been described *in vitro*, but the importance of these structures to pathogenesis is not yet understood (95, 97–100).

Oral challenge with virulent histomonads on day-of-hatch has previously induced histomonosis in turkeys (31), although the oral route in absence of vectors remains somewhat controversial in older birds. Recently, challenge with wild-type *H. meleagridis* before feeding on day-of-hatch induced disease regardless of oral or cloacal route, presumably due to the near-neutral pH in the proventriculus-ventriculus region allowing the histomonads to survive and parasitize the ceca (101). Interestingly, oral challenge with virulent *H. meleagridis* at day 21 did not induce histomonosis, further suggesting that the cloacal route rather than the oral route is the primary method for transmitting unprotected histomonads in older birds; however, the oral route should not be disregarded for young birds (101).

Tyzzer (102) indicated the survival of *H. meleagridis* within heterakid ova for 2 months during winter temperatures.

FIGURE 5 | Field case of histomonosis in a layer operation. Turkeys are especially susceptible to *Histomonas meleagridis* infection, although other gallinaceous birds such as chickens, pheasants, and peafowls can be affected. In this figure, a cage layer hen in a commercial operation with multiple ages and a fly problem developed clinical signs and lesions of histomonosis without the presence of *Heterakis* spp. (Created with BioRender.com).

Heterakids can thereby serve as primary transmitters for initial introduction of disease due to infected ova withstanding environmental conditions for long durations (18, 103–105). Histomonads are released when the infected *Heterakis* spp. larvae hatch in poultry (106, 107). Lifetime fecundity of *H. gallinarum* is regulated by both inverse density and density-dependent mechanisms (108). *Alphitobius diaperinus* (darkling beetle or lesser mealworm) function as environmental contaminants for accidental introduction of *H. meleagridis* into a flock rather than serving as a primary transmitter like *Heterakis* spp. (109). The importance of *A. diaperinus* as a reservoir is uncertain due to the persistence of *H. gallinarum* and *H. meleagridis* DNA within dead beetles and litter from depopulated houses even after long periods (109, 110). *Lumbricus* spp. (earthworms) are not required for completion of the heterakid larvae or histomonad life cycles, serving rather as paratenic hosts and mechanical vectors if consumed by poultry (12, 20, 21, 111).

PHYLOGENETIC AND MOLECULAR CHARACTERIZATIONS

Indirect and blocking ELISAs have been developed for detection of *H. meleagridis* but have not yet been rigorously tested for specificity or cross-reactivity to other related protozoa commonly found in field isolates (112, 113). An indirect sandwich ELISA has been used successfully to identify *H. meleagridis* infections in pullet and layer flocks (114). Other parasites such as *Tetratrichomonas gallinarum* and *Blastocystis* spp. may be present in field outbreaks and potentially confused with *H. meleagridis* (42, 115). Polymerase chain reaction (PCR) has been successfully utilized to detect *H. meleagridis* in samples and infected birds, as well as to differentiate from *T. gallinarum* and *Blastocystis* spp. (89, 116–120). DNA presence does not necessarily indicate active infection; therefore, diagnosis of histomonosis is recommended to include microscopy to confirm presence of the protozoa (78, 110, 115). *H. meleagridis* conforms similarly to other trichomonad parasites in structure and division; close phylogenetic relationships to *D. fragilis* and *Tritrichomonas foetus* were identified based on gene sequencing analysis of β-tubulin and small subunit rRNA genes (40, 121–123). Analysis of 18S rRNA and internal transcribed spacer (ITS)-1 sequences has demonstrated a clear distinction between *H. meleagridis* isolates and other trichomonads such as *D. fragilis* (98). Genetic sequencing and phylogenetic analysis of 5.8S rRNA and the flanking ITS-1 and ITS-2 regions revealed marked genetic diversity of *H. meleagridis* isolates (33). Furthermore, combinations of data obtained from Nanopore and Illumina sequencing platforms resulted in the assembly of genome sequences exhibiting gene deletions and truncations for two phenotypically different *H. meleagridis* isolates, indicating a difference in attenuated and virulent strains (124).

Analysis of 18S rRNA, α-actinin1, and *rpb1* genetic loci revealed two different phylogenetic clusters of *H. meleagridis* isolates in Europe and further identified two genotypes; in contrast, a probed sequence and partial 18S rRNA have displayed genetic similarity of six purportedly different isolates (33, 125). Biological relevance and incidence of these two distinct genotypes have not yet been fully elucidated (19, 125); although Grafl et al. (126) described a field outbreak of male turkeys with *H. meleagridis* genotype 2 infection as having severe typhlitis with limited hepatic lesions. Using micromanipulation, clonal cultures

of *H. meleagridis* and other protozoa have been established which enable researchers to better understand pathogenicity, morphology, and genetic differences between species (75). Mono-eukaryotic cultures have also been established from mixed field samples containing *H. meleagridis, T. gallinarum*, and *Blastocystis* spp., and these monocultures could potentially better mimic field strains as opposed to clonal cultures while removing the interference of other protozoa (127). Thirty-seven unique surface and intracellular antigens were identified through analysis of a cDNA library generated from a monoculture and screened against polyclonal anti-*H. meleagridis* rabbit sera (128). A cDNA library generated from a non-clonal culture resulted in the identification of 3,425 putative genes belonging to *H. meleagridis* (84). Hydrogenosome protein-coding sequences and three different α-actinin proteins (α-actinin1, α-actinin2, α-actinin3) were identified and shown to be immunogenic to turkeys and chickens (128, 129). Humoral immune response to *H. meleagridis* α-actinin1 and α-actinin3 was higher and induced sooner in specific-pathogen-free layer-type chickens as compared to meat-type chickens (68). Shotgun proteomics has been utilized to compare virulent and attenuated mono-eukaryotic monoxenic *H. meleagridis*; cysteine proteases were the predominant lytic molecules in the virulent exoproteome as compared to the attenuated isolate (130, 131). Mazumdar et al. (132) completed a *de novo* transcriptome sequencing study utilizing single-cell cloned virulent and attenuated isolates, demonstrating different gene families. Proteomic comparisons have detected expression differences including upregulation of stress response, peptidase, and metabolic proteins in a low-passaged virulent *H. meleagridis* isolate; whereas an attenuated strain had higher expression of cellular division proteins (133, 134).

CHEMOTHERAPY AND PROPHYLAXIS

Tyzzer (135) tested several trivalent arsenicals (including arsenious acid, atoxyl, neoarsphenamine, and tryparsamide) as chemotherapeutics against histomonosis, but with inconsistent results. Pentavalent arsenicals such as nitarsone (4-nitrophenyl-arsonic acid; Histostat-50^{TM}), carbasone (4-carbamylamino-phenylarsonic acid), and roxarsone (3-nitro-phenylarsonic acid) offered fewer toxicity concerns than the trivalent compounds for poultry but also exhibited a narrower chemotherapeutic index (16, 136). Carbasone was highly effective in prevention of a field isolate of *H. meleagridis* (136). Nitroimidazole compounds (including dimetridazole, metronidazole, ornidazole, and tinidazole) were effective *in vitro* at concentrations of $\geq$10 μg/ml and *in vivo* at 200 ppm in the feed, but were toxic if overdosed (137–141). Dimetridazole was highly effective for treating histomonosis and was rapidly metabolized and eliminated by turkeys with no detectable tissue residue (<0.02 ppm) following 3-day post-administration (142). Enheptin-T (2-amino 5-nitrothiazole) was used at 0.05% in the feed with effective prophylaxis against histomonosis, but average weights of turkeys were suppressed in direct proportion to drug inclusion (143). Nithiazide [1-ethyl-3-(5-nitro-2-thiazolyl) urea] was an effective therapeutic in turkeys when administered at 3-day post-infection and was somewhat better tolerated than enheptin-T (144). Benzimidazole compounds, such as albendazole and fenbendazole, were effective *in vivo* when provided prophylactically and mechanism of action was attributed to damage of heterakid larvae or histomonads residing in the cecal lumen (145).

Research with *H. meleagridis* waned around the 1970s, partly due to effective antihistomonal compounds alleviating disease outbreak, but research increased again in the early 2000s following the removal of effective drugs and feed additives from poultry production in the European Union and the United States which resulted in a re-emergence of disease due to lack of treatment options (6, 67, 146–148). The nitroimidazoles and nitrofurans were banned in the United States in 1987 and 1991, respectively (90, 149). Nitarsone was the last-remaining prophylactic drug for the treatment of histomonosis until the voluntary removal from the US market in late 2015 because of consumer carcinogenic concerns (147, 149–151). Despite occasional success with antihistomonal candidates *in vitro,* subsequent *in vivo* evaluations have failed to conclusively prevent or treat histomonosis (150, 152–156). Boric acid, deoxycholic acid, sodium chlorate, and sodium nitrate are among just a few chemoprophylaxis candidates with antimicrobial or antifungal properties that have been recently tested with *in vitro* evaluation showing significant antihistomonal properties but with no effective prophylaxis *in vivo* (154–156). The antibacterial properties of some candidate antihistomonal compounds are known to impact effectiveness *in vitro*, but histomonads can survive 48 h after destruction of xenic bacterial populations (16, 62, 70, 157). Further complicating the problem, *H. meleagridis* isolates have varied in susceptibility to candidate compounds *in vitro* and *in vivo* (30, 62, 70, 152, 158). Drug resistance was not previously known to occur with *H. meleagridis*; however, some isolates have developed partial resistance to nitarsone and metronidazole, further emphasizing the necessity of new solutions to prevent histomonosis and supporting the likelihood of different populations of protozoa and corresponding drug susceptibility (38, 159, 160). A comparatively reliable compound to replace the previously used dimetridazole and nitarsone drugs is critically needed, but mitigation of histomonosis remains elusive and inconsistent (6, 16). Adaptations likely need to occur for concentration and administration of compounds for *in vivo* protection, but effective *in vitro* evaluation is the initial key step to determining whether to devote resources toward a live animal study (150, 161). *In vitro* methods are useful for initially evaluating candidate chemoprophylactics, but emphasis is placed on *in vivo* evaluation against more than one isolate of *H. meleagridis* before concluding effectiveness.

Paromomycin, an aminoglycoside antibiotic that inhibits protein synthesis, has been effective prophylactically against histomonosis with the target site of action identified as a small subunit rRNA (162–164). Inclusion of paromomycin in the feed at 200 and 400 ppm also reduced *Clostridium perfringens* counts in excreta while reducing *H. meleagridis*-related mortalities under experimental conditions (163). Unfortunately, paromomycin seems limited to prophylactic rather than therapeutic properties,

as three commercial turkey flocks in Canada were not successful in reducing mortalities with paromomycin sulfate treatment in the feed (165). Taken together, paromomycin sulfate should be further evaluated as a prophylactic compound for in-feed or in-water administration to prevent *H. meleagridis* infection.

In absence of approved effective drugs or vaccines for histomonosis, the prevailing measure for disease prevention is to minimize exposure to *H. meleagridis*. Worm treatment programs and flock management to prevent *H. gallinarum* and accessory hosts such as earthworms and darkling beetles will help to reduce histomonosis incidence, since histomonads cannot survive for long durations if shed unprotected directly into the environment (15, 16). Limiting exposure to mechanical vectors such as rodents, insects, or contaminated litter is critical to reducing potential contamination. Prompt removal of infected birds and utilization of migration barriers are additional control strategies to prevent rapid horizontal transmission in turkey flocks, while de-worming options would be more appropriate to control histomonosis in chickens based on the differences in bird-to-bird transmission (26, 41, 69).

PHYTOCHEMICALS FOR PREVENTION OF HISTOMONOSIS

Phytogenic compounds offer great potential as alternatives to mitigate histomonosis and improve poultry health since the exclusion of antibiotics (166). Herbal products have received much interest for antihistomonal properties, but *in vitro* results are often encouraging while *in vivo* trials yield unsuccessful protection (30, 152, 158). Protophyt^{TM} and Natustat^{TM}, plant-derived proprietary combinations of herbal extracts, were successful antihistomonal products *in vitro* but generated only limited success in field trials when provided prophylactically (30, 158, 167–169). Further complicating the search and development of antihistomonal drugs, different monoculture strains of *H. meleagridis* have exhibited varied susceptibilities to natural organic compounds (70). Two proprietary blends of plant extract products containing unspecified amounts of *Capsicum* essential oils exhibited antihistomonal and antibacterial effects after only 48 h *in vitro*; furthermore, mode of action was suggested as cell membrane disruption directly on the histomonads rather than attributed to indirect effects of antibacterial reduction, but *in vivo* studies have not yet been conducted (170). Recently, a dietary supplement (adiCox^{SOL}PF) comprised of a proprietary mixture of herbal extracts was effective prophylactically and therapeutically against histomonosis in a turkey breeder flock (171). With increasing demand for organic-raised poultry, naturally derived plant compounds offer a certain attraction as they could potentially be utilized in both organic and traditional production facilities. Plant-based compounds are often relatively cheap to produce, leading to a greater likelihood for industry application (161).

Quinine, an alkaloid obtained from *Cinchona* tree bark, has been successfully utilized to combat malaria (172). Early researchers postulated its potential for treating histomonosis; however, researchers hypothesized that an antihistomonal compound would have to be active more than just locally within the intestines because *H. meleagridis* embeds within the cecal lining and migrates to hepatic tissue (2, 103). Tyzzer (135) observed no reduction in histomonosis following injection of unspecified levels of quinine into the veins or muscles of turkeys. Delaplane and Stuart (173) reported quinine sulfate to be ineffective against *H. meleagridis* infection but did not specify the dose or route of administration. Farmer (174) injected 0.1 ml of 10% quinine iodobismuthate with no apparent protection against histomonosis. Tyzzer and Fabyan (103) suggested that a possible reason for the failure of compounds utilized in human amebic infections to protect poultry from histomonosis could be due to histomonads exhibiting a predominantly flagellated form rather than solely an amoebic form, leading to some products being amebicidal but not antihistomonal. Ensuring delivery of chemoprophylactic candidates directly to the ceca is a challenge, and quinine, although recently shown to be an effective antihistomonal *in vitro*, may not have reached the ceca in sufficient concentration to impair the protozoa when evaluated *in vivo* (175). Previously, chickens recognized the bitter taste of quinine and reduced feed intake of diets containing more than 0.2% quinine, but threshold levels have not been established for turkeys (176). A 0.2% dietary inclusion of quinine was hypothesized to be maximum for turkeys as well; however, the days 0–10 body weight gain in the quinine diets was not different ($p > 0.05$) as compared to the basal diet (175). Turkeys may perceive the bitter taste of quinine differently from chickens and subsequently have higher threshold levels than 0.2%, but the impact to performance at higher inclusion levels is unknown. Other antimalarial compounds such as the herb *Artemisia annua* and plant extracts have been tested against *H. meleagridis* with limited success *in vitro* but no protection was transferred to birds when tested *in vivo* (152, 170).

IMMUNE RESPONSE TO *H. meleagridis* INFECTION

Turkeys and chickens recovered from *H. meleagridis* infection have shown a degree of natural resistance, although both species may retain histomonads sub-clinically and thereby serve as carriers (5, 177). Joyner (178) administered 0.05% dimetridazole in the water to *H. meleagridis*-infected turkeys, and the recovered turkeys were resistant to re-infection which suggested a level of acquired immunity. Protective immunity was observed in birds that recovered from histomonosis and were then subsequently re-infected with *H. meleagridis*, but further attempts with immunization have been inconsistent (18, 102, 103, 177, 179, 180). Sera recovered from immune birds failed to confer robust protection to histomonosis when injected into the peritoneum of naïve poultry that were subsequently challenged intracloacally with *H. meleagridis*-infected liver homogenate (180–182). Passive immunity (via peritoneal injection of antisera) or active immunity (via intramuscular or intraperitoneal injection of lysed clonal *H. meleagridis*) failed to protect against wild-type challenge (183, 184). Turkeys surviving *H. meleagridis* infection have exhibited resistance to re-infection while still maintaining

populations of the protozoa within the ceca (182). Humoral immunity does not seem to be the primary component of protective immunity to histomonosis, although antibodies may work in combination with local immunity initiated by leukocytes in the ceca (182).

Clarkson (181) reported that turkeys exhibited decreased albumin and elevated globulin concentrations at 12-day post-infection as compared to the non-challenged controls. Similarly, albumin concentrations greatly decreased by 9-day post-infection in chickens subjected to *H. meleagridis* infection, with normal levels of albumin and globulin fractions restored by 12-day post-infection, suggesting disease recovery (185). The immune barrier in purportedly histomonosis-resistant chickens was suggested to be limited to cecal epithelial tissue as *H. gallinarum* could disrupt and overcome any developed immunity (74). Natural and experimental *H. meleagridis* infection produced antibodies in both chickens and turkeys but transfer of antibodies to naïve birds did not successfully confer protection (180, 184). Subsequently, Clarkson (180) suggested that antibody production alone was not a good indicator of histomonosis recovery or immunity to re-infection. Antibody titers of passively immunized birds were increased compared to pre-immunized groups; however, no protection was induced against intracloacal infection with 3×10^5 *H. meleagridis*, possibly due to the experimental challenge dose not accurately mimicking a natural challenge, antibody levels lower than needed for protection, or more likely, serum antibodies not primarily responsible for protection against *H. meleagridis* infection (184). Immunoglobulin A (IgA) levels have been shown to increase throughout the intestine, while immunoglobulin G (IgG) levels particularly increased in the ceca following infection with an established clonal *H. meleagridis* isolate (186).

Heterophils begin to accumulate around histomonads following initial infection, but the protozoa secrete tissue-degrading enzymes to phagocytose leukocytes (44). Total numbers of heterophils increase throughout the body as *H. meleagridis* migrates to parasitize other tissues; other leukocytes involved include macrophages, giant cells, and plasma cells (44, 64, 119, 187). Once the histomonads invade the cecal submucosa or enter the portal blood, degenerating *H. meleagridis* can be observed within the gut-associated lymphoid tissue (44). Plasma levels of glutamic oxaloacetic transaminase can indicate cellular damage and this enzyme can increase in turkeys with liver and cecal damage from histomonosis (28, 29). CD4+ and CD8α+ T cells have been implicated in the immune response to histomonosis (188–190). Recently, populations of CD4+, CD8α+, and non-CD4+CD8α+ T cells in the liver and spleen of turkeys were induced following administration of attenuated *H. meleagridis* as a putative vaccine and subsequent virulent infection (191). Comparative study of chickens and turkeys indicated that vaccination with a monoxenic, clonal culture of live-attenuated *H. meleagridis* resulted in higher systemic immune response in turkeys as compared to chickens, with increased levels of interferon (IFN)-γ producing CD4+ T cells confirmed in the spleens of infected chickens as compared to turkeys (191). Increased T-helper cell type-1 (Th1) and type-2 (Th2) cytokine responses of IFN-γ and IL-13 occurred in chickens which were co-infected with *H. gallinarum* and *H. meleagridis* (192). Chickens developed a stronger pro-inflammatory innate immune response than turkeys, along with higher antibody levels, with specific increase in the Th2 response in cecal and liver tissues to mitigate infection (188). Despite the extracellular nature of *H. meleagridis* which would be expected to stimulate differentiation of Th2 cells, immune response to this pathogen was suggested to be dominated by Th1 rather than Th2 cells (190–193). Turkeys appeared to have a delayed and uncontrolled immune response as compared to chickens when infected with *H. meleagridis,* allowing greater tissue destruction and ultimately higher mortality in turkeys (194).

ATTEMPTED VACCINATION WITH ATTENUATED ISOLATES

Tyzzer (102) evaluated avirulent field strains of *H. meleagridis* for immunization against histomonosis, but inoculation of turkeys was required at a young age and constant re-infection was necessary to maintain a level of effective protection. Partial protection was conferred with an attenuated isolate against subsequent cloacal challenge with a virulent isolate; however, administration of histomonads as an immunization incorporated into *Heterakis* spp. ova and likewise challenged did not satisfactorily confer protection (195, 196). The resulting conclusion was that the low-virulent histomonads were not introduced in sufficient numbers via heterakid ova to successfully initiate immune response to protect against virulent challenge (195, 196). Tyzzer (102, 179) reported attenuation of *H. meleagridis* following repeated passage *in vitro* but attempts with immunization did not produce consistent protection. An isolate repeatedly passaged *in vitro* for 6 years resulted in loss of immunizing ability to chickens and turkeys (196). Further study observed a steady decline of immunizing ability of attenuated histomonads after 730, 766, and 1,000 passages *in vitro* (197). Specifically, passage 1,000 was non-pathogenic and had lost nearly all ability to confer protection to either chickens or turkeys against virulent challenge (197).

Long-term serial passaging *in vitro* places selective pressures on *H. meleagridis* and co-cultured bacterial populations. Freshly obtained field samples of histomonads could not grow in the limited bacterial populations of attenuated culture media; similarly, the attenuated protozoa were unable to survive with the field isolates of cecal bacteria (196). Importantly, *in vitro* attenuation of *H. meleagridis* occurred independently of bacterial populations in culture media (60). *In vitro* growth of *H. meleagridis* Hm-L1 strain at 41.5°C for 9 weeks resulted in low pathogenicity while histomonads stored in liquid nitrogen maintained their original virulence (198, 199). Serial *in vivo* passaging of the Hm-L1 attenuated strain from chicken-to-chicken or turkey-to-turkey restored the strain to original virulence (198, 199). Differences in virulence have been found within *H. meleagridis* isolates obtained from different geographical locations, in addition

to varied loss of pathogenicity following repeated passaging (131). Furthermore, subpopulations of serially passaged monocultures originating from the same parental isolate have shown a marked difference in virulence, supporting the idea of genetic mutation through repeated serial passaging *in vitro* (131). Long-term passaging *in vitro* (>290 serial passages) resulted in a phenotype shift toward greater tenacity of histomonad survival at lower temperatures and improved growth rates (37). Gross lesion scoring and histology samples have demonstrated the lowered pathogenicity and reduced ability of attenuated isolates to invade host tissues (200). After 295 serial passages *in vitro*, an avirulent strain of *H. meleagridis* parasitized only the cecal region with no translocation to other tissues in chickens or turkeys, while a virulent strain could be identified in cecal, hepatic, and lung tissues (200).

Vaccination attempts for histomonosis have yielded some success in controlled experimental conditions, but a histomonosis vaccine has not yet been developed for commercial application (91, 101, 164, 183, 194, 201–203). A clonal *in vitro* attenuated strain of *H. meleagridis* administered cloacally as a vaccine at day 14 protected turkeys which were subsequently challenged on day 42 with a virulent strain; in-contact turkeys from the vaccination were also resistant to subsequent infection (183). Furthermore, birds which were administered an attenuated clonal strain as a vaccine were negative for *H. meleagridis* DNA in the liver (183). Oral administration of *in vitro* attenuated *H. meleagridis* to turkeys at day-of-hatch has protected against subsequent wild-type challenge with no adverse effects to performance data during the vaccination phase; the oral route would be a preferable administration route for the poultry industry (91). Under experimental conditions, vaccination of layer chickens with attenuated histomonads prevented a drop in egg production upon virulent challenge and pathological histomonosis lesions were also reduced (203). *In vivo* serial passaging five times in chickens and turkeys did not revert virulence to an *in vitro* attenuated strain, demonstrating stability and safety of attenuated histomonads as vaccine candidates (204). An attenuated clonal strain (passage 295) induced cross-protective immunity in turkeys against subsequent challenge with heterologous virulent isolates; however, vaccination occurred at 1 day of age and a booster vaccination occurred at day 14, with challenge administration at 6 weeks of age (205). Repeated intracloacal passaging of *H. meleagridis* in turkeys produced an isolate of low virulence which was successfully used to induce protection against a virulent strain (206). Candidate vaccination isolates have been shown distinctly attenuated as indicated by lowered mortalities ($p < 0.05$), lowered lesion scores ($p < 0.05$), and similar body weight gain (BWG) ($p > 0.05$) as the non-challenged controls during vaccination phases (101). This information is consistent with previous research indicating attenuation of *H. meleagridis* following repeated *in vitro* passage (102, 131, 179, 196). Importantly, administration of non-clonal vaccination isolates on day 14 has conferred protection against challenge with homologous and heterologous virulent isolates; moreover, these conditions potentially better portrayed the field environment where turkeys are exposed to multiple isolates (101). More research remains necessary for histomonosis vaccine development and to elucidate practical methods for industry application.

FINAL REMARKS

Biosecurity measures to prevent exposure to *H. meleagridis* or vectors of this protozoa are important to reduce histomonosis incidence due to the absence of vaccines or approved drugs. Proper management practices are critical to reducing disease incidence, as birds experimentally reared in a non-challenged environment do not contract histomonosis. Although separate rearing of poultry (e.g., turkeys raised separately from chickens) can reduce disease incidence by limiting contact between asymptomatic carriers and susceptible hosts, an effective prophylactic or vaccination program is still greatly needed. Pairing *in vitro* and *in vivo* experiments is necessary to ensure effectiveness of candidate antihistomonal compounds.

Despite immunological research advancements, a histomonosis vaccine has not been developed for commercial application (164, 194, 202). Clonal *in vitro* attenuated histomonads have been administered orally or cloacally with efficacious protection in experimental settings against virulent challenge without negative performance impacts; however, evaluations have not yet occurred in field conditions against heterologous, multi-isolate challenges (91, 183, 203). Day-of-hatch oral vaccination with live-attenuated histomonads was previously reported as effective, but a booster vaccination was recommended at day 14 for established protection (91, 205). Recent vaccination experiments demonstrated that day-of-hatch administration of attenuated isolates either orally or cloacally did not protect turkeys against subsequent wild-type challenge (101), contrary to previously reported success with oral vaccination at this age (91, 205). Unfortunately, utilizing live histomonads would be difficult for industry application due to the required intracloacal administration, as well as the additional concerns of attenuation stability and inconsistent protective immunity (6, 9). In practicality, the administration of live-attenuated histomonads on a commercial scale with the current methodologies seems unlikely due to the high cost of cell culture propagation and application complexities, although the benefit to further develop a histomonosis vaccine would be tremendous (5, 202).

The overall review of literature reflects the difficulties in mitigating histomonosis, especially in recent years. Dietary inclusion of antihistomonal compounds such as quinine alone was not encouraging for prevention of *H. meleagridis* infection in turkeys, but vaccination appeared somewhat efficacious when live-attenuated histomonads were administered at day 14 via the cloacal route. Unfortunately, the protection against subsequent wild-type challenge of vaccinated turkeys was neither consistent nor robust throughout the literature. Further research should be conducted with phytochemicals as these compounds may offer a natural remedy for histomonosis that could be both economical for the industry and acceptable to the consumer.

Vaccination should be pursued further, especially to elucidate the administration route, dose, and age of bird. Taken together, this information is encouraging for immunity to histomonosis, but the administration of a vaccine and possible requirement for booster vaccination with the live-attenuated method is more experimentally interesting rather than industry applicable.

AUTHOR CONTRIBUTIONS

LB and CV developed the conceptualization and wrote the first draft of the manuscript. LB, VP-G, and GT-I conceptualized and created the figures. LB, GT-I, BG, BH, and CV participated in the design, analysis, presentation, and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Cushman S. *The Production of Turkeys*. Kingston, RI: Agricultural Experiment Station, Rhode Island College of Agriculture and Mechanical Arts (1893).
2. Smith T. An infectious disease among turkeys caused by protozoa (infectious entero-hepatitis). *USDA Bur Anim Ind Bull.* (1895) 8:3–27.
3. Tyzzer EE. The flagellate character and reclassification of the parasite producing "Blackhead" in turkeys: Histomonas (gen. nov.) meleagridis (Smith). *J Parasitol.* (1920) 6:124–31. doi: 10.2307/3271065
4. Farmer RK, Hughes DL, Whiting G. Infectious enterohepatitis (blackhead) in turkeys: a study of the pathology of the artificially induced disease. *J Comp Path.* (1951) 61:251–62. doi: 10.1016/S0368-1742(51)80025-0
5. Joyner LP, Davies SFM, Kendall SB. Chemotherapy of histomoniasis. In: *Experimental Chemotherapy, Vol. 1*. New York, NY: Academic Press (1963). doi: 10.1016/B978-1-4832-3178-5.50013-7
6. Hess M, Liebhart D, Bilic I, Ganas P. *Histomonas meleagridis*—New insights into an old pathogen. *Vet. Parasitol.* (2015) 208:67–76. doi: 10.1016/j.vetpar.2014.12.018
7. Clarke LL, Beckstead RB, Hayes JR, Rissi DR. Pathologic and molecular characterization of histomoniasis in peafowl (*Pavo cristatus*). *J Vet Diagn Invest.* (2017) 29:237–41. doi: 10.1177/1040638716687002
8. Davidson WR, Doster GL. *Blackhead Disease Does Not Really Cause Black Heads*. Edgefield, SC: National Wild Turkey Federation (1994).
9. Hess M, McDougald L. Histomoniasis (blackhead) and other protozoan diseases of the intestinal tract. In: Glisson JR, McDougald LR, Nolan LK, Suarez DL, Nair VL, Swayne E, editors. *Diseases of Poultry. 13th edn.* Ames, IA: Wiley-Blackwell (2013).
10. Kassai T, Cordero del Campillo M, Euzeby J, Gaafar S, Hiepe T, Himonas CA. Standardized nomenclature of animal parasitic diseases (SNOAPAD). *Vet Parasitol.* (1988) 29:299–326. doi: 10.1016/0304-4017(88)90148-3
11. Lund EE, Chute AM. Heterakis and histomonas infections in young peafowl, compared to such infections in pheasants, chickens, and turkeys. *J Wildl Dis.* (1972) 8:352–8. doi: 10.7589/0090-3558-8.4.352
12. Friend M, Franson JC, Ciganovich EA. *Field Manual of Wildlife Diseases: General Field Procedures and Diseases of Birds*. Madison, WI: USGS (1999).
13. Clark SR, Froebel L. *Turkey Industry Annual Report—Current Health and Industry Issues Facing the US Turkey Industry*. (2020). Retrieved from https://huvepharma.com/news/article/2020-turkey-industry-annual-report-current-health-and-industry-issues-facing-the-us-turkey-industry/
14. Graybill HW, Smith T. Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*. *J Exp Med.* (1920) 31:647–55. doi: 10.1084/jem.31.5.647
15. Tyzzer EE, Collier J. Induced and natural transmission of blackhead in the absence of Heterakis. *J Infect Dis.* (1925) 37:265–76. doi: 10.1093/infdis/37.3.265
16. McDougald LR. Blackhead disease (histomoniasis) in poultry: a critical review. *Avian Dis.* (2005) 49:462–76. doi: 10.1637/7420-081005R.1
17. Curtice C. *Further Experiments in Connection With the Blackhead Disease of Turkeys.* Agricultural Experiment Station of the Rhode Island College of Agriculture and Mechanic Arts (1907).
18. Tyzzer EE. Problems and observations concerning the transmission of blackhead infection in turkeys. *Proc Am Philos Soc.* (1932) 71:407–10.
19. Popp C, Hauck R, Balczulat S, Hafez HM. Recurring histomonosis on an organic farm. *Avian Dis.* (2011) 55:328–30. doi: 10.1637/9596-110810-Case.1
20. Cupo KL, Beckstead RB. Heterakis gallinarum, the cecal nematode of gallinaceous birds: a critical review. *Avian Dis.* (2019) 63:381–8. doi: 10.1637/0005-2086-63.3.381
21. Cupo KL, Beckstead RB. PCR detection of heterakis gallinarum in environmental samples. *Vet Parasitol.* (2019) 271:1–6. doi: 10.1016/j.vetpar.2019.05.011
22. Sorvari R, Naukkarinen A, Sorvari TE. Anal sucking-like movements in the chicken and chick embryo followed by the transportation of environmental material to the bursa of fabricius, caeca, caecal tonsils. *Poult Sci.* (1977) 56:1426–9. doi: 10.3382/ps.0561426
23. Hu J, Fuller L, McDougald LR. Infection of turkeys with *Histomonas meleagridis* by the cloacal drop method. *Avian Dis.* (2004) 48:746–50. doi: 10.1637/7152
24. McDougald LR, Fuller L. Blackhead disease in turkeys: direct transmission of *Histomonas meleagridis* from bird to bird in a laboratory model. *Avian Dis.* (2005) 49:328–31. doi: 10.1637/7257-081004R.1
25. Scanes CG, Pierzchala-Koziec K. Biology of the gastro-intestinal tract in poultry. *Avian Biol Res.* (2014) 7:193–222. doi: 10.3184/175815514X14162292284822
26. Hu J, McDougald LR. Direct lateral transmission of *Histomonas meleagridis* in turkeys. *Avian Dis.* (2003) 47:489–92. doi: 10.1637/0005-2086(2003)047<0489:DLTOHM>2.0.CO;2
27. Armstrong PL, McDougald LR. The infection of turkey poults with *Histomonas meleagridis* by contact with infected birds or contaminated cages. *Avian Dis.* (2011) 55:48–50. doi: 10.1637/9568-100710-Reg.1
28. Al-Khateeb GH, Hansen MF. Plasma glutamic oxalacetic transaminase as related to liver lesions from histomoniasis in turkeys. *Avian Dis.* (1973) 17:269–73. doi: 10.2307/1589208
29. Al-Khateeb GH, Hansen MF. Plasma enzymes as a measure of susceptibility of chickens and turkeys to infection with *Histomonas meleagridis*. *Avian Dis.* (1974) 18:507–14. doi: 10.2307/1589006
30. van der Heijden HMJF, Landman WJM. *In vivo* effect of herbal products against *Histomonas meleagridis* in turkeys. *Avian Pathol.* (2008) 37:45–50. doi: 10.1080/03079450701784883
31. Liebhart D, Grabensteiner E, Hess M. A virulent mono-eukaryotic culture of *Histomonas meleagridis* is capable of inducing fatal histomonosis in different aged turkeys of both sexes, regardless of the infective dose. *Avian Dis.* (2008) 52:168–72. doi: 10.1637/8107-090707-ResNote
32. Cepicka I, Hampl V, Kulda J. Critical taxonomic revision of parabasalids with description of one new genus and three new species. *Protistology.* (2010) 161:400–33. doi: 10.1016/j.protis.2009.11.005
33. Lollis L, Gerhold R, McDougald L, Beckstead R. Molecular characterization of *Histomonas meleagridis* and other parabasalids in the United States

using the 5.8S, ITS-1, and ITS-2 rRNA regions. *J Parasitol.* (2011) 97:610–5. doi: 10.1645/GE-2648.1
34. Bayon HP, Bishop A. Cultivation of *Histomonas meleagridis* from the liver lesions of a hen. *Nature.* (1937) 139:370–1. doi: 10.1038/139370b0
35. Bishop A. *Histomonas meleagridis* in domestic fowls (Gallus gallus). Cultivation and experimental infection. *Parasitology.* (1938) 30:181–94. doi: 10.1017/S0031182000025749
36. Hess M, McDougald LR. Histomoniasis (histomonoasis, blackhead disease). In: Swayne DE. Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL, editors. *Diseases of Poultry*. 14th edn. Ames, IA: Wiley-Blackwell (2020). p. 1223–30.
37. Gruber J, Ganas P, Hess M. Long-term *in vitro* cultivation of *Histomonas meleagridis* coincides with the dominance of a very distinct phenotype of the parasite exhibiting increased tenacity and improved cell yields. *Parasitology.* (2017) 144:1253–63. doi: 10.1017/S0031182017000646
38. Long PL, Current WL, Noble GP. Parasites of the christmas turkey. *Parasitol Today.* (1987) 3:360–6. doi: 10.1016/0169-4758(87)90241-9
39. Lund EE. Histomonas wenrichi n. sp. (*Madtigophora: Mastigamoebidae*), a nonpathogenic parasite of gallinaceous birds. *J Protzool.* (1963) 10:401–4. doi: 10.1111/j.1550-7408.1963.tb01696.x
40. Honigberg BM, Bennett CJ. Lightmicroscopic observations on structure and division of *Histomonas-meleagridis* (Smith). *J Protozool.* (1971) 18:687–97. doi: 10.1111/j.1550-7408.1971.tb03398.x
41. Fine PEM. Quantitative studies on the transmission of *Parahistomonas wenrichi* by ova of *Heterakis gallinarum*. *Parasitology.* (1975) 70:407–17. doi: 10.1017/S0031182000052185
42. Tyzzer EE. Developmental phases of the protozoon of "blackhead" in turkeys. *J Med Res.* (1919) 40:1–30.
43. Mielewczik M, Mehlhorn H, Al-Quraishy S, Grabensteiner E, Hess M. Transmission electron microscopic studies of stages of *Histomonas meleagridis* from clonal cultures. *Parasitol Res.* (2008) 103:745–50. doi: 10.1007/s00436-008-1009-1
44. Bleyen N, Mast J, De Gussem K, De Guseem J, De Gussem M, Godderis BM. *Histomonas meleagridis:* a new focus on a re-emerging protozoan parasite. In: LaMann GV, editor. *Veterinary Parasitology*. New York, NY: Nova Science Publishers (2010). p. 1–47.
45. Lesser E. Cultivation of *Histomonas meleagridis* in a modified tissue culture medium. *J Parasitol.* (1960) 6:686. doi: 10.2307/3275512
46. Hauck R, Armstrong PL, McDougald LR. *Histomonas meleagridis* (*Protozoa: Trichomonadidae*): analysis of growth requirements *in vitro. J Parasitol.* (2010) 96:1–7. doi: 10.1645/GE-1985.1
47. van der Heijden HMJF, Landman WJM. Improved culture of *Histomonas meleagridis* in a modification of dwyer medium. *Avian Dis.* (2007) 51:986–88. doi: 10.1637/8018-051007-REVIEWR.1
48. van der Heijden HMJF, McDougald LR, Landman WJM. High yield of parasites and prolonged *in vitro* culture of *Histomonas meleagridis*. *Avian Pathol.* (2005) 34:505–8. doi: 10.1080/03079450500368474
49. Gruber J, Pletzer A, Hess M. Cholesterol supplementation improves growth rates of *Histomonas meleagridis in vitro*. *Exp Parasitol.* (2018) 185:53–61. doi: 10.1016/j.exppara.2018.01.007
50. Chute MB, Chute AM. Freeze preservation of *Histomonas meleagridis*. *Poult Sci.* (1969) 48:2189–91. doi: 10.3382/ps.0482189
51. Honigberg BM, Dwyer DM. Maintenance of *Histomonas meleagridis* in liquid nitrogen. *J Protozool.* (1969) 16:18–9.
52. Gerhold RW, Lollis LA, Beckstead RB, McDougald LR. Establishment of culture conditions for survival of *Histomonas meleagridis* in transit. *Avian Dis.* (2010) 54:948–50. doi: 10.1637/9222-122209-ResNote.1
53. McDougald LR, Galloway RB. Blackhead disease: *in vitro* isolation of *Histomonas meleagridis* as a potentially useful diagnostic aid. *Avian Dis.* (1973) 17:847–50. doi: 10.2307/1589053
54. Hirsch DS. *Attempts at Axenic Cultivation of Histomonas meleagridis*. (M.S. Thesis), Kansas State University, Manhattan, KS (1975).
55. Lesser E. Replacement of serum in the cultivation of *Histomonas meleagridis*. *J Parasitol.* (1960) 46:271. doi: 10.2307/3275188
56. Lesser E. *In vitro* cultivation of *Histomonas meleagridis* free of demonstrable bacteria. *J Protozool.* (1961) 8:228–30. doi: 10.1111/j.1550-7408.1961.tb01208.x
57. Lesser E. Effect of amphotericin-B on *in vitro* growth of *Histomonas meleagridis*. *J Parasitol.* (1963) 49:329. doi: 10.2307/3276007
58. Lesser E. Studies on the *in vitro* growth of *Histomonas meleagridis* with three different mixed bacterial flora. *Proc Helm Soc Wash.* (1964) 31:263–4.
59. Lesser E. Studies on the *in vitro* growth of *Histomonas meleagridis* with single species of bacteria. *Proc Helm Soc Wash.* (1964) 31:365–6.
60. Ganas P, Liebhart D, Glösmann M, Hess C, Hess M. Escherichia coli strongly supports the growth of *Histomonas meleagridis*, in a monoxenic culture, without influence on its pathogenicity. *Int J Parasitol.* (2012) 42:893–901. doi: 10.1016/j.ijpara.2012.07.007
61. Harrison AP Jr, Hansen PA. The bacterial flora of the cecal feces of healthy turkeys. *J Bacteriol.* (1950) 59:197–210. doi: 10.1128/jb.59.2.197-210.1950
62. Berks G, Neal R. The effect of some drugs upon *Histomonas meleagridis in vitro*. *Ann Trop Med Parasitol.* (1952) 46:68–71. doi: 10.1080/00034983.1952.11685507
63. Cortes PL, Chin RP, Bland MC, Crespo R, Shivaprasad HL. Histomoniasis in the bursa of fabricius of chickens. *Avian Dis.* (2004) 48:711–5. doi: 10.1637/7175-030404R
64. Levine PP. Histomoniasis in a kidney of a turkey. *Cornell Vet.* (1947) 37:269–70.
65. Singh A, Weissenbock H, Hess M. *Histomonas meleagridis*: immunohistochemical localization of parasitic cells in formalin-fixed, paraffin-embedded tissue sections of experimentally infected turkeys demonstrates the wide spread of the parasite in its host. *Exp Parasitol.* (2008) 118:505–13. doi: 10.1016/j.exppara.2007.11.004
66. Sentíes-Cué G, Chin R, Shivaprasad HL. Systemic histomoniasis associated with high mortality and unusual lesions in the bursa of fabricius, kidneys, and lungs in commercial turkeys. *Avian Dis.* (2009) 53:231–8. doi: 10.1637/8363-051508-Reg.1
67. Spencer T. *Parasite Management for Natural and Organic Poultry: Blackhead in Turkeys*. ATTRA (2010). Available online at: https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.676.4349&rep=rep1&type=pdf
68. Lotfi A, Hauck R, Olias P, Hafez HM. Pathogenesis of histomonosis in experimentally infected specific-pathogen-free (SPF) layer-type chickens and SPF meat-type chickens. *Avian Dis.* (2014) 58:427–32. doi: 10.1637/10782-012814-Reg.1
69. Hu J, Fuller L, Armstrong PL, McDougald LR. *Histomonas meleagridis* in chickens: attempted transmission in the absence of vectors. *Avian Dis.* (2006) 50:277–9. doi: 10.1637/7431-090605R.1
70. Grabensteiner E, Arshad N, Hess M. Differences in the *in vitro* susceptibility of mono-eukaryotic cultures of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and *Blastocystis* sp. to natural organic compounds. *Parasitol Res.* (2007) 101:193–9. doi: 10.1007/s00436-007-0459-1
71. Chute AM, Lund EE, Wilkins GC. Comparative responses of white leghorn and new hampshire chickens to experimental infections with *Histomonas meleagridis* and *Heterakis gallinarum*. *Poult Sci.* (1976) 55:710–3. doi: 10.3382/ps.0550710
72. Lotfi A, Hafez HM. Susceptibility of different turkey lines to *Histomonas meleagridis* after experimental infection. *Parasitol Res.* (2009) 105:113–6. doi: 10.1007/s00436-009-1369-1
73. Metzler-Zebeli BU, Magowan E, Hollmann M, Ball MEE, Molnár A, Witter K, et al. Differences in intestinal size, structure, and function contributing to feed efficiency in broiler chickens reared at geographically distant locations. *Poult Sci.* (2018) 97:578–91. doi: 10.3382/ps/pex332
74. Lund EE. The progress of histomoniasis (blackhead) in turkeys as related to the size of the infective dose. *Poult Sci.* (1955) 34:127–30. doi: 10.3382/ps.0340127
75. Hess M, Kolbe T, Grabensteiner E, Prosl H. Clonal cultures of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and a *Blastocystis* sp. established through micromanipulation. *Parasitology.* (2006) 133:547–54. doi: 10.1017/S0031182006000758
76. Dolka B, Zbikowski A, Dolka Szeleszczuk IP. Histomonosis – an existing problem in chicken flocks in Poland. *Vet Res Commun.* (2015) 39:189–95. doi: 10.1007/s11259-015-9637-2
77. Nguyen DT, Bilic I, Jaskulska B, Hess M, Le DQ, Hua LNL, et al. Prevalence and genetic characterization of *Histomonas meleagridis* in chickens in Vietnam. *Avian Dis.* (2015) 59:309–14. doi: 10.1637/10964-102414-Reg

78. Daş G, Wachter L, Stehr M, Bilic I, Grafl B, Wernsdorf P, et al. Excretion of *Histomonas meleagridis* following experimentall co-infection of distinct chicken lines with *Heterakis gallinarum* and *Ascaridia galli*. *Parasit Vect.* (2021) 14:1–15. doi: 10.1186/s13071-021-04823-1
79. McDougald LR, Hu J. Blackhead disease (*Histomonas meleagridis*) aggravated in broiler chickens by concurrent infection with cecal coccidiosis (Eimeria tenella). *Avian Dis.* (2001) 45:307–12. doi: 10.2307/1592969
80. Chadwick E, Malheiros R, Oviedo E, Noboa HAC, Ospina GAQ, Wisaquillo MCA, et al. Early infection with *Histomonas meleagridis* has limited effects on broiler breeder hens' growth and egg production and quality. *Poult Sci.* (2020) 99:4242–8. doi: 10.1016/j.psj.2020.05.020
81. Bradley RE, Reid WM. *Histomonas meleagridis* and several bacteria as agents of infectious enterohepatitis in gnotobiotic turkeys. *Exp Parasitol.* (1966) 19:91–101. doi: 10.1016/0014-4894(66)90057-9
82. Springer WT, Johnson J, Reid WM. Histomoniasis in gnotobiotic chickens and turkeys: biological aspects of the role of bacteria in the etiology. *Exp Parasitol.* (1970) 28:383–92. doi: 10.1016/0014-4894(70)90106-2
83. Ganapathy K, Salamat MH, Lee CC, Johara MY. Concurrent occurrence of salmonellosis, colibaccillosis and histomoniasis in a broiler flock fed with antibiotic-free commercial feed. *Avian Pathol.* (2000) 29:639–42. doi: 10.1080/03079450020016000
84. Klodnicki ME, McDougald LR, Beckstead RB. A genomic analysis of *Histomonas meleagridis* through sequencing of a cDNA library. *J Parasitol.* (2013) 99:264–9. doi: 10.1645/GE-3256.1
85. Abdelhamid KM, Rychlik I, Hess C, Hatfaludi T, Crhanova M, Karasova D, et al. Typhlitis induced by *Histomonas meleagridis* affects relative but not the absolute *Escherechia coli* counts and invasion in the gut in turkeys. *Vet Res.* (2021) 52:1–12. doi: 10.1186/s13567-021-00962-6
86. Abdelhamid MK, Quijada NM, Dzieciol M, Hatfaludi T, Bilic I, Selberherr E, et al. Co-infection of chicken layers with *Histomonas meleagridis* and avian pathogenic *Escherichia coli* is associated with dysbiosis, cecal colonization and translocation of the bacteria from the gut lumen. *Front Microbiol.* (2020) 11:586437. doi: 10.3389/fmicb.2020.586437
87. Bilic I, Hess M. Interplay between *Histomonas meleagridis* and bacteria: mutualistic or predator-prey? *Trends Parasitol.* (2020) 36:232–5. doi: 10.1016/j.pt.2019.12.015
88. Collett SR. Principles of disease prevention, diagnosis, and control. In: Swayne E, Glisson JR, McDougald LR, Nolan LK, Suarez DL, Nair VL, editors. *Diseases of Poultry*. 13th ed. Ames, IA: Wiley-Blackwell (2013). p. 3–60.
89. Landman WJM, ter Veen C, van der Heijden HMJF, Klinkenberg D. Quantification of parasite shedding and horizontal transmission parameters in *Histomonas meleagridis*-infected turkeys determined by real-time quantitative PCR. *Avian Pathol.* (2015) 44:358–65. doi: 10.1080/03079457.2015.1058483
90. Jones RE, Rives DV, Fletcher OJ, Martin MP. Histomoniasis outbreaks in commercial turkeys in the southeastern United States: proximity of broiler breeder farms as a potential risk factor in disease development. *J Appl Poult Res.* (2020) 29:496–501. doi: 10.1016/j.japr.2019.12.006
91. Liebhart D, Windisch M, Hess M. Oral vaccination of 1-day-old turkeys with *in vitro* attenuated *Histomonas meleagridis* protects against histomonosis and has no negative effect on performance. *Avian Pathol.* (2010) 39:399–403. doi: 10.1080/03079457.2010.506906
92. Liebhart D, Hess M. Oral infection of turkeys with *in vitro*-cultured *Histomonas meleagridis* results in high mortality. *Avian Pathol.* (2009) 38:223–7. doi: 10.1080/03079450902912192
93. Reid WM. Etiology and dissemination of the blackhead disease syndrome in turkeys and chickens. *Exp Parasitol.* (1967) 21:249–75. doi: 10.1016/0014-4894(67)90087-2
94. Lotfi A, Abdelhab EM, Hafez HM. Persistence of *Histomonas meleagridis* in or on materials used in poultry houses. *Avian Dis.* (2012) 56:224–6. doi: 10.1637/9519-090910-ResNote.1
95. Daugschies A, Bangoura B, Lendner M. Inactivation of exogenous endoparasite stages by chemical disinfectants: current state and perspectives. *Parasitol Res.* (2013) 112:917–32. doi: 10.1007/s00436-013-3324-4
96. Badparva E, Kheirandish F. Epidemiology of pathogenic parasite *Histomonas meleagridis* in poultry in Lorestan province, western Iran. *J Parasit Dis.* (2017) 41:1040–3. doi: 10.1007/s12639-017-0931-5
97. Munsch M, Lotfi A, Hafez HM, Al-Quraishy S, Mehlhorn H. Light and transmission electron microscopic studies on trophozoites and cyst-like stages of *Histomonas meleagridis* from cultures. *Parasitol Res.* (2009) 104:683–9. doi: 10.1007/s00436-008-1246-3
98. Munsch M, Mehlhorn H, Al-Quraishy S, Lotfi AR, Hafez HM. Molecular biological features of strains of *Histomonas meleagridis*. *Parasitol Res.* (2009) 104:1137–40. doi: 10.1007/s00436-008-1299-3
99. Zaragatzki E, Hess M, Grabensteiner E, Abdel-Ghaffar F, Al-Rasheid KAS, Mehlhorn H. Light transmission electron microscopic studies on the encystation of *Histomonas meleagridis*. *Parasitol Res.* (2010) 106:977–83. doi: 10.1007/s00436-010-1777-2
100. Zaragatzki E, Mehlhorn H, Abdel-Ghaffar F, Rasheid KS, Grabensteiner E, Hess M. Experiments to produce cysts in cultures of *Histomonas meleagridis* – The agent of histomonosis in poultry. *Parasitol Res.* (2010) 106:1005–7. doi: 10.1007/s00436-010-1776-3
101. Beer LC, Graham BDM, Barros TL, Latorre JD, Tellez-Isaias G, Fuller AL, et al. Evaluation of live-attenuated *Histomonas meleagridis* isolates as vaccine candidates against wild-type challenge. *Poult Sci.* (2022) 101:101656. doi: 10.1016/j.psj.2021.101656
102. Tyzzer EE. Studies on histomoniasis, or "blackhead" infection, in the chicken and the turkey. *Daedalus.* (1934) 69:189–264. doi: 10.2307/20023041
103. Tyzzer EE, Fabyan M. A further inquiry into the source of the virus in blackhead of turkeys, together with observations on the administration of ipecac and of sulfur. *J Exp Med.* (1922) 35:791–812. doi: 10.1084/jem.35.6.791
104. Connell R. Enterohepatitis (blackhead) in turkeys. *Can J Comp Med.* (1950) 14:331–7.
105. McDougald LR. Intestinal protozoa important to poultry. *Poult Sci.* (1998) 77:1156–8. doi: 10.1093/ps/77.8.1156
106. Lee DL. The structure and development of *Histomonas meleagridis* (*Mastigamoebidae: Protozoa*) in the female reproductive tract of its intermediate host, *Heterakis gallinarum* (Nematoda). *Parasitology.* (1969) 59:877–84. doi: 10.1017/S0031182000070384
107. Waters CV III, Hall LD, Davidson WR, Rollor III EA, Lee KA. Status of commercial and noncommercial chickens as potential sources of histomoniasis among wild turkeys. *Wildl Soc Bull.* (1994) 22:43–9.
108. Daş G, Gauly M. Density related effects on lifetime fecundity of *Heterakis gallinarum* in chickens. *Parasit Vect.* (2014) 7:1–9. doi: 10.1186/1756-3305-7-334
109. Huber K, Gouilloud L, Zenner L. A preliminary study of natural and experimental infection of the lesser mealworm *Alphitobius diaperinus* (*Coleoptera: Tenebrionidae*) with *Histomonas meleagridis* (*Protozoa: Sarcomastigophora*). *Avian Pathol.* (2007) 36:279–82. doi: 10.1080/03079450701447317
110. Beckmann JF, Dormitorio T, Oladipupo SO, Terra MTB, Lawrence K, Macklin KS, et al. Heterakis gallinarum and *Histomonas meleagridis* DNA persists in chicken houses years after depopulation. *Vet Parasitol.* (2021) 298:109536. doi: 10.1016/j.vetpar.2021.109536
111. Lund EE, Wehr EE, Ellis DJ. Earthworm transmission of heterakis and histomonas to turkeys and chickens. *J Parasitol.* (1966) 52:899–902. doi: 10.2307/3276528
112. Windisch M, Hess M. Establishing an indirect sandwich enzyme-linked-immunosorbent-assay (ELISA) for the detection of antibodies against *Histomonas meleagridis* from experimentally infected specific pathogen-free chickens and turkeys. *Vet Parasitol.* (2009) 161:25–30. doi: 10.1016/j.vetpar.2008.12.014
113. van der Heijden HMJF, Stegeman A, Landman WJM. Development of a blocking-ELISA for the detection of antibodies against *Histomonas meleagridis* in chickens and turkeys. *Vet Parasitol.* (2010) 171:216–22. doi: 10.1016/j.vetpar.2010.03.028
114. Grafl B, Liebhart D, Windisch M, Ibesich C, Hess M. Seroprevalence of *Histomonas meleagridis* in pullets and laying hens determined by ELISA. *Vet Rec.* (2011) 168:160. doi: 10.1136/vr.c6479
115. Sulejmanovic T, Turblin V, Bilic I, Jaskulska B, Hess M. Detection of *Histomonas meleagridis* DNA in dust samples obtained from apparently healthy meat turkey flocks without effect on performance. *Avian Pathol.* (2019) 48:329–33. doi: 10.1080/03079457.2019.1599819
116. Hafez HM, Hauck R, Lüschow D, McDougald LR. Comparison of the specificity and sensitivity of PCR. nested PCR, and real-time

PCR for the diagnosis of histomoniasis. *Avian Dis.* (2005) 49:366–70. doi: 10.1637/7341-020805R.1
117. Huber K, Chauve C, Zenner L. Detection of *Histomonas meleagridis* in turkeys cecal droppings by PCR amplification of the small subunit ribosomal DNA sequence. *Vet Parasitol.* (2005) 131:311–6. doi: 10.1016/j.vetpar.2005.05.012
118. Grabensteiner E, Hess M. PCR for identification and differentiation of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and *Blastocystis* spp. *Vet Parasitol.* (2006) 142:223–30. doi: 10.1016/j.vetpar.2006.07.011
119. Reis JL Jr, Beckstead RB, Brown CC, Gerhold RW. *Histomonas meleagridis* and capillarid infection in a captive chukar (*Alectoris chukar*). *Avian Dis.* (2009) 53:637–9. doi: 10.1637/8678-030409-Case.1
120. Liu W, Peng J, Li F, Sun H, Ding Y, He J, et al. Identification of *Histomonas meleagridis* by *in vitro* microculture and polymerase chain reaction. *Rep Parasitol.* (2011) 1:1–6. doi: 10.2147/RIP.S18259
121. Gerbod D, Edgcomb VP, Noel C, Zenner L, Wintjens R, Delgado-Viscogliosi P, et al. Phylogenetic position of the trichomonad parasite of turkeys *Histomonas meleagridis* (Smith) Tyzzer, inferred from small subunit rRNA sequence. *J Eukaryot Microbiol.* (2001) 48:498–504. doi: 10.1111/j.1550-7408.2001.tb00185.x
122. Hauck R, Hafez HM. Partial sequence of the beta-tubulin of *Histomonas meleagridis* and the activity of benzimidazoles against *H*. meleagridis *in vitro*. *Parasitol Res.* (2009) 104:1183–9. doi: 10.1007/s00436-008-1309-5
123. Lynn EC, Beckstead RB. Identification of gene expression elements in *Histomonas meleagridis* using splinkerette PCR a variation of ligated adaptor PCR. *J Parasitol.* (2012) 98:135–41. doi: 10.1645/GE-2916.1
124. Palmieri N, Ramires MJ, Hess M, Bilic I. Complete genomes of the eukaryotic poultry parasite *Histomonas meleagridis*: Linking sequence analysis with virulence/attenuation. *BMC Genomics.* (2021) 22:753. doi: 10.1186/s12864-021-08059-2
125. Bilic I, Jaskulska B, Souillard R, Liebhart D, Hess M. Multi-locus typing of *Histomonas meleagridis* isolates demonstrates the existence of two different genotypes. *PLoS ONE.* (2014) 9:e92438. doi: 10.1371/journal.pone.0092438
126. Grafl B, Weise H, Bris JL, Liebart D, Bilic I, Hess M. Aberrant clinical appearance and pathomorphology noticed during an outbreak of histomonosis indicates a different pathogenesis of *Histomonas meleagridis* genotype 2. *Avian Dis.* (2015) 59:452–8. doi: 10.1637/11093-041715-Case.1
127. Pham ADN, Mast J, De Gussem JK, McDougald LR, Goddeeris BM. Establishing mono-eukaryotic *Histomonas meleagridis* cultures from *in vivo* infection contaminated with *Tetratrichomonas gallinarum* and *Blastocystis* spp. *Parasitology.* (2013) 140:1266–74. doi: 10.1017/S0031182013000723
128. Bilic I, Leberl M, Hess M. Identification and molecular characterization of numerous *Histomonas meleagridis* proteins using a cDNA library. *Parasitology.* (2009) 136:379–91. doi: 10.1017/S0031182008005477
129. Leberl M, Hess M, Bilic I. *Histomonas meleagridis* possesses three alpha-actinins immunogenic to its hosts. *Mol Biochem Parasitol.* (2010) 169:101–7. doi: 10.1016/j.molbiopara.2009.10.007
130. Mazumdar R, Nöbauer K, Hummel K, Hess M, Bilic I. Molecular characterization of *Histomonas meleagridis* exoproteome with emphasis on protease secretion and parasite-bacteria interaction. *PLoS ONE.* (2019) 14:e0212429. doi: 10.1371/journal.pone.0212429
131. Wei Z, Abraham M, Chadwick EV, Beckstead RB. *Histomonas meleagridis* isolates compared by virulence and gene expression. *Vet Parasitol.* (2020) 286:109233. doi: 10.1016/j.vetpar.2020.109233
132. Mazumdar R, Endler L, Monoyios A, Hess M, Bilic I. Establishment of a de novo reference transcriptome of *Histomonas meleagridis* reveals basic insights about biological functions and potential pathogenic mechanisms of the parasite. *Protist.* (2017) 168:663–85. doi: 10.1016/j.protis.2017.09.004
133. Monoyios A, Hummel K, Nöbauer K, Patzl M, Schlosser S, Hess M, et al. An alliance of gel-based and gel-free proteomic techniques displays substantial insight into the proteome of a virulent and an attenuated *Histomonas meleagridis* strain. *Front Cell Infect Microbiol.* (2018) 8:1–23. doi: 10.3389/fcimb.2018.00407
134. Monoyios A, Patzl M, Schlosser S, Hess M, Bilic I. Unravelling the differences: Comparative proteomic analysis of a clonal virulent and an attenuated *Histomonas meleagridis* strain. *Int J Parasitol.* (2018) 48:145–57. doi: 10.1016/j.ijpara.2017.08.017
135. Tyzzer EE. Arsenical compounds in the treatment of blackhead in turkeys. *J Exp Med.* (1923) 37:851–73. doi: 10.1084/jem.37.6.851
136. McDougald LR. Efficacy and compatibility of amprolium and carbarsone against coccidiosis and blackhead in turkeys. *Poult Sci.* (1979) 58:76–80. doi: 10.3382/ps.0580076
137. Lindquist WD. Some effects of paromomycin sulfate on blackhead in turkeys. *Am J Vet Res.* (1962) 23:1053–6.
138. McGuire WC, Moeller MW, Morehouse NF. The effect of dimetridazole on the growth and prevention of histomoniasis in poultry. *Poult Sci.* (1964) 43:864–71. doi: 10.3382/ps.0430864
139. Mitrovic M, Hoffer M, Schilknecht EG. Antihistomonal activity of 1,2-disubstituted 5-nitroimidazoles. *Antimicrob Agents Chemother.* (1968) 8:445–8.
140. Doneley B. Treating liver disease in the avian patient. *Semin Avian Exotic Pet Med.* (2004) 13:8–15. doi: 10.1053/S1055-937X(03)00053-7
141. Hu J, McDougald LR. The efficacy of some drugs with known antiprotozoal activity against *Histomonas meleagridis* in chickens. *Vet Parasitol.* (2004) 121:233–8. doi: 10.1016/j.vetpar.2004.02.022
142. Law GL, Mansfield GP, Muggleton DF, Parnell EW. Dimetridazole: absorption, excretion and metabolism in turkeys. *Nature.* (1963) 197:1024–5. doi: 10.1038/1971024a0
143. Seeger KC, Lucas WC, Tomhave AE. The use of enheptin-T in the control of enterohepatitis in turkeys. *Poult Sci.* (1950) 29:610–1. doi: 10.3382/ps.0290610
144. Cuckler AC, Malanga CM. Nithiazide II. Effect on enterohepatitis in turkeys. *Proc Soc Exp Biol Med.* (1956) 92:485–8. doi: 10.3181/00379727-92-22520
145. Hegngi FN, Doerr J, Cummings TS, Schwartz RD, Saunders G, Zajac A, et al. The effectiveness of benzimidazole derivatives for the treatment and prevention of histomonosis (blackhead) in turkeys. *Vet Parasitol.* (1999) 81:29–37. doi: 10.1016/S0304-4017(98)00233-7
146. Hauck R, Hafez HM. Experimental infections with the protozoan parasite *Histomonas meleagridis*: a review. *Parasitol Res.* (2013) 112:19–34. doi: 10.1007/s00436-012-3190-5
147. Regmi PR, Shaw AL, Hungerford LL, Messenheimer JR, Zhou T, Pillai P, et al. Regulatory considerations for the approval of drugs against histomoniasis (blackhead disease) in turkeys, chickens, and game birds in the United States. *Avian Dis.* (2016) 60:725–30. doi: 10.1637/11451-061516-Review.1
148. Liebhart D, Hess M. Spotlight on histomonosis (blackhead disease): a re-emerging disease in turkeys and chickens. *Avian Pathol.* (2020) 49:1–4. doi: 10.1080/03079457.2019.1654087
149. Clark S, Bailey A. Turkey industry annual report – current health and industry issues facing the US turkey industry. In: *Proceeding 119th Annual Meeting of the United States Animal Health Association Providence, Rhode Island*. (2015). p. 433–42. Available online at: http://www.usaha.org/upload/Proceedings/2015_Proceedings_web2.pdf
150. Grabensteiner E, Liebhart D, Arshad N, Hess M. Antiprotozoal activities determined *in vitro* and *in vivo* of certain plant extracts against *Histomonas meleagridis*, *Tetratrichomonas gallinarum*, *Blastocystis* sp. *Parasitol Res.* (2008) 103:1257–64. doi: 10.1007/s00436-008-1122-1
151. Hafez HM, Shehata AA. Turkey production and health: current challenges. *Ger J Vet Res.* (2021) 1:3–14. doi: 10.51585/gjvr.2021.0002
152. Thøfner ICN, Liebhart D, Hess M, Schou TW, Hess C, Ivarsen E, et al. Antihistomonal effects of artemisinin and artemisia annua extracts *in vitro* could not be confirmed by *in vivo* experiments in turkeys and chickens. *Avian Pathol.* (2012) 41:487–96. doi: 10.1080/03079457.2012.714459
153. Clark S, Kimminau E. Critical review: future control of blackhead disease (histomoniasis) in poultry. *Avian Dis.* (2017) 61:281–8. doi: 10.1637/11593-012517-ReviewR
154. Barros TL, Beer LC, Tellez G, Fuller AL, Hargis BM, Vuong CN. Research note: evaluation of dietary administration of sodium chlorate and sodium nitrate for *Histomonas meleagridis* prophylaxis in turkeys. *Poult Sci.* (2020) 99:1983–7. doi: 10.1016/j.psj.2019.11.055
155. Beer LC, Latorre JD, Rochell SJ, Sun X, Tellez G, Fuller AL, et al. Research note: evaluation of deoxycholic acid for antihistomonal activity. *Poult Sci.* (2020) 99:3481–6. doi: 10.1016/j.psj.2020.03.049
156. Beer LC, Vuong CN, Barros TL, Latorre JD, Tellez G, Fuller AL, et al. Research note: evaluation of boric acid as a chemoprophylaxis

candidate to prevent histomoniasis. *Poult Sci.* (2020) 99:1978–82. doi: 10.1016/j.psj.2019.12.003
157. Callait MP, Granier C, Chauve C, Zenner L. *In vitro* activity of therapeutic drugs against *Histomonas meleagridis* (2). *Poult Sci.* (2002) 81:1122–7. doi: 10.1093/ps/81.8.1122
158. van der Heijden HMJF, Landman WJM. *In vitro* effect of herbal products against *Histomonas meleagridis* in turkeys. *Vet Parasitol.* (2008) 154:1–7. doi: 10.1016/j.vetpar.2008.02.033
159. Abraham M, McDougald LR, Beckstead RB. Blackhead disease: reduced sensitivity of *Histomonas meleagridis* to nitarsone *in vitro* and *in vivo*. *Avian Dis.* (2014) 58:60–3. doi: 10.1637/10635-080913-Reg.1
160. Umar S, Khan MI, Ahmed S, Usman M, Younus M, Sarwar F, et al. *In vitro* and *in vivo* sensitivity of a flagellated protozoan, *Histomonas meleagridis*, to metronidazole and nitarsone. *Pak. J Zool.* (2016) 48:209–14. Available online at: https://www.proquest.com/scholarly-journals/vitro-vivo-sensitivity-flagellated-protozoan/docview/2010348719/se-2?accountid=8361
161. Zenner L, Callait MP, Granier C, Chauve C. *In vitro* effect of essential oils from *Cinnamomum aromaticum*, *Citrus limon* and *Allium sativum* on two intestinal flagellates of poultry, *Tetratrichomonas gallinarum* and *Histomonas meleagridis*. *Parasite.* (2003) 10:153–7. doi: 10.1051/parasite/2003102153
162. Bleyen N, De Gussem K, Pham ADN, Ons E, Gercen NV, Goddeeris BM. Non-curative, but prophylactic effects of paromomycin in *Histomonas meleagridis*-infected turkeys and its effect on performance in non-infected turkeys. *Vet Parasitol.* (2009) 165:248–55. doi: 10.1016/j.vetpar.2009.07.007
163. Hafez HM, Hauck R, Gad W, Gussem KD, Lotfi A. Pilot study on the efficacy of paromomycin as a histomonostatic feed additive in turkey poults experimentally infected with *Histomonas meleagridis*. *Arch Anim Nutr.* (2010) 64:77–84. doi: 10.1080/17450390903478851
164. Liebhart D, Ganas P, Sulejmanovic T, Hess M. Histomonosis in poultry: previous and current strategies for prevention and therapy. *Avian Pathol.* (2017) 46:1–18. doi: 10.1080/03079457.2016.1229458
165. Lin GW. Paromomycin sulfate treatment in histomoniasis outbreaks in three commercial turkey flocks in the fraser valley of British Columbia, Canada. *Avian Dis.* (2021) 65:592–8. doi: 10.1637/aviandiseases-D-21-00076
166. Puvača N, Brkić I, Jahić M, Nikolić SR, Radovi, ć G, et al. The effect of using natural or biotic dietary supplements in poultry nutrition on the effectiveness of meat production. *Sustainability.* (2020) 12:4373. doi: 10.3390/su12114373
167. Duffy CF, Sims MD, Power RF. Preliminary evaluation of dietary Natustat^{TM} versus Histostat® (nitarsone) for control of *Histomonas meleagridis* in broiler chickens on infected litter. *Int J Poult Sci.* (2004) 3:753–57. doi: 10.3923/ijps.2004.753.757
168. Duffy CF, Sims MD, Power RF. Evaluation of dietary Natustat^{TM} for control of *Histomonas meleagridis* in male turkeys on infected litter. *Avian Dis.* (2005) 49:423–5. doi: 10.1637/7344-022105R2.1
169. Hafez HM, Hauck R. Efficacy of a herbal product against *Histomonas meleagridis* after experimental infection of turkey poults. *Arch Anim Nutr.* (2006) 60:436–42. doi: 10.1080/17450390600884500
170. Hauck R, Hafez HM. Effect of coated plant extracts on *Histomonas meleagridis* and growth of bacteria *in vitro*. *Avian Dis.* (2007) 51:880–3. doi: 10.1637/7957-022707-REGR.1
171. Tykałowski B, Smiałek M, Kowalczyk J, Dziewulska D, Stenzel T, Koncicki A. Phytoncides in the prevention and therapy of blackhead disease and their effect on the turkey immune system. *J Vet Res.* (2021) 65:79–85. doi: 10.2478/jvetres-2021-0010
172. Uzor PE. Alkaloids from plants with antimalarial activity: a review of recent studies. *Evid Based Complement Alternat Med.* (2020) 2020:1–17. doi: 10.1155/2020/8749083
173. Delaplane JP, Stuart HO. (1935) *Blackhead (Entero-Hepatitis) Investigations*. State College, RI: Agricultural experiment station.
174. Farmer RK. Infectious enterohepatitis (blackhead) in turkeys: a study of the prophylactic and therapeutic value of certain compounds. *J Comp Pathol.* (1950) 60:294–310. doi: 10.1016/S0368-1742(50)80027-9
175. Beer LC, Hargis BM, Vuong CN. Research note: evaluation of quinine as a chemoprophylactic candidate against histomoniasis in turkeys. *Poult Sci.* (2021) 100:101469. doi: 10.1016/j.psj.2021.101469
176. Ueda H, Kaidou S. Aversion to quinine is associated with taste sensation in chicks. *J Poult Sci.* (2005) 42:254–62. doi: 10.2141/jpsa.42.254
177. Tyzzer EE, Fabyan M, Foot NC. Further observations on "blackhead" in turkeys. *J Infect Dis.* (1921) 29:268–86. doi: 10.1093/infdis/29.3.268
178. Joyner LP. Immunity to histomoniasis in turkeys following treatment with dimetridazole. *J Comp Pathol Ther.* (1963) 73:201–7. doi: 10.1016/S0368-1742(63)80023-5
179. Tyzzer EE. A study of immunity produced by infection with attenuated culture-strains of *Histomonas meleagridis*. *J Comp Patho Ther.* (1936) 49:285–303. doi: 10.1016/S0368-1742(36)80025-3
180. Clarkson MJ. Immunological responses to *Histomonas meleagridis* in the turkey and fowl. *Immunology.* (1963) 6:156–68.
181. Clarkson MJ. Progressive serum protein changes in turkeys infected with *Histomonas meleagridis*. *J Comp Path.* (1966) 76:387–97. doi: 10.1016/0021-9975(66)90059-4
182. Cuckler A. Coccidiosis and histomoniasis in avian hosts. In: Jackson GJ, Herman R, Singer I, editors. *Immunity to Parasitic Animals.* New York, NY: Appleton-Century-Crofts, Educational Division (1970). p. 371–97.
183. Hess M, Liebhart D, Grabensteiner E, Singh A. Cloned *Histomonas meleagridis* passaged *in vitro* resulted in reduced pathogenicity and is capable of protecting turkeys from histomonosis. *Vaccine.* (2008) 26:4187–93. doi: 10.1016/j.vaccine.2008.05.071
184. Bleyen N, Ons E, De Gussem M, Goddeeris BM. Passive immunization against *Histomonas meleagridis* does not protect turkeys from an experimental infection. *Avian Pathol.* (2009) 38:71–6. doi: 10.1080/03079450802641255
185. McDougald LR, Hansen MF. Research note: serum protein changes in chickens subsequent to infection with *Histomonas meleagridis*. *Avian Dis.* (1969) 13:673–7. doi: 10.2307/1588543
186. Windisch M, Hess M. Experimental infection of chickens with *Histomonas meleagridis* confirms the presence of antibodies in different parts of the intestine. *Parasite Immunol.* (2010) 32:29–35. doi: 10.1111/j.1365-3024.2009.01159.x
187. McGuire WC, Cavett JW. Blood studies on histomoniasis in turkeys. *Poult Sci.* (1952) 31:610–7. doi: 10.3382/ps.0310610
188. Powell FL, Rothwell L, Clarkson MJ, Kaiser P. The turkey, compared to the chicken, fails to mount an effective early immune response to *Histomonas meleagridis* in the gut. *Parasite Immunol.* (2009) 31:312–27. doi: 10.1111/j.1365-3024.2009.01113.x
189. Mitra T, Gerner W, Kidane FA, Wernsdorf P, Hess M, Saalmüller A, et al. Vaccination against histomonosis limits pronounced changes of B cells and T-cell subsets in turkeys and chickens. *Vaccine.* (2017) 35:4184–96. doi: 10.1016/j.vaccine.2017.06.035
190. Lagler J, Mitra T, Schmidt S, Pierron A, Vatzia E, Stadler M, et al. Cytokine production and phenotype of *Histomonas meleagridis*-specific T cells in the chicken. *Vet Res.* (2019) 50:107. doi: 10.1186/s13567-019-0726-z
191. Lagler J, Schmidt S, Mitra T, Stadler M, Wernsdorf P, Grafl B, et al. Comparative investigation of IFN-γ-producing T cells in chickens and turkeys following vaccination and infection with the extracellular parasite *Histomonas meleagridis*. *Dev Comp Immunol.* (2021) 116:103949. doi: 10.1016/j.dci.2020.103949
192. Schwarz A, Gauly M, Abel H, Daş G, Humburg J, Weiss ATA, et al. Pathobiology of *Heterakis gallinarum* mono-infection and co-infection with *Histomonas meleagridis* in layer chickens. *Avian Pathol.* (2011) 40:277–87. doi: 10.1080/03079457.2011.561280
193. Sharma JM, Rautenschlein S. Host factors for disease resistance. In: Swayne E. Glisson JR, McDougald LR, Nolan LK, Suarez DL, Nair VL, editors. *Diseases of Poultry*. 13th ed. Ames, IA: Wiley-Blackwell (2013). p. 47–74.
194. Mitra T, Kidane FA, Hess M, Liebhart D. Unravelling the immunity of poultry against the extracellular protozoan parasite *Histomonas meleagridis* is a cornerstone for vaccine development: a review. *Front Immunol.* (2018) 9:2518. doi: 10.3389/fimmu.2018.02518
195. Lund EE. Immunizing action of a nonpathogenic strain of histomonas against blackhead in turkeys. *J Protozool.* (1959) 6:182–5. doi: 10.1111/j.1550-7408.1959.tb03948.x
196. Lund EE, Augustine PC, Ellis DJ. Immunizing action of *in vitro*-attenuated *Histomonas meleagridis* in chickens and turkeys. *Exp Parasitol.* (1966) 18:403–7. doi: 10.1016/0014-4894(66)90041-5

197. Lund EE, Augustine PC, Chute AM. *Histomonas meleagridis* after one thousand *in vitro* passages. *J Eukaryot Microbiol.* (1967) 14:349–51. doi: 10.1111/j.1550-7408.1967.tb02007.x
198. Dwyer DM, Honigberg BM. Effect of *in vitro* cultivation on the virulence of a pathogenic strain of *Histomonas meleagridis*. *J Protozool*. (1969) 16:18–9.
199. Dwyer DM, Honigberg BM. Effect of certain laboratory procedures on the virulence of *Histomonas meleagridis* for turkeys and chickens. *J Parasitol.* (1970) 56:694–700. doi: 10.2307/3277714
200. Liebhart D, Zahoor M, Prokofieva I, Hess M. Safety of avirulent histomonads to be used as a vaccine determined in turkeys and chickens. *Poult Sci.* (2011) 90:996–1003. doi: 10.3382/ps.2010-01255
201. EFSA. Event report: Technical meeting on histomonosis in turkeys. *EFSA Supporting Pub*. (2013) 10:464. doi: 10.2903/sp.efsa.2013.EN-464
202. McAllister MM. Successful vaccines for naturally occurring protozoal diseases of animals should guide human vaccine research: a review of protozoal vaccines and their designs. *Parasitology.* (2014) 141:624–40. doi: 10.1017/S0031182013002060
203. Liebhart D, Sulejmanovic T, Grafl B, Tichy A, Hess M. Vaccination against histomonosis prevents a drop in egg production in layers following challenge. *Avian Pathol.* (2013) 42:79–84. doi: 10.1080/03079457.2012.760841
204. Sulejmanovic T, Liebhart D, Hess M. *In vitro* attenuated *Histomonas meleagridis* does not revert to virulence, following serial *in vivo* passages in turkeys or chickens. *Vaccine.* (2013) 31:5443–50. doi: 10.1016/j.vaccine.2013.08.098
205. Sulejmanovic T, Bilic I, Hess M, Liebhart D. An *in vitro* attenuated strain of *Histomonas meleagridis* provides cross-protective immunity in turkeys against heterologous virulent isolates. *Avian Pathol.* (2016) 45:46–53. doi: 10.1080/03079457.2015.1117057
206. Pham ADN, De Gussem JK, Goddeeris BM. Intracloacally passaged low-virulent *Histomonas meleagridis* protects turkeys from histomonosis. *Vet Parasitol.* (2013) 196:307–13. doi: 10.1016/j.vetpar.2013.03.008

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Feed Composition and Isolate of *Histomonas meleagridis* Alter Horizontal Transmission of Histomonosis in Turkeys. Proof of Concept

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Outbreaks of histomonosis in turkeys are typically initiated by the ingestion of contaminated embryonated eggs of *Heterakis gallinarum*, potentially present in earthworms and mechanical vectors. Once an outbreak is started, infected turkeys can transmit the disease by horizontal transmission. Factors influencing horizontal transmission of histomonosis are poorly understood. Replication of horizontal transmission in experimental conditions has not been consistent, presenting an obstacle in searching for alternatives to prevent or treat the disease. Two pilot experiments and three validation experiments were conducted in the present study. In pilot experiment 1, one isolate of *Histomonas meleagridis* (named Buford) was used. Turkeys were fed a low-nutrient density diet corn-soy based (LOW-CS) and raised in floor pens. In pilot experiment 2, another isolate of *H. meleagridis* was used (named PHL). Turkeys were fed a low-nutrient density diet with the addition of wheat middlings (LOW-WM) and raised in floor pens. In experiment 3, conducted on floor pens, both isolates and diets were used in different groups. In experiment 4, turkeys were raised on battery cages and only the PHL isolate was used. Both diets (LOW-WM and LOW-CS) were used, in addition to a diet surpassing the nutritional needs of young poults (turkey starter, TS). In experiment 5, conducted in battery cages, only the PHL isolate was used, and the LOW-WM and TS diets were in different groups. The horizontal transmission was achieved only with the PHL isolate from all experiments. The transmission rate varied among experimental diets, with the TS diet having the lowest transmission rate in experiments 4 and 5. Variation was observed between experiments and within experimental groups.

Keywords: intestinal health, enterohepatitis, protozoa, ceca, epidemiology

INTRODUCTION

The incidence of histomonosis, caused by the protozoa *Histomonas meleagridis*, has been increasing after the ban of nitroimidazoles and arsenicals as preventative and therapeutic measures (1). Outbreaks can lead to high mortality rates in turkey flocks (2–4). Turkeys become infected by ingesting contaminated *Heterakis gallinarum* eggs, but once a turkey is infected, the transmission to other turkeys can happen in the absence of *H. gallinarum* by direct contact (5), referred to in the present paper as horizontal transmission.

In the last decade, there has been an increase in research about histomonosis (2, 3, 6); however, some epidemiologic aspects remain unanswered, such as risk factors associated with the transmission of histomonosis and the lack of a model of infection by horizontal transmission. The replication of horizontal transmission of histomonosis has not been consistent in many research groups in the last years (6). One explanation is the duration of the trials, with experiments ending before turkeys could show lesions (6). In outbreaks of histomonosis in turkey flocks, it is common to have some variability, whereas, in some outbreaks, only females or only males were affected in a mixed flock house, typically separated by a wire mesh. Factors behind the variability in disease manifestation and incidence are unknown. Previous studies have reported the existence of two distinct clusters of *H. meleagridis* differing in prevalence in geographical locations and pathology (7) and variation in the expression of virulence factors and pathogenicity of different field isolates (8). Nevertheless, as far as we are aware, the impact of different isolates of *H. meleagridis* or the feed composition of the diet in the horizontal transmission of the disease has not been investigated. Hence, the purpose of this study was to evaluate the feed composition and isolates of *H. meleagridis* on the transmissibility of histomonosis in floor pens or battery cages.

MATERIALS AND METHODS

Bioethics

All animal handling procedures complied with the University of Arkansas Agricultural Institutional Animal Care and Use Committee (Animal Use Protocol #19118). Following inoculation with *H. meleagridis*, turkeys were monitored at least twice daily to evaluate and potentially euthanize terminally moribund animals. Severe clinical morbidity, with evidence of inability to ambulate, were euthanized and considered as mortalities.

Pathogen Culture and Challenge

The Buford isolate consists of a wild-type *H. meleagridis* from a field outbreak of histomonosis in layer pullets in the southern United States (9). The PHL isolate is also a wild-type *H. meleagridis*, isolated in 2017 from turkeys in the Northwest Arkansas, USA. Both isolates were cultured in Medium 199 (Lonza®, Walkersville, Maryland) supplemented with 10% horse serum (Gibco®, Penrose, Auckland, New Zealand) and 1.6 mg/mL rice powder (Arrowhead Mills®, Hereford, Texas), following previously described procedures (10). The 1mL vial cryogenically stored from each isolate was thawed and cultured in T-25 cell culture flasks (12.5mL culture volumes) approximately 1 week (Buford isolate) and 2 weeks (PHL isolate) before the inoculation. Cultures were incubated anaerobically at 40°C and passaged every 2 to 3 days. On the inoculation day for each experiment, histomonads were counted with a hemocytometer, adjusting the inoculum to 10^5 histomonads/dose using unsupplemented media. The volume of the inoculum varied between 250–500 μL between the trials, always administered via the intra-cloacal route, holding turkeys in an inverted position for 1 min after the inoculation to reduce the possibility that the turkeys would expel the material.

Animal Source and Husbandry

In all experiments, turkeys had *ad libitum* access to feed and water. All turkeys were female and were obtained from a commercial hatchery (Cargill, Gentry, AR, USA) for experiments 1, 2, and 3 and from another commercial hatchery (Butterball, Goldsboro, NC, USA) for experiments 4 and 5.

Experimental Design

Pilot Studies

In the present study, two pilot experiments were conducted with the intention of assessing the horizontal transmission of two different isolates of *H. meleagridis* and two diet compositions in turkeys.

In the first pilot study (Experiment 1), the inoculation of a typical isolate of *H. meleagridis* (Buford) and the use of a low-nutrient density diet with a reduction in the crude protein level would cause transmission of histomonosis in turkeys raised on floor pens. Pilot experiment 1 was conducted on floor pens (4.5 m^2), with wood shavings as the bedding material. Fifty day-of-hatch poults were randomly distributed to either a non-challenged control (NC) or horizontal transmission group (HT), both being fed a mashed low-nutrient density (LOW) diet, corn-soy based (CS) diets divided into two phases: the first CS diet (CS1) from day 10 to day 21 and the second CS diet (CS2) from day 21 to 45. The diets were formulated based on requirements for broilers (broiler starter and developer), with a lower protein content compared to a turkey diet commonly used in trials with turkeys conducted by our group, which surpasses the nutritional needs of young turkeys, referred to as turkey starter (TS). The composition of the diets is available in **Table 1**. The turkey starter diet was fed during the first 10 days of the poults' life. Seven out of twenty-five turkeys were directly inoculated on day 14 with the Buford isolate (10^5 histomonads/turkey), referred to as seeders. The experiment was terminated on day 45; mortality was monitored daily, and hepatic and cecal lesions were evaluated on a scale of 0–3 (10). The following experiments followed the same criteria of evaluation.

In the second pilot study (Experiment 2), we used a different isolate of *H. meleagridis*, named PHL, and a low-nutrient density diet with the addition of 20.5% wheat middlings provided by a commercial company that frequently reports outbreaks of histomonosis in pre-reproductive turkey hens. Sixty-eight day-of-hatch poults were randomly distributed to either a non-challenged control group ($n = 34$) or a horizontal transmission

TABLE 1 | Composition of the experimental diets.

Ingredient (%)	Low-nutrient density diet corn soy based (LOW-CS)		Diet surpassing the nutritional needs of young turkeys (TS)	Low-nutrient density diet with wheat middlings (LOW-WM)
	Corn-soy 1 (CS1)	Corn-soy 2 (CS2)		
Corn	57.90	75.64	43.33	61.75
Soybean meal	30.23	19.09	42.24	13.20
Wheat middlings	-	-	-	20.50
Animal protein concentrate[§]	5.00	0	7.50	-
Poultry fat	3.58	1.00	3.40	-
Limestone	1.10	1.59	0.66	-
Calcium	-	-	-	1.52
Monocalcium phosphate	-	-	-	2.13
Dicalcium phosphate	1.10	1.57	1.52	-
Salt	0.40	0.41	0.24	0.25
Bicarbonate	-	-	-	0.20
Methionine	0.20[1]	0.20[1]	0.38[1]	0.16[3]
Lysine	-	-	0.42[2]	0.02[4]
L-threonine	-	-	0.11	-
Vitamin/mineral premix	0.20/0.10[†]	0.20/0.10[†]	0.15[†]	0.23[e]
Choline chloride (60%)	0.20	0.20	0.05	0.02[$]
Enzymes[¶]	-	-	-	0.02
Calculated composition (%)				
Crude protein	22	15	28	14
AME (kcal/kg)	3,098	3,082	3,020	2,800
Total Ca	1.27	1.07	1.49	1.15
Available phosphorus	0.56	0.43	0.76	0.58
Dig TSAA	0.82	0.67	1.06	0.55
Dig Lys	1.02	0.69	1.64	0.62
Dig Thr	0.68	0.49	0.96	0.43
Dig Ile	0.79	0.56	1.01	0.49
Dig Val	0.91	0.66	1.12	0.59
Dig Trp	0.22	0.15	0.28	0.14
Dig Arg	1.34	0.88	1.75	0.80

[§]*Composition: crude protein, 57%; crude fat, 8.5%; calcium, 7.94%; phosphorus, 3.59%; sodium, 0.49%; potassium, 0.38%; chloride, 0.73%; cysteine, 1%; methionine, 0.71%; lysine, 3.13%; histidine, 0.91%; tryptophan, 0.34%; threonine, 1.97%; arginine, 3.78%; isoleucine, 1.88%; leucine, 3.71%; phenylalanine, 2.09%; valine, 2.77% (H.J. Baker's ProPlus 57%).*

[†]*Supplied per kg of feed by vitamin premix (0.2%): Vitamin A, 61,740 IU; vitamin D3, 44,100 ICU; vitamin E, 441 IU; vitamin B12, 0.1 mg; menadione, 12 mg; riboflavin, 52.9 mg; D-pantothenic acid, 79.4 mg; niacin, 308.6 mg; folic acid, 7.1 mg; pyridoxine, 22 mg; thiamine, 12.3 mg; biotin, 0.7 mg. Mineral premix (0.1%): calcium, 767 mg; total phosphorus, 0.8 mg; potassium, 1.2 mg; sodium, 1.2 mg; magnesium, 1.0 mg; sulfur, 1,228 mg; iron, 15 mg; zinc, 100 mg; manganese, 100 mg; copper, 15 mg; iodine, 1.2 mg; selenium, 0.3 mg.*
Supplied per kg of feed by vitamin and mineral premix (0.15%): Vitamin A, 13,227 IU; vitamin D3, 6,613 ICU; vitamin E, 66 IU; calcium, 51 mg; manganese, 124.5 mg; zinc, 124.5 mg; copper, 7.5 mg; iodine, 2.1 mg; selenium, 0.3 mg.

[e]*Supplied per kg of feed by Vitamin and mineral premix (0.225%) Vitamin A, 13,230 IU; vitamin D3, 66,100 ICU; vitamin E, 100 IU; vitamin B12, 0.0248 mg; vitamin E EQ, 100 mg; biotin, 0.33 mg; menidione, 4 mg; riboflavin, 15 mg; d-pantothenic acid, 24.26 mg; niacin, 88.2 mg; folic acid, 1.1 mg; pyridoxine, 6.9 mg; thiamine, 2.21 mg; manganese, 125.1 mg; chelated manganese, 40 mg; zinc, 125.1 mg; chelated zinc, 40 mg; iron, 2.1 mg; copper, 7.5 mg; iodine, 2.1 mg; selenium, 0.3 mg.*

[$]*Choline chloride (70%).*

[¶]*Rovabio® Advance Phy L: endo-1,4-β-xylanase ≥ 6,250 VU/ml, endo-1,3(4)-β-glucanase ≥ 4,300 VU/ml, arabinofuranosidase ≥ 23,000 VU/ml, 6-phytase ≥5,000 FTU/ml.*

[1]*DL-methionine.*

[2]*L-lysine HCl.*

[3]*Methionine hydroxy analog (88% methionine).*

[4]*BIOLYS-77® (60% lysine).*

group ($n = 34$). Ten of the 34 poults in the HT group were directly inoculated (seeders) on day 18 with only the PHL isolate (10^5 histomonads/turkey). Poults were fed the mashed turkey starter diet for the first 14 days, and on day 15, a pelleted LOW diet containing 20.5% wheat-middlings (WM) was introduced until the end of the experiment (day 52). The experiment was conducted on floor pens. The composition of the diet is available in **Table 1**. The experiment was terminated on day 52; mortality was monitored daily, with an evaluation of the lesions.

Validation Studies

Based on the findings of the pilot studies, three experiments were conducted to validate the preliminary data as described below.

In Experiment 3, also conducted in floor pens, two LOW diets (CS and WM) with the same formulation used in the first two experiments were introduced after the first week of the poults' lives: WM diet (WM, d7-38) and CS diets (CS1, d7-21, and CS2, d21-38). The diets were similar to those in the first two experiments, but in different batches. All poults were fed the turkey starter diet during the first seven days. The two previously mentioned isolates of *H. meleagridis* were tested: Buford or PHL. Day-of-hatch poults were randomly distributed to one of eight groups: 1) NC, fed a LOW diet, CS-based (NC-CS, $n = 45$); 2) NC, LOW diet, WM based (NC-WM, $n = 50$); 3) positive control (PC), all turkeys directly inoculated with the Buford isolate, fed a LOW diet, WM based (PC-Buford-WM, $n = 50$); 4) PC, all turkeys directly inoculated with the PHL isolate, fed a LOW diet, WM based (PC-PHL-WM, $n = 50$); 5) HT with the Buford isolate, fed a LOW diet, WM based (HT-Buford-WM, $n = 45$); 6) HT with the Buford isolate, fed a LOW diet, CS-based (HT-Buford-CS, $n = 45$); 7) HT with the PHL isolate, fed a LOW diet, WM based (HT-PHL-WM, $n = 45$); or 8) HT with the PHL isolate, fed a LOW diet, CS-based (HT-PHL-CS, $n = 45$). Due to a space limitation, we did not include positive controls with the CS diet. On day 10, 14 of 45 poults in the HT groups were directly inoculated with 10^5 histomonads/turkey and all turkeys ($n = 50$/group) in the PC groups. Mortality was recorded daily in both experiments. The PC-Buford-WM was terminated on day 24, 14 days post infection (d.p.i.), the PC-PHL-WM on day 36 (26 d.p.i.), and the remaining groups on day 38; mortality was monitored daily, and hepatic and cecal lesions were evaluated on a scale of 0–3. Bodyweight gain (BWG) from day 7 to 38 was measured only on the NC groups because the PC-Buford-WM group had to be terminated on day 24, and the PC-PHL-WM was terminated on day 36. For those groups, BWG was measured from day 7 to day 21.

In Experiment 4, turkeys were raised in battery cages. The cage floor was covered with heavy paper from day 10 to day 25. The paper was changed daily. Three diets were tested: in addition to the two previously LOW diets (WM and CS), the diet surpassing the nutritional needs of young turkeys (turkey starter, TS) was used throughout the experiment for two groups. The TS diet was administered to all groups for the first seven days, then the WM diet was introduced on day 7 until termination (d30) (groups 2 and 5), or the CS diet was divided into two phases (CS1 and CS2), or the TS the whole period (groups 1 and 4). The CS1 was administered from day 7 to day 21 and CS2 from day 21 to termination (d30) (groups 3 and 6). One-hundred ninety-two poults were randomly divided into the following groups ($n = 8$ poults/cage, 4 replicates): 1) NC, fed the TS diet (NC-TS); 2) NC, fed a LOW diet, WM based (NC-WM); 3) NC, fed a LOW diet, CS-based (NC-CS); 4) HT group, fed the TS diet (HT-TS); 5) HT group, fed a LOW diet, WM based (HT-WM); or 6) HT group, fed a LOW diet, CS-based (HT-CS). Only the PHL isolate was used; on day 9, 2 of 8 poults were directly inoculated (seeders) with 10^5 histomonads/turkey. Body weight was recorded on day 7 and at termination (day 30) to calculate BWG. Mortality was recorded daily with an evaluation of lesions.

In Experiment 5, turkeys were again raised in battery cages, following the abovementioned practices. For this trial, only two diets were used: the LOW diet, WM-based with the addition of 3% of celite as a filler, and the corn-soy diet surpassing the nutritional needs of young turkeys (TS), mashed. The TS diet was administered to all groups for the first seven days, then the WM diet was introduced on day 7 until termination (d29) (groups 2 and 4) or the TS diet for the whole period (groups 1 and 3). Two hundred forty poults were randomly allocated into 4 groups ($n = 10$/cage, 6 replicates): 1) NC, fed the TS diet (NC-TS); 2) NC, fed a LOW diet, WM based (NC-WM); 3) HT group, fed the TS diet (HT-TS); or 4) HT group, fed a LOW diet, WM based (HT-WM). On day 7, only 8 poults were kept in each cage. On day 9, 2 of 8 poults were directly inoculated (seeders) with 10^5 histomonads/turkey from the PHL isolate. Body weight was recorded on day 7 and at termination (day 27) to calculate BWG, and feed consumption was recorded. Mortality was recorded daily with an evaluation of lesions.

Statistical Analysis

Mortality and frequency of lesions were compared with all possible combinations using the chi-square test of independence to determine significance in experiment 3, 4 and 5 ($P < 0.05$). Bodyweight gain data were subjected to multi-way analysis of variance for the randomized design using SAS's General Linear Models procedure. Means were separated with the Duncan test and considered significant at $P < 0.05$. Data were reported as mean ± SE. In experiment 3, for BWG each bird was the experimental unit. In experiments 4 and 5, the average of each cage was considered the experimental unit for performance data and individual birds for lesions scores. For all experiments, turkeys succumbing to infection or which were euthanized were subjected to lesion scoring.

RESULTS

Table 2 shows a summary of the results of the five horizontal transmission experiments in turkeys fed with different feed compositions and challenged with two different isolates of *Histomonas meleagridis*.

In pilot experiment 1, no horizontal transmission was observed (**Table 2**). In pilot experiment 2, the horizontal transmission was achieved, with 30.4% (7 out of 23) of the contacts presenting lesions in the ceca and/or liver, with only one mortality. Mortality of the seeder turkeys started on day 39 (21 d.p.i.), reaching 40% on day 44 (26 d.p.i.) when all seeders were humanely euthanized (**Table 2**). The trial was terminated on day 52 (34 d.p.i.).

In Experiment 3, no horizontal transmission was observed in turkeys fed with WM and challenged with either PHL or Buford strain of *H. meleagridis*. Interestingly, turkeys fed with CS and challenged with the PHL isolate showed 57% of horizontal transmission in the contacts (**Table 2**). Moreover, this group had 23.3% (7 out of 30) mortality in the contact turkeys (**Table 2**). The positive control groups PC-Buford-WM and PC-PHL-WM

TABLE 2 | Summary of the results of the horizontal transmission, evaluated as the % frequency of lesions in ceca and/or liver in contact turkeys for all five experiments evaluating different feed compositions and isolates of *Histomonas meleagridis*.

Experiment	Diet	Isolate	Number of turkey seeders/Number of turkey contacts	% Horizontal transmission in contacts	% Mortality rate (seeders / total)	% Mortality rate (contacts / total)
Pilot experiment 1 (Floor pen)	CS[1]	Buford	7/18	0 %	100 % (7/7)	0 % (0/18)
Pilot experiment 2 (Floor pen)	WM[2]	PHL	10/23	30.4 % (7/33)	40 % (4/10)	4.3 % (1/23)
Experiment 3 (Floor pen)	WM CS	Buford or PHL	14/30	57 % (17/30) PHL-CS[a] 0 % (0/30) PHL-WM[b] 0 % (0/30) Buford-WM[b] 0 % (0/30) Buford-CS[b]	42.9 % (6/14) PHL-CS 42.9 % (6/14) PHL-WM 64.3 % (9/14) Buford-WM 57.1 % (8/14) Buford-CS	23.3 % (7/30) PHL-CS[a] 0 % (0/30) PHL-WM[b] 0 % (0/30) Buford-WM[b] 0 % (0/30) Buford-CS[b]
Experiment 4 (Battery cages)	WM CS TS[3]	PHL	2/6	100 % (23/23) WM[a] 83.3 % (20/24) CS[b] 45.8 % (11/24) TS[c]	12.5 % (1/8) WM[a] 0 % CS[b] 0 % TS[b]	0 % (0/6) WM 0 % (0/6) CS (0 %) (0/6) TS
Experiment 5 (Battery cages)	WM TS	PHL	2/6	61.1 % (22/36) WM[a] 16.7 % (6/36) TS[b]	41.6 % (5/12) WM 4.2 % (2/12) TS	2 % (1/36) WM[a] 0 % (0/6) TS[b]

[1] *CS: low nutrient density diet, corn-soy based.*
[2] *WM: low nutrient density diet, wheat middlings based.*
[3] *TS: diet surpassing the nutritional requirements of young turkeys, turkey starter diet.*
[a–c] *Values within columns in each Experiment with different superscripts differ significantly ($P < 0.05$).*

had mortality rates of 63.3 and 33.3%, respectively, with mortality of turkeys on the PC-Buford-WM group starting at 10 d.p.i. and having a sharp increase until 14 d.p.i., while mortality on the group PC-PHL-WM started 15 d.p.i., prolonging until 26 d.p.i. (**Figure 1**). Mortality in the seeders of the HT-Buford-WM group began at 11 d.p.i., 15 d.p.i. in the PC-PHL-WM group, 16 and 21 d.p.i. in the groups HT-PHL-WM and HT-PHL-CS, respectively. Mortality of the seeder turkeys in the HT groups was 42.9% in the HT-PHL-CS and HT-PHL-WM groups, 57.1% in HT-Buford-CS, and 64.3% in HT-Buford-WM (**Table 2**, $P > 0.05$). From day 7 to 21, the groups NC-WM and NC-CS had a similar BWG, differing from the group PC-Buford-WM and PC-PHL-WM (**Table 3**). From day 7 to 38, the group NC-WM had a higher BWG than the group NC-CS (**Table 3**).

In Experiment 4, the horizontal transmission was observed in all groups fed with different diet compositions (HT-TS, HT-WM, HT-CS). However, the group fed the turkey starter diet (HT-TS) had a lower percentage of contacts ($P > 0.05$) with lesions compared to both low-nutrient density diets (**Table 2**). In one of the four replicate cages of the group HT-TS, no contacts had cecal nor hepatic lesions, while both seeders presented cecal lesions. No hepatic lesions were observed in both seeders and contacts from all groups. Only one seeder from the HT-WM group died (**Table 2**). Mortalities were not observed in the other groups. From day 7 to 30, the group NC-WM had the highest BWG, followed by the NC-TS, HT-WM, HT-TS, NC-CS, and HT-CS (**Table 3**).

In Experiment 5, the horizontal transmission was observed in both groups (HT-TS and HT-WM). Agreeing with the previous experiment, a lower transmission level was observed in the group fed the turkey starter diet (HT-TS; **Table 2**). Four of six replicate cages of the HT-TS group had no contacts with lesions in the ceca or liver, although both seeders developed severe lesions, except one in one cage where only one seeder had lesions. The contacts of two cages of the HT-WM did not develop lesions in the ceca and liver. Two seeders died in the HT-TS group and five seeders and one contact died in the HT-WM group. The BWG from day 7 to 27, the group NC-TS had the highest BWG, followed by HT-TS, NC-WM, and HT-WM. Feed intake followed a similar pattern, being higher in the NC-TS and HT-TS groups, followed by NC-WM, and HT-WM (**Table 3**).

DISCUSSION

In the present study, variation was observed between experiments conducted under similar conditions. In Experiment 3, we could not achieve horizontal transmission using the same isolate (PHL) and diet (low-nutrient density wheat middlings diet) used in the pilot experiment 2. In addition to that, variability was observed within treatments in experiments 4 and 5 conducted in battery cages, with the horizontal transmission not being observed in some cages, although having the same conditions, agreeing with the variability of horizontal transmission observed in other studies (11).

Armstrong and McDougald (11) investigated the rate of transmission of histomonosis between turkeys exposed to directly inoculated turkeys (referred to as seeders) or to contaminated cages, where directly inoculated turkeys were previously present. The authors also compared the transmission rate between turkeys raised on bare-wire cage floors, paper-covered cage floors, or on floor pens with pine shavings. Differences were not detected in the rate of transmission between

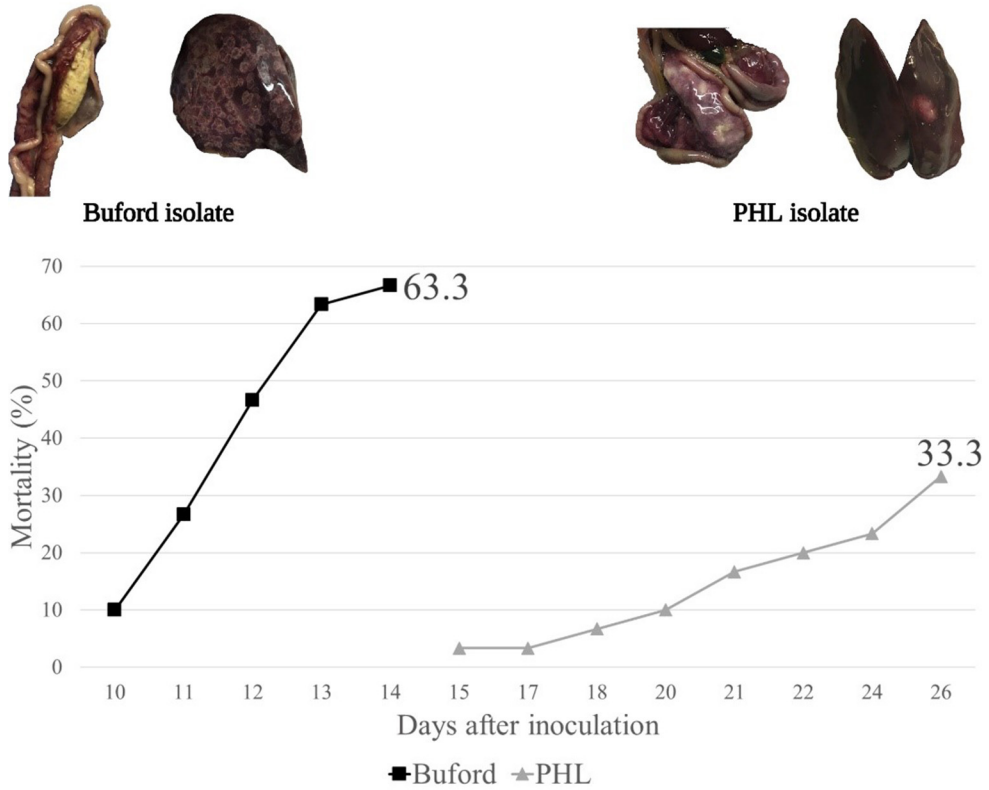

FIGURE 1 | Mortality of turkeys directly inoculated (intra-cloacal) with two different isolates of *Histomonas meleagridis* (Buford or PHL) in experiment 3. Created with BioRender.com.

groups of turkeys placed on cages covered with paper or on floor pens with pine shavings. A higher rate of transmission was observed with turkeys that had directly contacted seeders, while turkeys exposed to contaminated cages had a lower infection rate. Interestingly, in one of three experiments conducted in similar conditions, having two seeder birds and six contact turkeys in battery cages with the floor covered, contact turkeys were not infected (11). Still, the seeders developed severe lesions of histomonosis, similarly to what was observed in some cages of experiments 4 and 5 of the present study. The variation in infection rate was not explained by the authors. McDougald and Fuller (12) compared the horizontal transmission rate of histomonosis in turkeys on battery cages. Each cage had a total of eight poults, with two, three, or four of them being directly inoculated with *H. meleagridis*, and each treatment had three replicates. The horizontal transmission was achieved in all groups, with 72.2, 80.0, and 75.0% of the contacts positive for histomonosis in the groups with two, three, and four seeder turkeys, respectively. The authors also investigated the impact of the length of exposure on contamination. Poults were exposed to seeders birds (two seeders and six contacts) for one, two, three, or four days. Each treatment had two replicates. Contact turkeys presented lesions at a rate of 16.7, 100, 87.5, and 100% when exposed to seeder turkeys for 1, 2, 3, or 4 days, respectively. The authors presented the results as the average of the replicates, not stating if variation was observed between replicates. Nevertheless, no information about the diets was provided (12). In a study by Landman and colleagues (13), ten turkeys were directly inoculated with *H. meleagridis* and 30 turkeys were exposed to them for 14 days. Seeders and contact turkeys were 14 days old. All contact turkeys were positive for histomonosis (mortality or lesions) at the end of the experiment. Only one study was reported without replication. Turkeys were fed a standard turkey feed containing 2292 cal/gram and 25.8% crude protein, but once again, no dietary ingredients were reported (13).

Variability in the incidence and outcome of histomonosis in outbreaks in turkey farms is also commonly reported, with only one house within a farm with multiple houses being affected; only one sex in mixed flocks, or only one section within a house (14, 15). Sulejmanović and colleagues (15) reported three outbreaks of histomonosis in turkey houses where toms and hens were raised together but in different compartments separated by a wire mesh. In the three outbreaks, only toms were severely affected by histomonosis. At the same time, female turkeys were infected, detected by the presence of antibodies evaluated by ELISA but did not manifest clinical signs. The presence of histomonads was also confirmed in high numbers in dust samples by PCR. The authors hypothesized that the gut microbiota and a variation in the immune response between males and females could be responsible for the difference in the disease outcome

TABLE 3 | Evaluation of body weight gain (BWG) or feed intake (FI) of turkeys inoculated with two different isolates of *Histomonas meleagridis* (Buford or PHL) and fed different experimental diets in validation experiments 3, 4 and 5.

Experiment 3	BWG (d7-21)	BWG (d7-38)
NC[1]−WM[2]	233.1 ± 2.65[a]	870.9 ± 16.31[a]
NC-CS[3]	233.6 ± 5.89[a]	610 ± 11.97[b]
PC[4]-Buford-WM	176.7 ± 11.86[c]	ND
PC-PHL-WM	210.1 ± 6.95[b]	ND
Experiment 4	**BWG (d7-30)**	
NC-TS	701.0 ± 10.44[b]	ND
NC-WM	834.0 ± 20.86[a]	ND
NC-CS	524.7 ± 16.03[c]	ND
HT-TS	586.0 ± 22.63[c]	ND
HT-WM	599.7 ± 29.02[c]	ND
HT-CS	425.0 ± 15.91[d]	ND
Experiment 5	**BWG, g (d9-27)**	**FI, g (d9-27)**
NC-TS	497.7 ± 7.48[a]	751.2 ± 21.96[a]
NC-WM	307.5 ± 8.07[c]	608.3 ± 10.61[b]
HT-TS	442.5 ± 20.09[b]	761.3 ± 40.51[a]
HT-WM	215.0 ± 22.22[d]	561.7 ± 24.26[b]

[1]*NC: non-challenge control.*
[2]*WM: low nutrient density diet, wheat middlings based.*
[3]*CS: low nutrient density diet, corn-soy based.*
[4]*PC: positive control (all turkeys directly inoculated on day 10 with the Buford or PHL isolates, 10^5 histomonads/turkey, intracloacally).*
ND, not determined; d, day.
Data express as Mean ± SE. [a–c]Values within columns with different superscripts differ significantly ($P < 0.05$).

(15). Unfortunately, the stocking density was not reported in the two compartments of the house, nor the composition of the diets nor if the diets were different among males and females (15).

It is still not clear if histomonosis can be transmitted by contacting fresh feces in the litter or only by direct cloacal contact between turkeys and which factors would affect the survival of histomonads in the litter. Lotfi and colleagues (16) showed that histomonads could survive 9 h in turkey feces and non-chlorinated water, raising the possibility that the protozoa can be transmitted by contact with the litter and other environmental sources. In the present study, both pilot studies (Experiments 1 and 2) and Experiment 3 were conducted in floor pens with wood shavings as bedding material. Although it was not quantified, in pilot Experiment 2, litter moisture was apparently higher than in Experiment 3 in the floor pen of the group HT-WM. Moreover, in Experiment 3, a difference was noticed in the quality of the feces between groups fed the LOW diets, the corn-soy based or the wheat-middlings based, with turkeys from the HT-PHL-CS group presenting watery feces. Interestingly, that was the only group that experienced horizontal transmission. It is noteworthy that although the same diet formulation for the wheat middling diet was used in experiments 2, 3, 4, and 5, the nutritional composition of the diets varied and that in experiment 5, the WM diet had the addition of 3% of celite as a filler (**Supplementary Table 1a**). Another hypothesis, although not evaluated, is that the diets used in the different experiments had ingredients with different levels of mycotoxins. It is known that mycotoxins interact with the intestinal microbiota, possibly leading to dysbiosis (17), which could potentially favor the development of histomonosis. Only one study investigated horizontal transmission of histomonosis in turkeys fed a diet containing ingredients contaminated with aflatoxins (18). The influence of other mycotoxins in histomonosis is unknown and requires further investigation.

Histomonas meleagridis has a particular relationship with bacteria (19–21). Some studies suggest that bacteria can serve as a food source or provide specific compounds necessary for *H. meleagridis* survival (22–24). In other intestinal protozoa affecting human beings, such as *Entamoeba histolytica* and *Trichomonas vaginalis*, the bacteria can affect the virulence and adhesion of the protozoa (25, 26). In the case of *H. meleagridis*, the protozoa are probably influenced by the intestinal microbiota and the litter microbiota (22–24, 27). There is a direct correlation between gut and litter microbiota, and both are the reflection of the interaction between feed, ventilation, air quality, water quality, gender, among others (22–24, 27). One hypothesis is that depending on the bacteria and consequently by-products of bacterial fermentation present in the litter and ceca, the ability of *H. meleagridis* to adhere and invade host cells can be impaired. *Histomonas meleagridis* is a pleomorphic microorganism, assuming a rounded, flagellated form on the cecal lumen, transitioning to amoeboid during the invasion of tissues (28, 29). Under challenging conditions, the shape and behavior of histomonads can change to a cyst-like stage (28–30). The role of this cyst-like stage in the infectivity and transmission of histomonosis is unknown. One hypothesis is that the ability to invade tissue, causing infection, is reduced in these stages. Callait-Cardinal and colleagues (14) evaluated factors impacting the incidence and severity of histomonosis in free-range turkey flocks in France and the authors observed an interaction between hygiene and litter quality to the presence and severity of histomonosis. The authors hypothesized that higher levels of moisture in the building, caused by diarrhea, poor hygiene, and wet litter, could increase the contact between turkeys and their excreta.

To remove the variability of the litter, experiments 4 and 5 were conducted in battery cages with heavy paper covering the floor, allowing contact of turkeys with excreta. Nevertheless, variability was observed within treatments, which are puzzling findings. It could be hypothesized that the fecal moisture was different within treatments, impacting the survival or activation of histomonads; however, the position of each replicate cage in the room was randomized, therefore, a ventilation effect is not probable. Water consumption was not measured, but comparing the feed consumption of experiment 5, there was a low variation within treatments, suggesting that it is unlikely the possibility of some cages having a lower water consumption.

Interestingly, the BWG of turkeys eating the LOW, WM diet was higher than the BWG of turkeys eating the turkey starter in the experiment. The same was not observed in Experiment

5. In experiment 5, poor poult quality was observed, with 4.2% seven-day mortality. All groups were fed the turkey starter diet during the first seven days and poor poult quality reflected on the overall performance, as can be observed comparing the BWG of experiments and 5. The effect on BWG does not seem to be associated with transmission of histomonosis since, in experiment 4, turkeys in the HT-TS group had no difference in BWG compared to the HT-WM group; however, the HT-TS had a lower number of contacts presenting lesions. Poor poult quality has been linked with the severity of histomonosis (31).

Regarding the two isolates of *H. meleagridis* used in the present study, we were not able to achieve horizontal transmission with the Buford isolate, only with the PHL isolate. The Buford isolate was recovered from layer pullets around 20 years ago and supplied to us by Dr. Lorraine Fuller, University of GA, Athens. It is possible that although the isolate is still able to cause clinical disease and that initially, it led to horizontal transmission in experiments conducted by other research groups (personal communication); the isolate lost its ability to infect other turkeys by direct transmission during sequential *in vitro* passages during the years. The PHL isolate is contemporary, and it was isolated from turkeys. The Buford isolate is more virulent than the PHL isolate, causing the formation of cecal cores and inflammation of the ceca, together with severe hepatic lesions, and having a shorter incubation period compared with the PHL isolate. The PHL isolate causes severe typhlitis, much more severe than the one caused by the Buford isolate, sometimes leading to perforation of the cecal wall and peritonitis. Although the present study did not evaluate the genetic variation between the isolates Buford and PHL, based on the mortality rate, clinical signs, and lesions, it can be assumed that the isolates belong to different genetic clusters. In outbreaks in commercial flocks, usually, a combination of isolates of *H. meleagridis* can be involved in an infection, potentially explaining the variability in disease manifestation (32).

To conclude, we were able to reproduce horizontal transmission of histomonosis in four out of five experiments, more consistently on battery cages with the floors covered with paper and with diets with low-nutrient density. Transmission of histomonosis is multifactorial and not fully understood. Further studies are needed to investigate the role of litter moisture, diets, and morphological stages of *H. meleagridis* on the transmissibility of histomonosis.

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 University of Arkansas Agricultural Institutional Animal Care and Use Committee (Animal Use Protocol #19118).

AUTHOR CONTRIBUTIONS

TB and BH designed the experiments with inputs from EM, CV, JL, GT-I, and SR. TB conducted the experiments with assistance from RC, CV, JL, and GT-I. EM and SR assisted with the formulation of the experimental diets. TB, CV, RC, JL, and GT-I conducted termination of experiments and evaluated lesions. TB conducted the data analysis and wrote the manuscript with the support of all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.937102/full#supplementary-material

REFERENCES

1. Regmi PR, Shaw AL, Hungerford LL, Messenheimer JR, Zhou T, Pillai P, et al. Regulatory considerations for the approval of drugs against histomoniasis (blackhead disease) in turkeys, chickens, and game birds in the United States. *Avian Dis.* (2016) 60:725–30. doi: 10.1637/11451-061516-Review.1
2. McDougald LR. Blackhead disease (histomoniasis) in poultry: a critical review. *Avian Dis.* (2005) 49:462–76. doi: 10.1637/7420-081005R.1
3. Hess M, Liebhart D, Bilic I, Ganas P. *Histomonas meleagridis*—New insights into an old pathogen. *Vet Parasitol.* (2015) 208:67–76. doi: 10.1016/j.vetpar.2014.12.018
4. Liebhart D, Ganas P, Sulejmanovic T, Hess M. Histomonosis in poultry: previous and current strategies for prevention and therapy. *Avian Pathol.* (2017) 46:1–18. doi: 10.1080/03079457.2016.1229458
5. Hu J, McDougald LR. Direct lateral transmission of histomonas meleagridis in Turkeys. *Avian Dis.* (2003) 47:489–92. doi: 10.1637/0005-2086(2003)047[0489:DLTOHM]2.0.CO;2
6. Hauck R, Hafez HM. Experimental infections with the protozoan parasite Histomonas meleagridis: a review. *Parasitol Res.* (2013) 112:19–34. doi: 10.1007/s00436-012-3190-5
7. Bilic I, Jaskulska B, Souillard R, Liebhart D, Hess M. Multi-locus typing of Histomonas meleagridis isolates demonstrates the existence of two different genotypes. *PLoS ONE.* (2014) 9:e92438. doi: 10.1371/journal.pone.0092438

8. Wei Z, Abraham M, Chadwick EV, Beckstead RB. *Histomonas meleagridis* isolates compared by virulence and gene expression. *Vet Parasitol.* (2020) 286:109233. doi: 10.1016/j.vetpar.2020.109233
9. Hauck R, Armstrong PL, McDougald LR. *Histomonas meleagridis* (Protozoa: Trichomonadidae): analysis of growth requirements *in vitro*. *J Parasitol.* (2010) 96:1–7. doi: 10.1645/GE-1985.1
10. Beer LC, Vuong CN, Barros TL, Latorre JD, Tellez G, Fuller AL, et al. Research Note: Evaluation of boric acid as a chemoprophylaxis candidate to prevent histomoniasis. *Poult Sci.* (2020) 99:1978–82. doi: 10.1016/j.psj.2019.12.003
11. Armstrong PL, McDougald LR. The infection of turkey poults with *Histomonas meleagridis* by contact with infected birds or contaminated cages. *Avian Dis.* (2011) 55:48–50. doi: 10.1637/9568-100710-Reg.1
12. McDougald LR, Fuller L. Blackhead disease in turkeys: direct transmission of Histomonas meleagridis from bird to bird in a laboratory model. *Avian Dis.* (2005) 49:328–31. doi: 10.1637/7257-081004R.1
13. Landman WJM, ter Veen C, van der Heijden HMJF, Klinkenberg D. Quantification of parasite shedding and horizontal transmission parameters in Histomonas meleagridis -infected turkeys determined by real-time quantitative PCR. *Avian Pathol.* (2015) 44:358–65. doi: 10.1080/03079457.2015.1058483
14. Callait-Cardinal MP, Gilot-Fromont E, Chossat L, Gonthier A, Chauve C, Zenner L. Flock management and histomoniasis in free-range turkeys in France: description and search for potential risk factors. *Epidemiol Infect.* (2010) 138:353–63. doi: 10.1017/S0950268809990562
15. Sulejmanović T, Grafl B, Bilić I, Jaskulska B, Hess M, PCR. and serology confirm the infection of turkey hens and their resilience to histomonosis in mixed flocks following high mortalities in toms. *Parasit Vectors.* (2019) 12:228. doi: 10.1186/s13071-019-3482-z
16. Lotfi AR, Abdelwhab EM, HafezA HM. Persistence of *Histomonas meleagridis* in or on Materials Used in Poultry Houses. *Avian Dis.* (2012) 56:224–6. doi: 10.1637/9519-090910-ResNote.1
17. Guerre P. Mycotoxin and Gut Microbiota Interactions. *Toxins.* (2020) 12:769. doi: 10.3390/toxins12120769
18. Fudge C, Olivia Wedegaertner, Cupo K, Sigmon C, Edens F, Beckstead R, et al. Understanding the transmission of Histomonas meleagridis in turkeys. In: *2022 International Poultry Scientific Forum Abstract Book*. 2022 Jan 24-45, Atlanta, Georgia (2022). p. 11–12.
19. Ganas P, Liebhart D, Glösmann M, Hess C, Hess M. *Escherichia coli* strongly supports the growth of *Histomonas meleagridis*, in a monoxenic culture, without influence on its pathogenicity. *Int J Parasitol.* (2012) 42:893–901. doi: 10.1016/j.ijpara.2012.07.007
20. Mazumdar R, Endler L, Monoyios A, Hess M, Bilic I. Establishment of a *de novo* reference transcriptome of *Histomonas meleagridis* reveals basic insights about biological functions and potential pathogenic mechanisms of the parasite. *Protist.* (2017) 168:663–85. doi: 10.1016/j.protis.2017.09.004
21. Bilic I, Hess M. Interplay between *Histomonas meleagridis* and bacteria: mutualistic or predator–prey? *Trends Parasitol.* (2020) 36:232–5. doi: 10.1016/j.pt.2019.12.015
22. Dumas MD, Polson SW, Ritter D, Ravel J, Gelb J, Morgan R, et al. Impacts of poultry house environment on poultry litter bacterial community composition. *PLoS ONE.* (2011) 6:e24785. doi: 10.1371/journal.pone.0024785
23. Pan D, Yu Z. Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes.* (2014) 5:108–19. doi: 10.4161/gmic.26945
24. Adhikari B, Tellez-Isaias G, Jiang T, Wooming B, Kwon YM. Comprehensive survey of the litter bacterial communities in commercial turkey farms. *Front Vet Sci.* (2020) 7:596933. doi: 10.3389/fvets.2020.596933
25. Burgess SL, Gilchrist CA, Lynn TC, Petri WA. Parasitic protozoa and interactions with the host intestinal microbiota. *Infect Immun.* (2017) 85:e00101–17. doi: 10.1128/IAI.00101-17
26. Hinderfeld AS, Simoes-Barbosa A. Vaginal dysbiotic bacteria act as pathobionts of the protozoal pathogen *Trichomonas vaginalis*. *Microb Pathog.* (2020) 138:103820. doi: 10.1016/j.micpath.2019.103820
27. Bindari YR, Moore RJ, Van TTH, Hilliar M, Wu SB, Walkden-Brown SW, et al. Microbial communities of poultry house dust, excreta and litter are partially representative of microbiota of chicken caecum and ileum. *PLoS ONE.* (2021) 16:e0255633. doi: 10.1371/journal.pone.0255633
28. Munsch M, Lotfi A, Hafez HM, Al-Quraishy S, Mehlhorn H. Light and transmission electron microscopic studies on trophozoites and cyst-like stages of *Histomonas meleagridis* from cultures. *Parasitol Res.* (2009) 104:683–9. doi: 10.1007/s00436-008-1246-3
29. Gruber J, Ganas P, Hess M. Long-term *in vitro* cultivation of *Histomonas meleagridis* coincides with the dominance of a very distinct phenotype of the parasite exhibiting increased tenacity and improved cell yields. *Parasitology.* (2017) 144:1253–63. doi: 10.1017/S0031182017000646
30. Zaragatzki E, Hess M, Grabensteiner E, Abdel-Ghaffar F, Al-Rasheid KAS, Mehlhorn H. Light and transmission electron microscopic studies on the encystation of *Histomonas meleagridis*. *Parasitol Res.* (2010) 106:977–83. doi: 10.1007/s00436-010-1777-2
31. Chadwick E, Beckstead R. Two blackhead disease outbreaks in commercial turkey flocks were potentially exacerbated by poor poult quality and coccidiosis. *Avian Dis.* (2020) 64:522–4. doi: 10.1637/aviandiseases-D20-00052
32. Lund EE, Wehr EE, Ellis DJ. Earthworm transmission of *Heterakis* and *Histomonas* to turkeys and chickens. *J Parasitol.* (1966) 52:899. doi: 10.2307/3276528

Conflict of Interest: EM is employed by Cargill Turkeys LLC.

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Saponin-Rich Plant Premixture Supplementation Is as Efficient as Ionophore Monensin Supplementation Under Experimental *Eimeria spp* Challenge in Broiler Chicken

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For decades avian coccidiosis prevention was based on the use of synthetic coccidiostats. However, their intensive use led to the development of resistance phenomena. In addition, societal demand is increasing for antibiotic-free animal products. Thus, there is a need for a natural and efficient solution for coccidiosis management. Saponin-rich plants, like *Yucca schidigera* and *Trigonella foenum-graecum,* are promising tools for coccidiosis management. This study assessed the effects of supplementing broiler chickens with a commercial blend of these two plants (NorponinXO2) under an experimental *Eimeria* challenge and compared their effects to monensin supplementation. Three trials were performed. For each trial, chickens were divided into 4 groups, untreated uninfested control (UUC), infested untreated control (IUC), infested supplemented with 120 ppm of Monensin in feed (PM), and infested supplemented with 250 ppm of Norponin XO2 in the feed (PX). Chickens were raised in cages; experimental infestation was performed on d14. On d21, intestinal lesions (ILs) scores and growth performances were recorded. A statistical study was carried out on each trial, as well as data from the 3 trials. Experimental infestation reduced in a significant way final body weight in IUC broilers compared to UUC broilers. This loss was numerically compensated by PM and PX treatment. As expected, intestinal lesions were almost absent in the UUC group; however, broilers from the IUC group showed a higher intestinal lesion occurrence. Supplementations with Monensin and NPXO were able to reduce intestinal lesions occurrence. These results suggest that NPXO supplementation is as efficient as Monensin in managing coccidiosis.

Keywords: cocccidia, Eimeria spp, gut, broiler–chicken, saponin, fenugeek, Yucca (Yucca schidigera)

INTRODUCTION

As broiler production intensified, several breaks in productivity appeared; Coccidiosis is among these breaks. This disease is caused by an apicomplexan parasite of the genus *Eimeria* (1). Animal infestation by the parasite seriously impairs broilers' growth performances (reduced body weight and feed efficiency) and negatively impacts their health status and welfare (2). In addition to negatively impacting the health and welfare of chickens, coccidiosis is a disease with a serious economic impact on poultry producers. This negative impact is linked to the cost of chemoprevention and loss due to decreased animal growth performances (3, 4). In a recent study, Blake et al. estimate the cost of coccidiosis at 0,21US$/bird (5).

The use of synthetic ionophores, like monensin in the chemoprevention of coccidiosis, helped the poultry industry to reach high levels of productivity while preventing coccidiosis (Chapman, 2009). However, this intensive use of these synthetic molecules for decades led to the development of resistant strains of *Eimeria* worldwide (6–10). Moreover, residues of these molecules in animal products and/or the environment are a serious issue (11–13). Facing these new challenges, poultry producers are looking for an efficient tool to add to their global strategy in managing coccidiosis (14). Plant and plant-extract feed additives are among the interesting approaches used to control coccidiosis in broiler flocks (15). The Saponins, thanks to their ability to disrupt cellular membranes, are a promising approach to managing coccidiosis in broiler chickens (16, 17). However, the perception of the effectiveness of these solutions by poultry producers is not always as positive as conventional solutions based on synthetic molecules according to a recent market survey (internal data). The reasons evoked by the professionals during this survey to explain this perception are mainly the rarity of data evidencing their efficacy using usual experimental methods and their mode of action. This highlights the need to generate data using experimental infestation methods to evaluate new solutions for coccidiosis management. The objective of this study was to evaluate and compare the effectiveness of feed supplementation with saponin-rich plant premixture (Norponin XO2®) to ionophore monensin under various experimental *Eimeria* challenges.

MATERIALS AND METHODS

Experimental Design, Products, and Animal Management

Three trials were carried out in three experimental facilities. The same experimental design was applied in each facility. The trials respectively took place in Belgium (Wolvenhof, Poulpharm animal site), the U.S.A (Willington, Colorado, COLORADO QUALITY RESEARCH, INC.), and Spain (Murcia, IMASDE Campus de Espinardo). These three centers were chosen to consolidate the results by maximizing the diversification of the breeding system (chick origin/strain, feed material, and operators). The trials were conducted according to the principles of GCP (2000) Guidelines on Good Clinical Practice for Clinical Trials for Registration of Veterinary Medicinal Products (VICH) and met appropriate current quality standards indicated by European Food Safety Authority. The experimental protocols used in this study were approved by the competent authorities of the country for each trial. Chickens were randomly distributed to either one of four treatments: Untreated Uninfested Control, Infested Untreated Control, Infested and supplemented with recommended inclusion rate in the feed of monensin (120 ppm), Infested and supplemented with the recommended inclusion rate in the feed of Norponin XO2® (250 ppm), and a premixture of saponin-rich plants. Broilers were raised to the age of 21 days in cages. For the supplemented groups, the supplementation started on the first day of the experiment. Feed and water were distributed *ad libitum* for all trials within the 3 experimental facilities. The experimental conditions (sex, strain, the number of animals, the number of replicates, and the size of the cage) are specified in **Table 1**.

TABLE 1 | Specificity of experimental design for each trial.

Country	Spain	USA	Belgium
Breed	Ross 308	Cobb 500	Ross 308
Sex	Male	Male & Female	Male
Number of Chicken	160	224	192
Chicken per cage	5	8	8
Light program	18H/24H	24H/24H	18h/24H
Temperature	32°C	32°	32°C

Monensin was purchased from Elanco (Greenfield, Indiana, USA) and Norponin XO2® from Nor-Feed SAS (Beaucouzé, France). The basal diets were formulated to meet or exceed the nutrient requirements recommended by the breed suppliers (Aviagen and Cobb) (**Table 2**).

Experimental Eimeria Challenge

For the experimental challenge with wild-type *Eimeria spp*, oocytes from the field were used. As *Eimeria. spp* genetic background/virulence was proven to vary depending on geographical region (18), the number of sporulated oocysts per bird in the inoculum was defined by a preliminary dose titration study using susceptible broilers. This preliminary study aimed to determine the number of oocysts needed to obtain infestations leading to homogeneous lesions between the different trials and similar to those encountered in the field. Once the number of sporulated oocysts was determined, broilers were infested with sporulated oocysts in suspension by oral gavage at D14. The number of sporulated oocysts/birds is shown in **Table 3**. Chicks within the UUC group were gavaged with the same volume of distilled water.

Data Collection

Broilers were weighed per cage on days 1 and 21 to estimate BW. Feed intake was measured daily. These data were used to estimate average daily gain (**ADG**), average daily feed intake (**ADFI**), Feed Conversion Ratio (**FCR**), and European Production Efficiency Factor [**EPEF**, (19)]. EPEF is defined as follows:

TABLE 2 | Diet composition and nutrient levels (as-feed basis) according to age and trial.

Country	Belgium	Belgium	Spain	Spain	USA
Feed	Starter	Grower	Starter	Grower	Starter/grower
Age (d)	1–14	15–21	1–14	15–21	1–21
Nutrient Level					
AME_n (kcal/kg)	2 958	3 109	3 152	3 266	3150
Crude protein	216.9	201.9	217.6	192.7	220.0
Lysin (g/kg)	12.7	11.9	13.6	12.0	13.5
Met (g/kg)	5.6	5.2	6.3	5.3	7.1
Met+Cys (g/kg)	9.2	8.7	10.0	8.6	10.1
Ca (g/kg)	9.0	7.2	9.0	9.1	9.6
P (g/kg)	6.4	5.4	6.6	6.3	5.9

$$EPEF = \frac{viability\ (\%) \times BW\ (kg)}{age\ (d) \times FCR\ (kg\ feed\ /\ kggain)} \times 100 \quad (1)$$

Dead chicks were counted and removed daily. Broilers were euthanized at 21 days old to proceed to intestinal lesions scoring according to the scoring system published by Johnson and Reid (20).

Statistical Analysis

Data were analyzed using R (21) with *emmeans* and *nnet* packages. All quantitative data were analyzed using the following model:

$$Y_{ijk} = Treatment_i + Trial_j + Treatment : Trial_{ij} + \epsilon_{ijk} \quad (2)$$

where Y_{ijk} was a dependent variable of a repetition k, within treatment *i* and trial j. Treatment, trial, and their interaction were fixed factors, due to the low number of levels within each factor (22). All observations were weighted according to the number of broilers they represented. Body weight gain (**BWG**), ADG, ADFI, FCR, and EPEF were analyzed using a linear model. Results are reported as means and standard error of the means (mean ± SEM). Individual survival analysis and scores for the intestinal lesions were analyzed using logistic regression, results being reported as odds ratio (**OR**) to the base level and confidence interval (CI) [OR (lower confidence interval—upper confidence interval)]. Values for OR are given in scientific notation as they may be highly variable. The considered base level was IUC treatment for both cases, with a 0 score of lesions and being alive for survival analysis as standard. This allowed us to ensure that inoculation was successful in comparison to UUC treatment, and to assess the effects of both PX and PM treatments. Belgium was randomly chosen as a basal level for the trial effect. Survival analyses were analyzed using the same model as for quantitative data when the interaction was removed for the analysis of lesion scores to avoid error due to levels of scores not being presented for the given trial and treatment. A *P*-value of <0.05 was used to indicate statistical significance, and between 0.05 and 0.1 was used to indicate a tendency.

TABLE 3 | Inoculum composition.

	E. acervulina/bird	*E. maxima*/bird	*E. tenella* / bird
Belgium	52 000	10 000	17 500
Spain	65 000	6 500	15 000
USA	100 00	50 000	75 000

RESULTS

Growth Performances

Initial BWs were not significantly different between treatments within a trial (P = 0.75 for trial x treatment interaction). The global analysis of from the 3 trials showed that BWs at the end of trials was higher ($P < 0.05$) in the UUC group than in the IUC group, with PX and PM being intermediate but not significantly different from other treatments ($P > 0.10$) (**Table 1**). The difference at the trial level was only numerical without statistical significance (**Table 5**). There was no significant difference due to treatments either in global analysis or trial level for ADG (P = 0.16), ADFI (P = 0.57), FCR (P = 0.24). or EPEF (P = 0.57) or showing similar growth performances results between treatments (**Tables 4**, **5**). There was a tendency for ADFI (P = 0.10) for chicks within PX treatment to have a lower feed intake in comparison to chicks in PM treatment (48 ± 1.44 g vs. 52.8 ± 1.46 g), but this did not affect BWG or FCR. The mortality rate was very low, around 4%, whatever the considered effects. Survival analysis using logistic regression showed no differences in death OR due to treatment effect ($P = 0.17$), trial effect ($P = 0.29$), or their interaction ($P = 0.53$). This was due to the low number of deaths within each treatment. Survival rates were always higher than 90%, whatever the treatment or the trial.

Lesion Scores

Odds of the apparition of lesions (Score 0 vs. Score 1 or higher) were affected by experimental treatments (**Figure 1**). Apparition of lesions due to *E. acervulina* was significantly lower for the UUC treatment ($P < 0.001$) in comparison to the IUC treatment. The OR was 3.21e-2 [1.25e-2–7.55e-2]. Odds of the apparition of the lesion were similar to IUC treatment for PM and PX groups, with

TABLE 4 | Growth performances global analysis.

	Body Weight Gain (kg)	FCR	EPEF
UUC	0,84 ± 0,04 [a]	1,39 ± 0,02 [a]	269 ± 15 [a]
IUC	0,80 ± 0,03 [b]	1,42 ± 0,02 [a]	261 ± 12 [a]
PM	0,82 ± 0,03 [ab]	1,39 ± 0,02 [a]	273 ± 15 [a]
PX	0,81 ± 0,04 [ab]	1,41 ± 0,03 [a]	272 ± 16 [a]

Values expressed as mean ± SEM [statistical group].
Body weight gain, FCR, Feed conversion ratio; EPEF, European poultry efficiency factor; UUC, Untreated uninfested control; IUC, Infested Untreated control; PM, Infested monensin treated; PX, infested Norponin XO treated.

TABLE 5 | Growth performance per trial.

		Body Weight Gain (kg)	FCR	EPEF
Belgium	UUC	1,13 ± 0,04 [a]	1,33 ± 0,02 [a]	378 ± 20 [a]
	IUC	1,03 ± 0,02 [a]	1,35 ± 0,02 [a]	348 ± 13 [a]
	PM	1,08 ± 0,03 [a]	1,30 ± 0,03 [a]	376 ± 26 [a]
	PX	1,10 ± 0,02 [a]	1,33 ± 0,02 [a]	395 ± 11 [a]
Spain	UUC	0,76 ± 0,03 [a]	1,47 ± 0,02 [a]	219 ± 6 [a]
	IUC	0,72 ± 0,01 [a]	1,53 ± 0,03 [a]	211 ± 8 [a]
	PM	0,76 ± 0,01 [a]	1,53 ± 0,03 [a]	220 ± 7 [a]
	PX	0,70 ± 0,03 [a]	1,56 ± 0,05 [a]	202 ± 13 [a]
USA	UUC	0,73 ± 0,02 [a]	1,35 ± 0,01 [a]	244 ± 8 [a]
	IUC	0,72 ± 0,01 [a]	1,36 ± 0,01 [a]	249 ± 6 [a]
	PM	0,72 ± 0,01 [a]	1,33 ± 0,01 [a]	254 ± 6 [a]
	PX	0,74 ± 0,02 [a]	1,35 ± 0,01 [a]	255 ± 5 [a]

Values expressed as mean ± SEM [statistical group].
Body weight gain, FCR, Feed conversion ratio; EPEF, European poultry efficiency factor; UUC, Untreated uninfested control; IUC, Infested Untreated control; PM, Infested monensin treated; PX, infested Norponin XO treated.

respectively OR of 4.17e-1 [1.77e-1–9.38e-1] and 5.35e-1 [2.25e-1–1.23e+0], even OR for PM treatment was significantly lower. The trial effect was significant ($P < 0.001$), but there was no statistical difference when comparing OR.

Odds of apparition of lesions due to *E. maxima* were lower for UUC, PM, and PX groups in comparison to IUC group, with respective OR of 3.68e-3 [6.91e-4–1.46- 2], 1.30e-1 [3.34e-2–4.22-1] and 2.86e-1 [7.23e-2–9.44e-1]. Trial effects were also highly significant ($P < 0.01$), with higher odds of lesion apparition in the USA and Spain trials, with OR of 8.39e+0 [2.99e+0–2.83 e+1] and 3.89e+1 [1.28e+1–1.53e+2], respectively.

Treatment and trial effects significantly affected the odds of apparition of lesions due to *E. tenella* ($P < 0.001$). Like for the two other types of lesions, UUC treatment had the lowest OR, with a value of 1.52e-2[5.15e-3–3.97 e-2]. PM and PX groups also had lower odds of apparition than IUC group, with respective OR of 1.94e-1 [7.75e-2–4.49e-1] and 3.34e-1 [1.4e-1–8.41e-1]. The USA and Spain trials had higher OR of lesions apparition, with values of 3.01e0 [1.58e0–5.92e0] and 4.49e0 [1.99e0–1.08e1], respectively.

Analysis of the score of the lesions showed significant effects of trial and treatments ($P < 0.001$), whatever the considered type of lesion. Estimations and confidence intervals for each modality of treatment effect and each level of lesions are summarized in **Figure 1**. UUC treatment always had the lowest OR, whatever the level or type of lesions. For a score of 3+, estimations of UUC treatment were inefficient, infinite, or had a very large confidence interval, as this score was never observed for this treatment, whatever the trial. PM and PX had similar results on the score of lesions, reducing the odds of a higher score, 2 or 3+, in comparison to IUC treatment.

DISCUSSION

Successful completion of an *Eimeria. spp* challenge is important for the interpretation of treatment results. In this study, we can state that the *Eimeria. spp* challenge was successful as evidenced by the significant decrease in final body weight between infested and non-infested chickens, even though the effects on other performance parameters, FCR, and EPEF, were not statistically significant. Most interestingly, the appearance of intestinal lesions was higher in infested chickens compared to non-infested chickens. The drop in final body weight caused by the experimental challenge in the IUC broilers tended to be compensated by the supplementation of monensin and saponin-rich plant premixture globally. At the trial level, there were some disparities (**Table 5**). However, this global compensation was only numerical and not statistically significant. It is well-known and documented that feed supplementation with monensin compensates for the loss of performance due to coccidiosis in broiler chicken (23). However, data remain scarce concerning the effect of the saponin-rich plant, *Yucca schidigera,* and *Trigonella foenum greacum* in *Eimeria. Spp* challenge conditions. The few available data report different results and conclusions. Our results are in line with recently published data concerning the use of saponin-rich plants (*Quillaja.s*) by the Bafundo team and those published by Saeed et al. (*Yucca.s*). (24, 25). On the other hand, a recent study shows that saponin-rich plant supplementation (*Yucca.s*) does not have a significant compensation effect on the loss of performance of chickens infested by *Eimeria* (26); thus, contradicting obtained data from the present study. An element that could explain this variability of results could be that the concentration of active compounds in the saponin-rich plants used is different from one study to another. Indeed, the concentration of these active compounds can be more or less high according to several parameters (type of plant, part of the plant used, harvesting period, etc.). In addition, unlike some cited studies, we used a formulation of 2 saponin-rich plants, namely, *Yucca.s* and *Trigonella. f.g.,* thus, making the comparison of the obtained results quite difficult to the available scientific literature. This fact also highlights the importance of transparency and standardization in active compounds when natural plant-based products are used in animal nutrition. (27). The complexity of the parasites and the pathophysiology of coccidiosis infection can also play an important role in the observed variability of the results. In

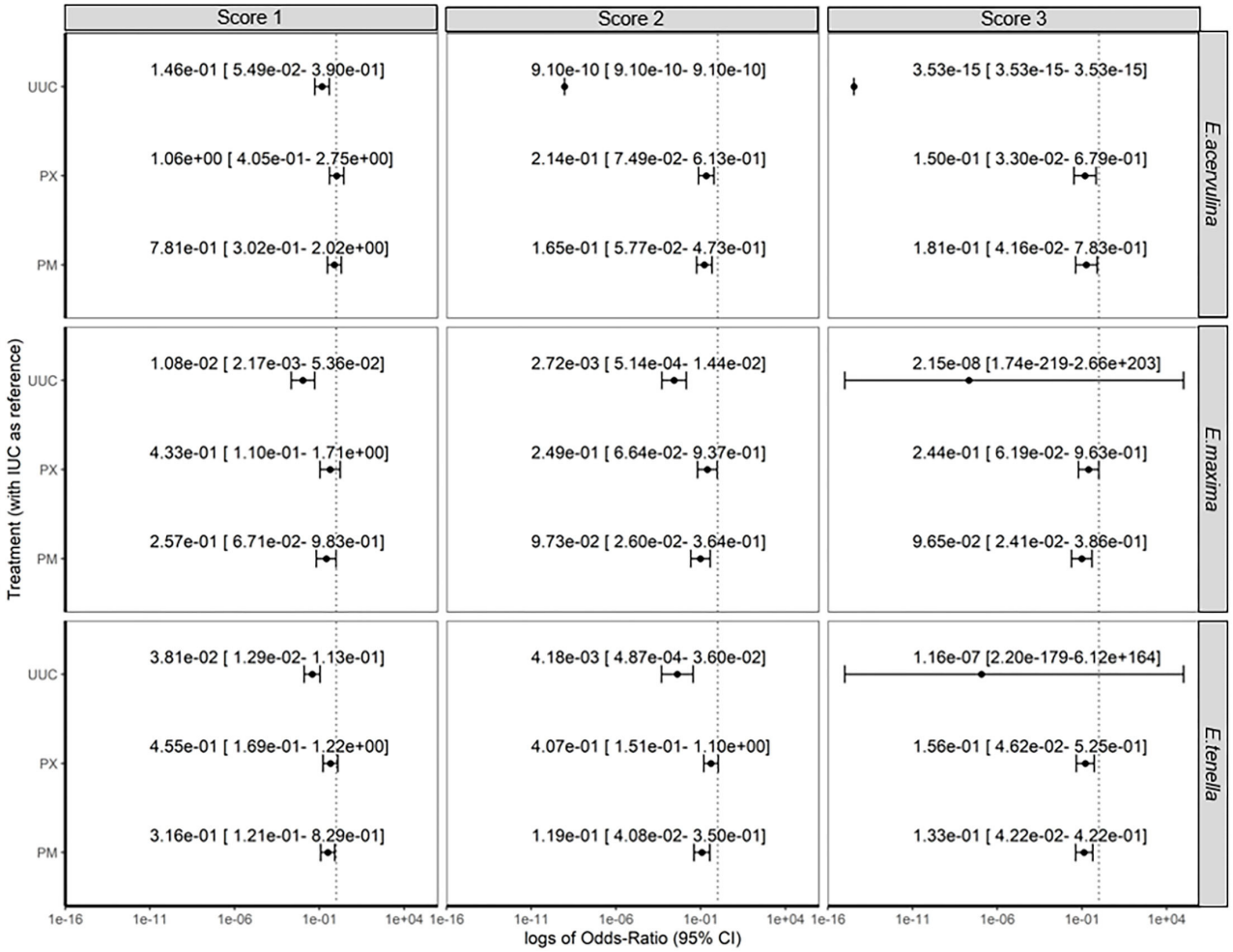

FIGURE 1 | Odds of the apparition of lesions.

this study, we observed that both treatments (Monensin and Norponin XO2) reduced the appearance of intestinal lesions due to experimental *Eimeria* infestation. If these observations are well-documented for monensin supplementation in chicken (28, 29), data dealing with saponins deserve to be reinforced. The decrease in the occurrence of intestinal lesion scores for the saponin-rich plant treatment can be explained by the direct action of saponins. Indeed, saponins have the property to disrupt the cellular membrane of the parasite, thanks to their permeabilization effects (30). Interacting with the parasite and disrupting its membrane leading to the loss of its homeostasis could be a possible mechanism of action of saponin. Another mechanism of action of saponins can be the inhibition of the invasion step of the parasite. Felici et al. (31) evidenced the fact that saponins can inhibit the invasion process of the parasite. However, the cited studies are mainly *in vitro* studies that did not consider the possible degradation of the natural active compounds of saponin-rich plants. Moreover, in addition to saponin, these plants contain other actives compounds like flavonoids that certainly plays a role in the observed effect on animal and intestinal lesions. Thus, making the investigation of the mechanism of action of saponin-rich plants quite challenging. More studies are needed to better understand the mechanism of action behind the observed effects. Nevertheless, these experimental results showed that a 100% plant-based solution can be as efficient as a conventional coccidiostat in managing coccidiosis; thus, offering more agility for broiler chicken producers. Particulary, studies have shown that the use of alternative solutions to chemoprevention helps to restore the effectiveness of molecules, such as monensin and duclazuril (32, 33). Therefore, introducing a plant-based solution and coccidiosis vaccines could help to solve the resistance problem observed and described all over the world. However, we believe that in addition to the efficient and natural solution, there

is a need to rethink coccidiosis management. Parameters like nutritional management (34), biosecurity, and chicken strain selection could help to raise chickens while limiting the use of synthetic and/or ionophore coccidiostats.

This study evidenced the fact that a natural solution formulated from *Yucca schidigera* and *Trigonella foenum-graecum* (Norponin XO2) is as efficient as a monensin supplementation in managing coccidiosis in an experimental-infestation model. These results should be confirmed in the field. Altogether, these elements will certainly help to achieve the ultimate goal of producing a sustainable broiler chicken at a reasonable cost for the continuously growing number of humans on earth.

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 IMASDE and POULPHARME Commity.

AUTHOR CONTRIBUTIONS

PC and CM reviwed the article. MB wrote the article. Statistical analysis made by PG and CM. Experiments were setup by MB. PC and MB made the experimental design. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Shivaramaiah C, Barta JR, Hernandez-Velasco X, Téllez G, Hargis BM. Coccidiosis: recent advancements in the immunobiology of *Eimeria* species, preventive measures, and the importance of vaccination as a control tool against these apicomplexan parasites. *Vet Med (Auckl).* (2014) 5:23–34. doi: 10.2147./VMRR.S57839
2. Butterworth A, Weeks C. The impact of disease on welfare. In: Duncan I, HawkinP, editors. *The Welfare of Domestic Fowl and Other Captive Birds. Animal Welfare, Vol. 9.* Dordrecht: Springer (2010). p. 189–218. doi: 10.1007/978-90-481-3650-6_8
3. Dalloul RA, Lillehoj HS. Poultry coccidiosis: recent advancements in control measures and vaccine development. *Expert Rev Vaccines.* (2006) 5:143–63. doi: 10.1586/14760584.5.1.143
4. Chapman HDA. Landmark contribution to poultry science–prophylactic control of coccidiosis in poultry. *Poult Sci.* (2009) 88:813–5. doi: 10.3382/ps.2008-00316
5. Blake DP, Knox J, Dehaeck B, Huntington B, Rathinam T, Ravipati V, et al. Re-calculating the cost of coccidiosis in chickens. *Vet Res.* (2020) 51:115. doi: 10.1186./s13567-020-00837-2
6. Jeffers TK, Acervulina E, Maxima E. Incidence and anticoccidial drug resistance of isolants in major broiler-producing areas avian. *Diseases.* (1974) 18:331–42. doi: 10.2307/1589101
7. Jeffers TK. *Eimeria* tenella: incidence, distribution, and anticoccidial drug resistance of isolants in major broiler-producing areas. *Avian Dis.* (1974) 18:74–84. doi: 10.2307/1589244
8. Chapman HD. Drug resistance in avian coccidia (a review). *Vet Parasitol.* (1984) 15:11–27. doi: 10.1016/0304-4017(84)90106-7
9. Peek HW, Landman WJ. Resistance to anticoccidial drugs of Dutch avian *Eimeria spp*. field isolates originating from 1996, 1999 and 2001. *Avian Pathol.* (2003) 32:391–401. doi: 10.1080/0307945031000121149
10. Peek HW, Landman WJ. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. *Vet Q.* (2011) 31:143–61. doi: 10.1080/01652176.2011.605247
11. Li P, Wu Y, Wang Y, Qiu J, Li, Y. Soil behaviour of the veterinary drugs lincomycin, monensin, and roxarsone and their toxicity on environmental organisms. *Molecules.* (2019) 24:24244465. doi: 10.3390./molecules24244465
12. Mainero Rocca L, Gentili A, Pérez-Fernández V, Tomai, P. Veterinary drugs residues: a review of the latest analytical research on sample preparation and LC-MS based methods. *Food Addit Contam Part A Chem Anal Control Expo Risk Asses.* (2017) 34:766–84. doi: 10.1080/19440049.2017.1298846
13. Mortier L, Huet AC, Charlier C, Daeseleire E, Delahaut P, Van Peteghem C. Incidence of residues of nine anticoccidials in eggs. *Food Addit Contam.* (2005) 22:1120–5. doi: 10.1080/02652030500199355
14. Quiroz-Castañeda RE, Dantán-González E. Control of avian coccidiosis: future and present natural alternatives. *Biomed Res Int.* (2015) 2015:430610. doi: 10.1155./2015/430610
15. El-Shall NA, El-Hack MEA, Albaqami NM, Khafaga AF, Taha AE, Swelum AA, et al. Phytochemical control of poultry coccidiosis: A review poultry *Science.* (2022) 101:101542. doi: 10.1016/j.psj.2021.101542
16. Bozkurt M, Giannenas I, Küçükyilmaz K, Christaki E, Florou-Paneri P. An update on approaches to controlling coccidia in poultry using botanical extracts. *Br Poult Sci.* (2013) 54:713–27. doi: 10.1080/00071668.2013.849795
17. Roussel P, Arnaiz V, Rodríguez V, García JM, Honorio Javes C, Chicoteau P. Replacing the coccidiostats: defining cost-effective surrogates. In: *Planta Medica International Open.* Verlag KG: Georg Thieme (2017).
18. Chengat Prakashbabu B, Venkatachalam T, Limon G, Kundu K, Kumar S, Garg R, et al. *Eimeria* species occurrence varies between geographic regions and poultry production systems and may influence parasite genetic diversity. *Vet Parasitol.* (2017) 233:62–72. doi: 10.1016/j.vetpar.2016.12.003
19. Marcu A, Vacaru-Opris L, Dumitrescu G, Ciochina LP, Marcu A, Nucula M, et al. The influence of the genotype on economic efficiency of broiler chickens growth scientific papers animal science and biotechnologies. *Anim Sci Biotechnol.* (2013) 46:339–46.
20. Johnson J, Reid WM. Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens. *Exp Parasitol.* (1970) 28:30–6. doi: 10.1016/0014-4894(70)90063-9
21. R Core Team. *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing, Vienna, Austria (2008).
22. Bell A, Fairbrother M, Jones K. Fixed and random effects models: making an informed choice. *Qual Quant.* (2019) 53:1051–74. doi: 10.1007/s11135-018-0802-x
23. Chapman HD, Jeffers TK, Williams RB. Forty years of monensin for the control of coccidiosis in poult.ry *Poult Sci.* (2010) 89:1788–801. doi: 10.3382/ps.2010-00931
24. Saeed M, Arain MA, Naveed M, Alagawany M, Abd El-Hack ME, Bhutto ZA, et al. Yucca schidigera can mitigate ammonia emissions from manure and promote poultry health and production. *Environ Sci Pollut Res.* (2018) 25:35027–33. doi: 10.1007/s11356-018-3546-1
25. Bafundo KW, Gomez L, Lumpkins B, Mathis GF, McNaughton JL, Duerr I, et al. Concurrent use of saponins and live coccidiosis vaccines: the influence of a quillaja and yucca combination on anticoccidial effects

and performance results of coccidia-vaccinated broilers. *Poult Sci.* (2020) 100:100905. doi: 10.1016/j.psj.2020.12.010
26. Oelschlager ML, Rasheed MSA, Smith BN, Rincker MJ, Dilger RN. Effects of Yucca schidigera-derived saponin supplementation during a mixed *Eimeria* challenge in broilers. *Poult Sci.* (2019) 98:3212–22. doi: 10.3382/ps/pez051
27. Scheurer W, Spring P, Maertens, L. Effect of 3 dietary phytogenic products on production performance and coccidiosis in challenged broiler chickens. *J Appl Poult Res*. (2013) 22:591–9. doi: 10.3382/japr.2013-00726
28. Moraes PO, Cardinal KM, Gouvêa FL, Schroeder B, Ceron MS, Lunedo R, et al. Comparison between a commercial blend of functional oils and monensin on the performance and microbiota of coccidiosis-challenged broilers. *Poult Sci.* (2019) 98:5456–64. doi: 10.3382/ps/pez345
29. Bampidis V, Azimonti G, Bastos ML, Christensen H, Dusemund B, Kos Durjava M, et al. Safety and efficacy of ElancobanR G200 (monensin sodium) for chickens for fattening, chickens reared for laying and turkeys. *EFSA J.* (2019) 17:5891. doi: 10.2903./j.efsa.2019.5891
30. Lorent J, Le Duff CS, Quetin-Leclercq J, Mingeot-Leclercq MP. Induction of highly curved structures in relation to membrane permeabilization and budding by the triterpenoid saponins, α- and δ-Hederin. *J Biol Chem.* (2013) 288:14000–17. doi: 10.1074/jbc.M112.407635
31. Felici M, Tugnoli B, Ghiselli F, Massi P, Tosi G, Fiorentini L, et al. In vitro anticoccidial activity of thymol, carvacrol, and saponins. *Poult Sci.* (2020) 99:5350–5. doi: 10.1016/j.psj.2020.07.035
32. Chapman HD, Jeffers TK. Vaccination of chickens against coccidiosis ameliorates drug resistance in commercial poultry production. *Int J Parasitol Drugs Drug Resist.* (2014) 4:214–7. doi: 10.1016/j.ijpddr.2014.10.002
33. Peek HW, Landman WJ. Higher incidence of *eimeria spp* field isolates sensitive for diclazuril and monensin associated with the use of live coccidiosis vaccination with paracox^{TM}-5 in broiler farms. *Avian Dis.* (2006) 50:434–9. doi: 10.1637/7486-121205R.1
34. Rochell SJ, Parsons CM, Dilger R. Effects of *Eimeria* acervulina infection severity on growth performance, apparent ileal amino acid digestibility, and plasma concentrations of amino acids, carotenoids, and α1-acid glycoprotein in broilers. *Poult. Sci.* (2016) 95:1573–81. doi: 10.3382/ps/pew035

Conflict of Interest: MB and PC work in the R&D departement of Nor Feed commercialize products based on sapoinns.

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.

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Potential of ferritin 2 as an antigen for the development of a universal vaccine for avian mites, poultry red mites, tropical fowl mites, and northern fowl mites

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Introduction: Poultry red mites (PRMs, *Dermanyssus gallinae*), blood-sucking ectoparasites, are a threat to the poultry industry because of reduced production caused by infestation. In addition, tropical fowl mites (TFMs, *Ornithonyssus bursa*) and northern fowl mites (NFMs, *Ornithonyssus sylviarum*) are hematophagous, distributed in various regions, genetically and morphologically close to PRMs, and cause similar problems to the poultry industry. Vaccine approaches have been studied for PRM control, and several molecules have been identified in PRMs as candidates for effective vaccine antigens. The development of an anti-PRM vaccine as a universal vaccine with broad efficacy against avian mites could improve the productivity of poultry farms worldwide. Molecules that are highly conserved among avian mites and have critical functions in the physiology and growth of mites could be ideal antigen candidates for the development of universal vaccines. Ferritin 2 (FER2), an iron-binding protein, is critical for the reproduction and survival of PRMs and has been reported as a useful vaccine antigen for the control of PRMs and a candidate for the universal vaccine antigen in some tick species.

Method and results: Herein, we identified and characterized FER2 in TFMs and NFM. Compared with the sequence of PRM, the ferroxidase centers of the heavy chain subunits were conserved in FER2 of TFMs and NFMs. Phylogenetic analysis revealed that FER2 belongs to clusters of secretory ferritins of mites and other arthropods. Recombinant FER2 (rFER2) proteins from PRMs, TFMs, and NFMs exhibited iron-binding abilities. Immunization with each rFER2 induced strong antibody responses in chickens, and each immune plasma cross-reacted with rFER2 from different mites. Moreover, mortality rates of PRMs fed with immune plasma against rFER2 from TFMs or NFMs, in addition to PRMs, were higher than those of control plasma.

Discussion: rFER2 from each avian mite exhibited anti-PRM effects. This data suggests that it has the potential to be used as an antigen candidate for a universal vaccine against avian mites. Further studies are needed to access the usefulness of FER2 as a universal vaccine for the control of avian mites.

KEYWORDS

poultry red mite, tropical fowl mite, northern fowl mite, ferritin 2, vaccine

Introduction

The poultry red mite (PRM, *Dermanyssus gallinae*), is a harmful ectoparasite for poultry and is prevalent worldwide (1). Blood feeding by PRMs leads to reduced animal welfare and serious economic losses on poultry farms. Tropical fowl mites (TFMs, *Ornithonyssus bursa*) and northern fowl mites (NFMs, *Ornithonyssus sylviarum*) are ectoparasites of untamed birds (2), and are widely spread as key pests of poultry because of their introduction to farms *via* wild birds (3). Once invaded, these mites can persist long-term in farm facilities and poultry (4). PRMs feed on the host blood for a short period, mainly at night, and leave the hosts after blood feeding, residing in cracks and crevices for the rest of the time (5). In contrast, TFMs and NFMs are parasitic on the hosts throughout their life cycle (6). PRMs are widespread worldwide, with >46% of poultry farms in China and Japan (7) and 90% of the layer industry in Europe being affected (8). TFMs are cosmopolitan in tropical and subtropical countries (7, 9). Although the issues caused by NFMs are not uniform across the world, they are included as key pests in the poultry industry in North America, Brazil, Australia, and Asia (7, 10–12).

The success of acaricide treatment is hindered by the selection of mites resistant to acaricides owing to prolonged or improper application on farms (13). Because of the diminished efficacy of commercially available acaricides, they may have short residual action on mites. Consequently, the subclinical stages of mites or their eggs could enable a cycle of mite repopulation on farms (8). Some natural products, such as essential oils and plant derivatives, have been studied for their non-chemical acaricidal effects (14, 15). However, they may contain some active ingredients and may be harmful to humans and animals (16). Currently, vaccine-based control strategies are considered promising. Several recombinant protein-based anti-PRM vaccines have been reported by our research group (17–21) and other groups (22–25). Immunization with anti-PRM vaccines induces antigen-specific immunoglobulin (Ig)-Y in chickens, leading to reduced PRM survival in *in vivo* or *in vitro* studies. However, vaccine efficacy has not been sufficient for practical use in farms (23, 26). Therefore, the search for more effective antigens against PRMs is required, and antigens with broad protective efficacies across avian mites are more suitable for reducing the economic losses on poultry farms in various areas than those with limited protective efficacies.

For research on anti-tick vaccine development, molecules that are in direct contact with the host during blood feeding and are required to create an environment for blood feeding (exposed antigens) and molecules that are not exposed to the host and have essential physiological functions for the mites (concealed antigens) are considered vaccine candidates (27). Similar to the strategy of anti-tick vaccines, the development of anti-mite vaccines should focus on molecules involved in the key physiological functions of avian mites. As blood meal is the nutrient source for blood-sucking ectoparasites, including avian mites, the molecules involved in blood digestion and acquisition of essential nutrients could be suitable candidates for vaccine antigens with a broad spectrum across hematophagous avian mites. Iron is an essential nutrient for blood-feeding ectoparasites; however, the excessive presence of iron could be toxic. Therefore, iron homeostasis must be precisely controlled in the blood-feeding ectoparasites. Ferritin (FER), an iron-binding protein, is involved in iron homeostasis in most organisms (28). Two types of FER, FER1 and FER2, have been identified in ticks (29, 30), and both are fundamentally involved in blood feeding, reproduction, iron transport, and antioxidant defense (31). FER1 plays a role in intracellular iron storage and serves as an antioxidant by sequestering excess intracellular iron, whereas FER2 is a secreted ferritin that plays a role in the transportation of iron to peripheral tissues (29). In PRMs, two FERs have been identified, and their detrimental effects on survival, reproduction, and blood digestion have been demonstrated by RNA interference (RNAi) assays. Furthermore, both ferritins showed acaricidal potentials as vaccine antigens; importantly, the survival rate of PRMs fed with the plasma of chickens immunized with rFER2 (rDg-FER-1 in the original study) was significantly reduced compared with those of the rFER1-immunized group (rDgFER-2 in the original study) (25). Therefore, the potential of FER2 as a vaccine antigen has induced our interest in the development of a universal vaccine with broad-spectrum efficacy across mite species.

Development of a universal vaccine is of significant importance in veterinary practice. The successful application of universal vaccines offers cost-effectiveness by reducing the number of vaccine antigens, because there is no requirement to prepare species-specific antigens. A vaccine using Bm86, which has the potential to cross-react with different species of ticks, has been highlighted as a benefit to the livestock industry (32, 33). Additionally, glutathione S-transferase (34, 35), FER2 (27, 36), and subolesin (37) have been reported as vaccine antigen candidates for cross-species universal vaccines. However, the number of tick species for which the vaccine showed efficacy is limited and its effectiveness at various developmental stages has not yet been established (38). As for the control of avian mites, the development of vaccines against PRMs has progressed, whereas there are no reports of vaccine development against other avian mites, such as TFMs and NFMs, to the best of our knowledge. Therefore, the development of a cross-protective vaccine could be a sustainable management strategy for avian hematophagous mites on poultry farms, and it may save the economic losses on poultry farms and improve the cost-effectiveness of commercial production. Thus, in the present study, we aimed to investigate the potential of FER2 as a common antigen for developing a universal vaccine for avian mites. We identified the *FER2* genes from TFMs and NFMs, evaluated the iron-binding ability of each rFER2, and investigated the cross-reactivity of immune plasmas against each rFER2 with the rFER2 of different mites to assess the acaricidal potential of immunization with rFER2 for avian mites. Additionally, the acaricidal effects on PRMs by the immune plasmas from TFMs and NFMs were assessed.

Material and methods

Sample availability, RNA extraction, and complementary DNA synthesis

PRMs were collected into a TubeSpin Bioreactor 600 bottle (TPP Techno Plastic Products AG, Trasadingen, Switzerland) from

egg-laying farms contaminated with PRMs in Japan and transferred to the laboratory at 4°C. PRMs were kept at 25°C for a week without blood feeding, designated as starved PRMs, and stored at 5°C for further use. TFMs and NFMs, collected in the Republic of the Union of Myanmar (Burma), which were morphologically and genetically characterized in a previous study (39), were used for analysis in this experiment. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of isolated RNA using PrimeScript Reverse Transcriptase (Takara Bio Inc., Shiga, Japan) and 200 pmol of oligo (dT)18 primer (Hokkaido System Science, Hokkaido, Japan). The synthesized cDNAs was treated with DNase I (Invitrogen) to remove unwanted DNA.

Identification of *ferritin 2* genes from TFMs and NFMs

To determine the open reading frames (ORFs) of *FER2* from TFMs and NFMs, partial segments were amplified using primers designed based on the conserved region of *FER2* from *Dermanyssus gallinae* (HZ459285) and *Varroa destructor* (XM022808086). Primers used in this study are listed in Supplementary Table 1. The amplified fragments were cloned into a pMD20 vector (Takara Bio Inc.). Nucleotide sequences were analyzed using the CEQ GeXP automated sequencer (Beckman Coulter Inc., Brea, CA, USA). The primers used for the 3′ and 5′ RACE polymerase chain reaction (PCR) amplifications were designed based on the partial sequences of *FER2*. We conducted 3′ and 5′ RACE PCR using the RACE system (Invitrogen) according to the manufacturer's instructions. The PCR products were separated by agarose gel electrophoresis, purified, and cloned into the pGEMT-Easy vector (Promega, Madison, WI, USA).

Genetic characterization of *FER2*

Homologies of *FER2* genes of TFMs and NFMs with reported sequences (Supplementary Table 2) from the National Center for Biotechnology Information gene bank were compared using the Basic Local Alignment Search Tool program. We constructed a phylogenetic tree using the nucleotide sequences of the *FER2* genes of arthropods, including other mites, ticks, and chickens, and their sequences, using MEGA X software (40). These sequences were aligned using the MUSCLE (codon) option. A maximum-likelihood phylogenetic tree was constructed using the same software with 1,000 bootstrap replicates and a discrete gamma distribution (+G) to improve the tree topology.

Expression and purification of recombinant ferritin 2 proteins

The coding regions of *FER2* genes were amplified with Taq polymerase (Takara Bio Inc.) using specific primers containing the sites of NdeI and XhoI for introduction into the pET19b vector (Merck & Co., Inc., Rahway, NJ, USA) (Supplementary Table 1). The amplified fragments were cloned into the pET19b vector (Merck) and transformed into *Escherichia coli* strain Rosetta-gami B (DE3, pLysS) (Merck). We generated N-terminal His-tagged rFER2 proteins of PRMs, TFMs, and NFMs using the *E. coli* expression system, termed as rFER2 PRM, rFER2 TFM, and rFER2 NFM, respectively. For rFER2 PRM generation, the reference sequence of FER2 (HZ459284) from Japan was used. Recombinant protein expression and purification were performed according to the manufacturer's instructions. The cell pellets were fractionated with BugBuster solution (Merck), and the insoluble fractions were solubilized in the buffer containing 0.3% N-lauroylsarcosine, 50-mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) (Merck) (pH 11.0). Recombinant proteins were purified from insoluble fractions using Ni Sepharose™ 6 Fast Flow resin (GE Healthcare, Chicago, IL, USA) according to the manufacturer's instructions. The recombinant proteins were eluted with 0.3% N-lauroylsarcosine, 50-mM CAPS (pH 11.0) containing 250-mM imidazole (Nacalai Tesque, Tokyo, Japan). The purified FER2 proteins were refolded by dialysis against a 10-mM Tris-HCL (pH 8.5) buffer containing 0.1-mM DL-dithiothreitol (Merck) at 4°C overnight. The purity of the recombinant proteins was analyzed using 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The concentration of recombinant proteins was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin (BSA) as the standard, according to the manufacturer's instructions.

Iron binding assay

A ferrozine-based iron-binding assay was performed to analyze the iron-binding ability of rFER2 proteins (31). Different concentrations of rFER2 were dissolved in 954 μL of double distilled water and mixed with 20 μL of 1-M HEPES (pH 7.0) and 1 μL of 40-mM $Fe_2(NH_4)_2(SO_4)_2$. After incubation at 30°C for 30 min, 20 μL of 10-mM ferrozine (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 30 min. Each mixture (300 μL) was transferred to 3 wells of a microplate. The absorbance was measured at 570 nm using a microplate reader (Corona Electric, Hitachinaka, Japan). For the analyses, we used 2.5, 5, and 10 μg/mL of each rFER2 protein, apoferritin from equine spleen (Sigma-Aldrich) as the positive control, and BSA (Merck) as the negative control. All values were indicated as means, and error bars indicate standard deviations.

Immunization of chickens with rFER2 proteins

Sixteen chickens were randomly allocated to four groups: rFER2 PRM, rFER2 TFM, and rFER2 NFM immunization groups

and the control group. To generate immune plasma, four chickens per group were subcutaneously immunized with 20 μg of each rFER2 mixed with the Freund incomplete adjuvant (FUJIFILM Wako Pure Chemical Corporation) at 3 weeks of age. The chickens were boosted at 3 weeks after the first immunization with 20 μg of recombinant proteins with the same adjuvant. As the control, four chickens were immunized with phosphate-buffered saline (PBS) and mixed with the same adjuvant. Three weeks after the second immunization, heparinized blood was collected from each chicken, and immune plasma was isolated.

Western blotting

Western blotting was performed to ascertain the antibody responses to each rFER2 vaccination and analyze the cross-reactivity of each immune plasma. The rFER2 proteins were electrophoresed on 13% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes (Merck). The membranes were blocked with 3% skim milk at 4°C overnight. Membranes were incubated with isolated immune plasma (1:1,000) at 25°C for 1 h and washed 3 times with PBST. The membranes were incubated with an anti-chicken IgY peroxidase rabbit antibody (1:10,000) (Sigma-Aldrich) at 25°C for 1 h and washed 3 times with PBST. Finally, the signal was detected using the Immobilon Western Chemiluminescent HRP Substrate (Merck).

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was performed to determine the antibody titers of the immune plasma against each rFER2. Briefly, 100 ng/well of each rFER2 protein was coated onto an ELISA plate (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) with a carbon-bicarbonate buffer (pH 9.8) at 4°C overnight. The plate was washed 3 times with PBST and blocked with PBST-containing 1% BSA at 37°C for 2 h. After blocking, diluted immune plasma was added to each well and incubated at 25°C for 30 min. The wells were then washed with PBST 5 times and incubated with anti-chicken IgY[IgG](H+L)-HRP (goat; Bethyl Laboratories, Inc., Montgomery, TX, USA) at 37°C for 1 h. The reaction was detected by adding TMB One Component HRP Microwell Substrate (Bethyl Laboratories, Inc.) to each well, followed by incubation at 37°C for 15 min. After adding 100 μL of 0.18 M H_2SO_4 to stop the reaction, the absorbance was measured at 450 nm in a microplate reader (Corona Electric).

In vitro feeding assay

Fresh heparinized blood was collected from healthy chickens maintained at the Field Science Center for Northern Biosphere, Hokkaido University and incubated at 40°C before use. Plasma samples from chickens unimmunized or immunized with each rFER2 were pooled separately for each group. Plasma from heparinized fresh blood was replaced with each pool of plasma. Starved PRMs of mixed developmental stages were collected into *in vitro* feeding devices (17), and blood feeding was performed for 4 h at 40°C in dark and humid conditions with shaking at 100 rpm. Only blood-fed PRMs were collected using Pasteur pipettes and maintained at 25°C in 60% humidity throughout the monitoring period. The number of dead PRMs in each group was counted daily for a week, and the acaricidal effects of immune plasma against rFER2 were evaluated based on the survival rate of PRMs (the number of dead PRMs / the number of blood-fed PRMs).

Statistical analysis

In the iron-binding assay, statistical comparisons were performed using the Kruskal–Wallis test and the Steel–Dwass comparison test; asterisks indicate significant differences ($^{*}P < 0.05$ and $^{**}P < 0.01$). To compare PRM mortality between the immunized and control groups after *in vitro* feeding, we generated Kaplan–Meier curves and performed a log-rank test with Bonferroni corrections on multiple comparisons. Additionally, the Fisher exact test was performed to compare the mortality of PRMs between the groups on each day. All statistical analyses were performed using EZR, an easy-to-use software based on R and R commander (41). Moreover, the odds ratios and 95% confidence intervals were calculated. $P < 0.01$ for log-rank test and $P < 0.05$ for Fisher exact test were considered statistically significant.

Results

Identification and genetic characterization of the *FER2* genes in TFMs and NFMs

The nucleotide sequences of the ORFs of *FER2* genes from TFMs (LC752110) and NFMs (LC752111) consisted of 588 bp with 195 amino acids and signal peptides at positions 1–17. The *FER2* genes of TFMs and NFMs showed 99.0% homology with each other and 65.0% homology with those of PRMs (Supplementary Figure 1; Table 1). In addition, the ferroxidase centers, which are iron-binding sites for the oxidation of Fe(II) in heavy-chain (H) ferritin, were completely conserved with those of PRMs. To genetically characterize the *FER2* genes of TFMs and NFMs, we constructed a phylogenetic tree using the sequences

TABLE 1 Comparison of sequences of ferritin 2 from poultry red mites, northern fowl mites, and tropical fowl mites.

		Amino acid sequence (%)		
		PRM	TFM	NFM
Nucleotide sequence (%)	PRM	–	65.0	66.0
	TFM	67.0	–	99.0
	NFM	68.0	99.0	–

PRM, poultry red mite; NFM, northern fowl mite; TFM, tropical fowl mite.

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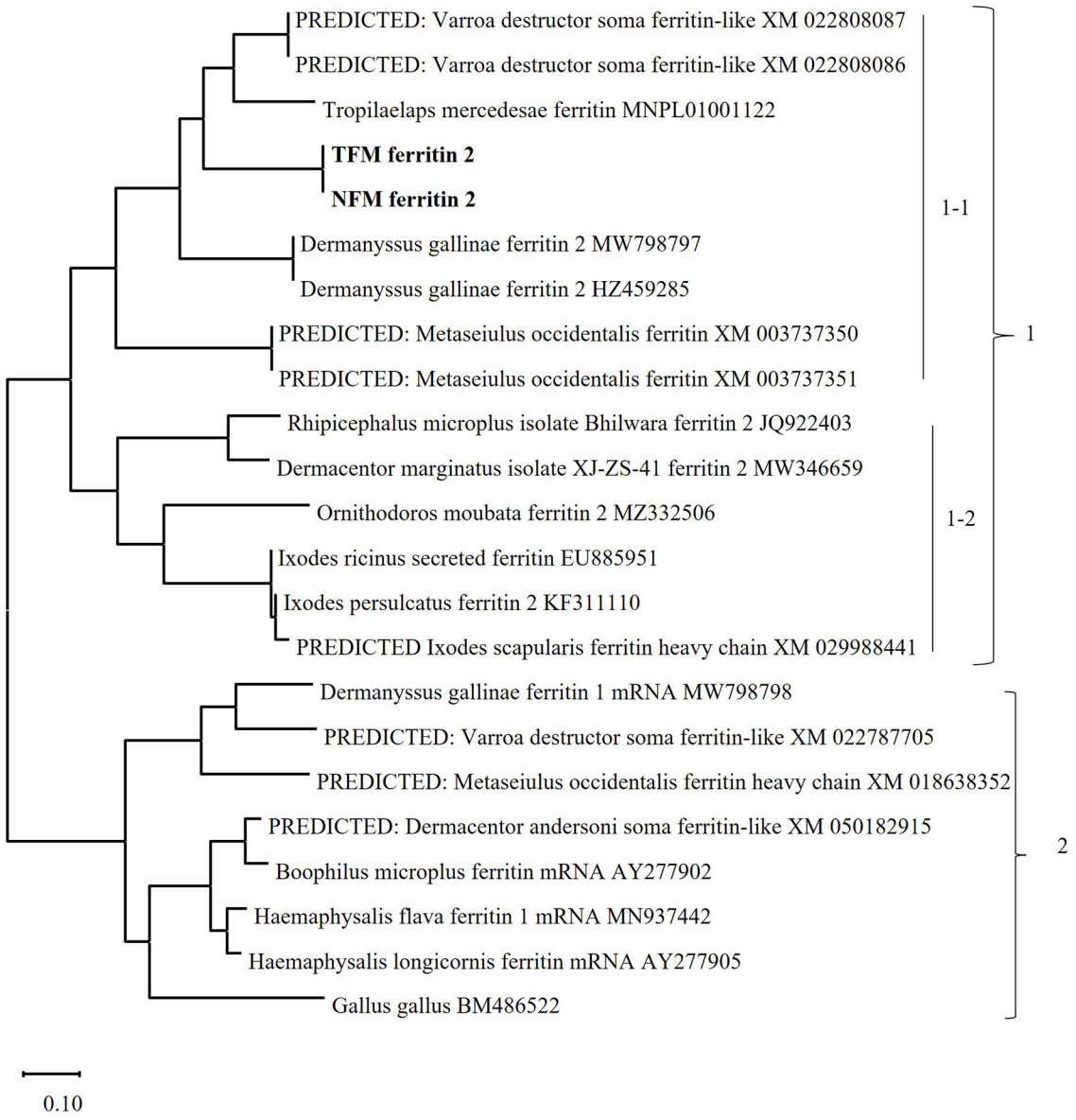

FIGURE 1
Phylogenetic analysis of the *ferritin* (*FER*) genes from poultry red mites (PRM), tropical fowl mite (TFM), northern fowl mite (NFM), arthropods, including other mites and ticks, and chickens. The phylogenetic tree was constructed using the maximum-likelihood method with MEGA X software. The numbers on the right indicate the clusters. Cluster 1 includes secretory types of *FER2* genes, and is divided into 2 subclusters. Subcluster 1-1: The secretory types of *FER2* genes of mites are classified into this subcluster, and the *FER2* genes of PRMs, TFMs (bold), and NFMs (bold) belong to this cluster. Subcluster 1-2: This cluster consist of secretory types of *FER2* genes from ticks. Cluster 2: The intracellular *FER* genes of mites, ticks, and chickens are classified into this cluster.

of *FER* genes from mites, including PRMs, ticks, and chickens (Figure 1). The *FER2* genes of TFMs and NFMs were distinctly classified into cluster 1, consisting of secreted *ferritin* (*FER2*) genes, and were most closely related to the secreted *ferritin* genes of PRM, *Varroa destructor*, and *Tropilaelaps mercedesae* in subcluster 1-1 consisting of secreted *ferritin* genes of mites. The subcluster 1-2 included secreted *ferritin* genes in ticks. Cluster 2 included intracellular *ferritin* (*FER1*) genes from PRMs and other mites, ticks, and chicken. Thus, *FER2* genes of mites, including TFMs and NFMs, clearly belong to the secretory type of ferritins.

Iron binding ability of FER2 proteins of PRMs, TFMs, and NFMs

The whole regions of FER2 from PRMs, TFMs, and NFMs, excluding the signal peptides, were generated as recombinant proteins fused with His-tag, termed as rFER2 TFM, rFER2 NFM, and rFER2 PRM. The recombinant proteins were purified from the insoluble fractions by affinity chromatography, and their purities were confirmed by SDS-PAGE and Western blotting (Figures 2A, B). To assess whether each rFER2 has iron-binding ability, we conducted a ferrozine-based colorimetric assay. The

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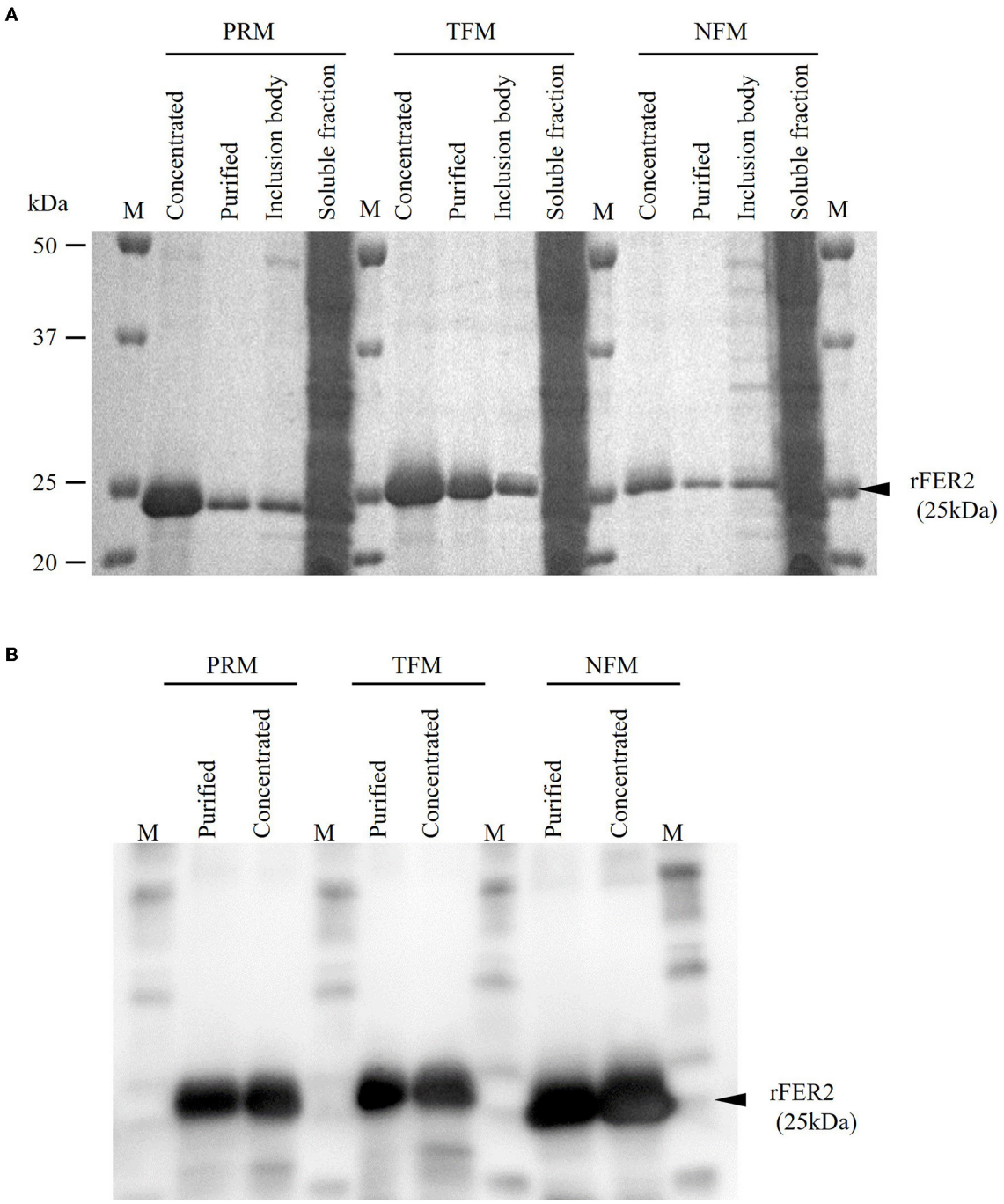

FIGURE 2
Expression and purification of recombinant ferritin 2 (rFER2) proteins. The ferritin 2 from poultry red mites (PRMs), tropical fowl mite (TFMs), and northern fowl mites (NFMs) were expressed and purified as recombinant proteins fused with histidine tag, and named as rFER2 PRM, rFER2 TFM, and rFER2 NFM, respectively. rFER2 from each mite was expressed in *Escherichia coli* and purified from the inclusion body fraction by affinity chromatography. The purity of rFER2 was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis **(A)** and Western blotting **(B)**. M, Marker (Precision Plus Protein™ All Blue Prestained Protein Standards; Bio-Rad, Hercules, CA, USA).

absorbance of rFER2 proteins in PRMs, TFMs, and NFMs decreased in a dose-dependent manner, similar to that of apoferritin, the positive control (Figure 3). Thus, these results showed that rFER2 proteins of PRMs, TFMs, and NFMs have iron-binding abilities.

Cross-reactivities of antibodies produced by the immunization with rFER2

To examine the potential of rFER2 as a universal vaccine antigen against avian mites, immune plasma was isolated from

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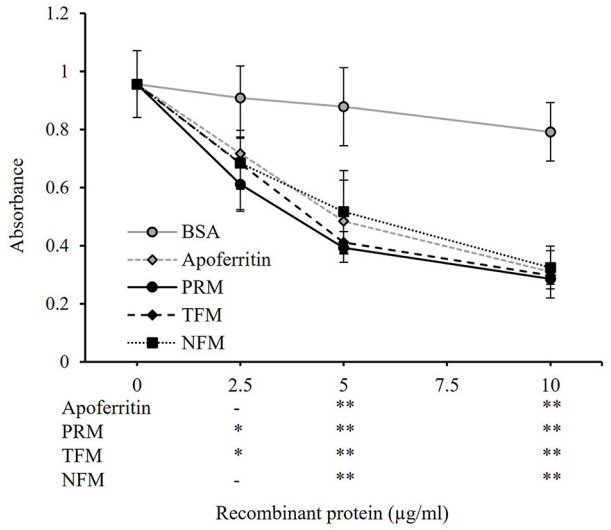

FIGURE 3
Iron-binding ability of recombinant ferritin 2 (rFER2) proteins. The iron-binding ability of rFER2 proteins from poultry red mite, tropical fowl mite, and northern fowl mite was assessed by a ferrozine-based colorimetric assay using different concentrations of each rFER2. Ferrozine was used as an indicator agent. Bovine serum albumin and horse apoferritin were used as the negative and positive controls, respectively. The *x*-axis indicates the amounts of rFER2 proteins used in this assay. Error bars indicate standard deviations. Statistical differences are shown as a comparison to bovine serum albumin (BSA). Asterisks indicate significant differences ($^{*}P < 0.05$ and $^{**}P < 0.01$).

TABLE 2 Antibody titers in plasma samples from chickens immunized with recombinant ferritin 2.

Group	Chicken	Antibody titer
Control	C1	<2,000
	C2	<2,000
	C3	<2,000
	C4	<2,000
Immunized-rFER2 PRM	PRM1	32,000
	PRM2	128,000
	PRM3	128,000
	PRM4	64,000
Immunized-rFER2 TFM	TFM1	128,000
	TFM2	128,000
	TFM3	64,000
	TFM4	64,000
Immunized-rFER2 NFM	NFM1	64,000
	NFM2	32,000
	NFM3	16,000
	NFM4	32,000

PRM, poultry red mite; NFM, northern fowl mite; TFM, tropical fowl mite; rFER2, recombinant ferritin 2.

chickens immunized with each rFER2. As shown in Table 2, increased production of antibodies in the immunized groups was confirmed. Western blotting revealed the presence of chicken IgY specific to rFER2 in the immune plasma (Figure 4). Additionally, the immune plasma against each rFER2 reacted with all rFER2 proteins, including those from different mites. However, the intensity of the signals was slightly different when the reactivity of immune plasma against rFER2 PRM was compared between rFER2 PRM and those of TFMs and NFMs, and vice versa. These results suggest that rFER2s have potential as common vaccine antigens in avian mites.

Assessment of acaricidal activity of the plasmas from chickens immunized with each rFER2

To assess the acaricidal effects of each FER2, we performed *in vitro* feeding assays and monitored the mortality of PRMs fed immune plasmas against each FER2. In this study, we used PRMs for *in vitro* feeding assays because of the limited distribution of NFMs and the absence of TFMs in Japan. To examine the acaricidal effects, we compared mortality between the immunized and control groups using the Fisher exact and log-rank tests. In this study, we used pooled plasma of chickens from each immunized group and found that the antibody titer of each group was different. According to a previous report, the acaricidal effects could depend on the antibody titer (21). Therefore, we only compared the mortality between the control group and each immunized group. The *in vitro* feeding assays were performed twice. In experiment 1, the mortality rate of PRMs reached 40.55, 32.03, and 39.34% in immunized groups of rFER2 PRM, rFER2 TFM, and rFER2 NFM, respectively, at 7 days post-feeding; moreover, according to the Fisher exact test, we observed significant differences in the mortality of PRMs fed the immune plasma against rFER2 PRM at 2–7 days post-feeding, and 3–7 days post-feeding in the immunized group of rFER2 TFM and rFER2 NFM, compared with those of the control group (Table 3). Kaplan–Meier curves revealed that the survival rate of PRMs fed with the immune plasma against each rFER2 was significantly lower than that of the control group (Figure 5A). Similar results were recorded in experiment 2. The survival rate of PRMs fed with immune plasmas against each rFER2 was significantly decreased (Figure 5B), and significant differences in the mortality rates were observed within 3–7 days post-feeding in the immunized groups of rFER2 PRM and rFER2 NFM and within 4–7 days post-feeding in the immunized group of rFER2 TFM (Table 4). Thus, the immune plasma against each rFER2 exhibited acaricidal effects on PRMs. Therefore, rFER2 could be a candidate antigen for the development of a universal vaccine across avian mites.

Discussion

Vaccine approaches have been focused on as a method for controlling PRMs to overcome the diminished effectiveness of acaricides and the selection of acaracide-resistant mites on poultry farms, in addition to their cost-effectiveness, low toxicity to

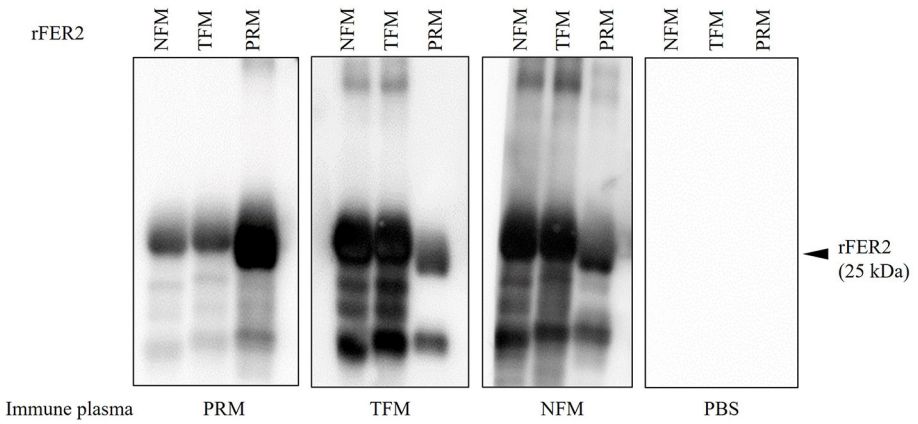

FIGURE 4
The production of specific antibodies in the plasma from chickens immunized with recombinant ferritin 2 (rFER2) proteins. Four chickens per group were immunized with rFER2, separately. The plasmas were isolated from each immunized chicken, and the production of antibodies specific to rFER2 in the plasma were detected by Western blotting. The cross-reactivities of immune plasmas with each rFER2 were tested by Western blotting. The arrowhead indicates the predicted molecular weight of rFER2 (~25 kDa).

TABLE 3 Mortality of PRMs fed plasma from chickens immunized with recombinant ferritin 2 from different species of mites (experiment 1).

	Days post-feeding						
Control group ($n = 103$)							
No. of dead PRMs post-feeding	2	4	4	6	9	10	14
Mortality (%)	1.94	3.88	3.88	5.82	8.73	9.71	13.59
Immunized group (rFER2 PRM, $n = 143$)							
No. of dead PRMs post-feeding	11	22	25	33	40	50	58
Mortality (%)	7.69	15.38	17.48	23.07	27.97	34.96	40.55
Chi-square	2.89	7.206	9.38	12.096	12.707	19.363	19.75
P-value	0.079	0.003*	1.06E-03*	1.77E-04*	1.67E-04*	4.3E-06*	4.12E-06*
Odds ratio	4.188	4.476	5.214	4.823	4.035	4.969	4.312
95% confidence interval	0.88–39.69	1.45–18.45	1.72–21.31	1.89–14.69	1.80–9.98	2.32–11.67	2.18–9.02
Immunized group (rFER2 TFM, $n = 103$)							
No. of dead PRMs post-feeding	4	9	13	17	20	26	33
Mortality (%)	3.88	8.73	12.62	16.50	19.41	25.24	32.03
Chi-square	0.171	1.313	4.103	4.894	4.013	7.574	8.931
P-value	0.683	0.251	0.0401*	0.0252*	0.0437*	5.39E-03*	2.56E-03*
Odds ratio	2.033	2.36	3.554	3.178	2.505	3.123	2.981
95% confidence interval	0.28–22.96	0.63–10.85	1.05–15.51	1.13–10.31	1.02–6.61	1.36–7.73	1.42–6.52
Immunized group (rFER2 NFM, $n = 61$)							
No. of dead PRMs post-feeding	2	7	10	13	17	21	24
Mortality (%)	3.27	11.47	16.39	21.31	27.87	34.42	39.34
Chi-square	1.6E-04	2.419	6.1603	7.521	9.125	13.699	37.437
P-value	0.629	0.102	8.35E-03*	4.56 E-03*	1.77 E-03*	1.56E-04*	6.18E-10*
Odds ratio	1.705	3.184	4.803	4.336	3.997	4.829	9.628
95% confidence interval	0.12–24.11	0.77–15.51	1.31–22.03	1.43–14.81	1.54–11.04	1.97–12.60	4.30–22.70

*$P < 0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite.

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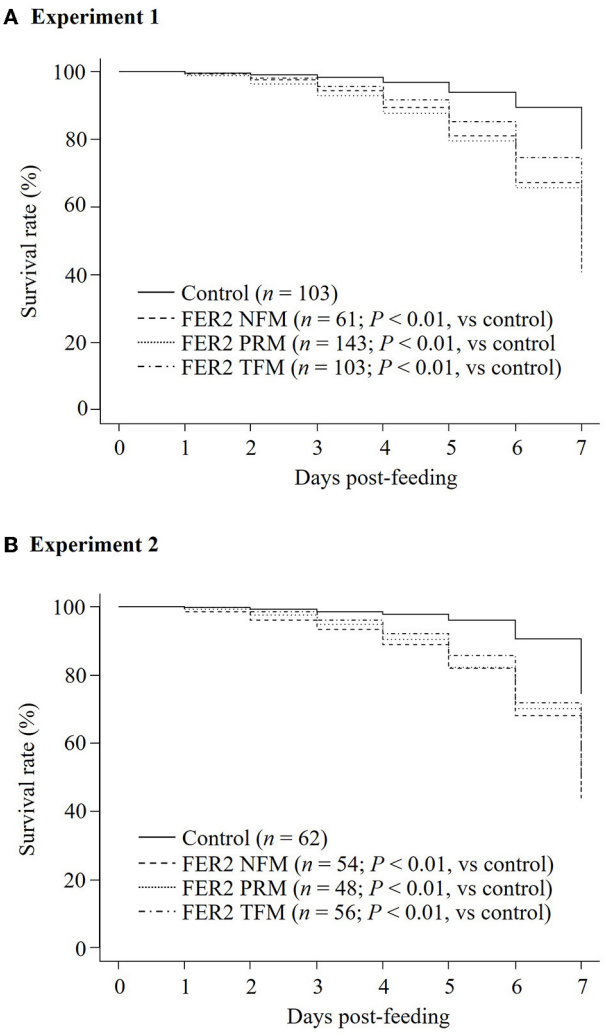

FIGURE 5
Assessment of the acaricidal potential of plasma obtained from chickens immunized with recombinant ferritin 2 (rFER2) proteins. Artificial blood feeding to poultry red mite (PRMs) was performed by the *in vitro* feeding assay. The survival rates of PRMs fed with immune plasmas or control plasma were monitored daily for a week. The *in vitro* feeding assays were performed 2 times. The total number of PRMs used in this study is as follows: **(A)** experiment 1: fed with immune plasma: $n = 143$ (rFER2 PRM), $n = 103$ [rFER2 tropical fowl mite (TFM)], and $n = 61$ [rFER2 northern fowl mite (NFM)] and fed with control plasma: $n = 103$; **(B)** experiment 2: fed with immune plasma: $n = 48$ (rFER2 PRM), $n = 56$ (rFER2 TFM), and $n = 54$ (rFER2 NFM) and fed with control plasma: $n = 62$. The number of dead PRMs were recorded and plotted on the graph to generate Kaplan–Meier survival curves. Statistical analysis was performed using a log-rank test with Bonferroni corrections on multiple comparisons. $P < 0.01$ was considered statistically significant between each immunized and control group.

the environment, and long-lasting action (42). However, other hematophagous mites, TFMs and NFMs, which are genetically similar to PRMs, cause problems similar to those of PRMs on poultry farms. Therefore, the development of a vaccine with broad protection efficacy against these avian mites could be a promising approach for their control in the poultry industry. The molecules involved in the critical physiological functions of avian mites are suitable vaccine antigens. FER2 is involved in iron transport and is critical for blood feeding and reproduction in ticks; moreover, FER2 is highly immunogenic and a useful candidate for anti-tick vaccines with broad protective efficacy across some tick species (27, 30, 43). FER2 has also been reported as a crucial molecule for the survival, reproduction, and blood digestion of PRMs. Moreover, acaricidal effects on PRMs by immunization have been demonstrated (25). Herein, we genetically identified and characterized *FER2* genes from TFMs and NFMs. In addition, the recombinant FER2 proteins from PRMs, TFMs, and NFMs were shown to be iron-binding proteins. Moreover, immune plasmas against each rFER2 showed cross-reactivity with rFER2 of different mites and acaricidal effects on PRMs, even when we used immune plasmas against rFER2 of TFMs and NFMs. Collectively, FER2 could be used as a vaccine antigen with protective efficacy against avian mites.

Genetic analysis showed that the *FER2* genes of PRMs, TFMs, and NFMs belonged to the cluster of secretory ferritins and were distinct from the cluster of intracellular ferritins. The secretory FER2 proteins of avian mites include signal peptides at the N-terminus. Similar to ferritins in vertebrates, ferritins of insects consist of H subunits containing ferroxidase centers (iron-binding sites) and light-chain (L) subunits containing amino acid residues with ferrihydrite nucleation centers (44). In this study, the ferroxidase centers of FER2 of avian mites were completely conserved, showing H-type subunits that are highly functional for catalytic activity in Fe(II) oxidation. We also observed the iron-binding abilities of rFER2-PRM, rFER2-TFM, and rFER2-NFM. Two types of ferritins have been identified in ticks (31, 45), and unlike FER1, FER2 has been recognized as a secretory protein in the tick hemolymph (29). Two types of ferritin have been identified in PRMs (25). The *FER2* gene of PRMs is expressed in all developmental stages, and RNAi analysis revealed critical functions for survival and reproduction (25). Unfortunately, in the present study, we could not elucidate the expression patterns of the *FER2* genes in the developmental stages of TFMs and NFMs and could not perform RNAi analysis on TFMs and NFMs because of the limited availability of TFM and NFM samples in Japan. However, we confirmed that the immune plasma against rFER2 of TFMs and NFMs cross-reacted with PRM rFER2 and exhibited anti-PRM effects. Collectively, these data suggest that the FER2 proteins identified in this study are secretory ferritins and have essential roles in the physiology of avian mites.

Host blood is an essential source of nutrients required for the growth and reproduction of hematophagous ectoparasites. During a single feed, a PRM can suck ~0.2 μL of the host blood (16) and is exposed to a large amount of iron. Excessive exposure to non-heme iron after hemoglobin digestion in midguts could be toxic to mites. Similar to the mechanisms in ticks (46), FERs are considered to play a role in iron homeostasis, although iron metabolism remains poorly understood in avian mites. According to a previous report, silencing of FER1 and FER2 affects feeding and oviposition in ticks, and FER2 depletion is linked to FER1 expression and altered iron homeostasis in ticks (31). Therefore, FER2 has been targeted as an effective vaccine candidate for ticks (27, 30, 43). Knockdown of FER1 and FER2 led to decreased blood digestion and oviposition, and increased mortality in PRMs; moreover, a significant increase in mortality of PRMs were recorded by immunization of chickens

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TABLE 4 Mortality of PRMs fed plasma from chickens immunized with recombinant ferritin 2 from different species of mites (experiment 2).

	Days post-feeding						
	1	2	3	4	5	6	7
Control group ($n = 62$)							
No. of dead PRMs post-feeding	1	2	2	2	3	7	11
Mortality (%)	1.61	3.23	3.23	3.23	4.84	11.29	17.74
Immunized group (rFER2 PRM, $n = 48$)							
No. of dead PRMs post-feeding	2	5	7	9	13	14	17
Mortality (%)	4.17	10.42	14.58	18.75	27.08	29.17	35.47
Chi-square	0.051	1.296	3.257	5.622	9.055	4.499	3.571
P-value	0.579	0.236	0.039*	0.0096*	0.0019*	0.027*	0.047*
Odds ratio	0.629	3.449	5.049	6.808	7.175	3.199	2.52
95% confidence interval	0.13–158.83	0.53–37.84	0.9–52.18	1.31–68.15	1.8–41.94	1.08–10.37	0.97–6.81
Immunized group (rFER2 TFM, $n = 56$)							
No. of dead PRMs post-feeding	1	4	7	9	12	18	20
Mortality (%)	1.79	7.14	12.5	16.07	21.43	32.14	35.71
Chi-square	2.37E-31	0.299	2.396	4.325	5.879	6.464	0.023
P-value	1	0.421	0.0832	0.0243*	0.0109*	0.0069*	0.036*
Odds ratio	1.108	2.292	4.237	5.668	5.292	3.679	2.555
95% confidence interval	0.01–88.41	0.31–26.31	0.76–43.61	1.09–56.31	1.32–30.97	1.31–11.49	1.02–6.69
Immunized group (rFER2 NFM, $n = 54$)							
No. of dead PRMs post-feeding	3	5	8	10	13	18	20
Mortality (%)	5.56	9.26	14.81	18.52	24.07	33.33	37.04
Chi-square	0.424	0.942	3.559	5.722	7.436	7.042	4.546
P value	0.337	0.248	0.0432*	0.0118*	0.0055*	0.00601*	0.0221*
Odds ratio	3.551	3.033	5.149	6.716	6.143	3.881	2.703
95% confidence interval	0.28–191.19	0.47–33.16	0.96–52.02	1.33–66.14	1.55–35.7	1.38–12.16	1.07–7.11

*$P < 0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite.

with rFER2 (rDg-FER1 in the original study) (25). In the present study, we observed the anti-PRM effects of rFER2 from TFMs and NFMs, and all immune plasmas cross-reacted with FER2 proteins of different mites. Therefore, these findings suggest the usefulness of FER2 as a vaccine antigen against TFMs and NFMs, and highlight FER2 as a candidate for the development of a universal vaccine against avian mites.

The development of a cross-protective vaccine for multi-tick species has been emphasized because of the non-uniform distribution of ticks worldwide. Cattle can get infected by various ticks due to their preferences, and the usefulness of common antigens with broad protective efficacy against different ticks has been reported (27, 30, 43, 47). Our research group has introduced a similar concept for avian mites, which poses a serious problem to the poultry industry. In addition to the development of anti-PRM vaccines, we are extending our work to determine the usefulness of anti-PRM vaccines for different avian mites. In this study, the immune plasma of chickens against each rFER2 cross-reacted with rFER2 proteins of different mites, and immune plasma against TFMs and NFMs showed acaricidal effects on PRMs by *in vitro* feeding. The development of a universal vaccine could be cost-effective for commercial production. The potential application of this kind of vaccine in poultry farms could prevent economic losses in production. However, there is a limitation in evaluating the acaricidal effects on TFMs and NFMs. To demonstrate the acaricidal effects of immune plasmas on TFMs and NFMs, *in vitro* assays using TFMs and NFMs for the assessment of vaccine antigens must be established, and challenge trials on chickens immunized with each mite are required to develop an effective universal vaccine for controlling avian hematophagous mites. Moreover, it is difficult to assess if immunization with rFER2 contributes to the improved economics based on the *in vitro* data; therefore, field trials are required to precisely evaluate the impact of vaccination with FER2. In addition, the search for other common antigens and the combined use of multiple antigens as a cocktail vaccine could further enhance the acaricidal effects on PRMs, TFMs, and NFMs.

Conclusions

In the present study, we characterized FER2 from PRMs, TFMs, and NFMs and investigated its acaricidal effects as a vaccine antigen to assess the potential application of a universal vaccine across avian mites. The amino acid residues crucial for the oxidization of Fe(II) were conserved among the three species, and all rFER2 proteins tested showed iron-binding ability. Most importantly, the immune plasmas against rFER2 of PRMs, TFMs, and NFMs cross-reacted with rFER2 from different mites and exhibited acaricidal effects on PRMs, even in assays using immune plasmas against rFER2 of TFMs and NFMs. Thus, FER2 may be a useful vaccine antigen for avian mites. Further studies are needed to assess the usefulness of FER2 as a universal vaccine against avian mites.

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

All animal experiments were performed in accordance with the guidelines and regulations of the Faculty of Veterinary Medicine, Hokkaido University, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and approved by the Institutional Animal Care and Use Committee of Hokkaido University (approval number: 20–0051).

Author contributions

SW and SM conceived of and designed the study, analyzed the data, and drafted the manuscript. SW, SF, HS, JS, YM, LH, and SB conducted the experiments. SM, TO, NM, TS, EO, AT, SK, and KO provided intellectual input, laboratory materials, reagents, and analytical tools. All authors have reviewed and approved the final manuscript.

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Conflict of interest

TS, EO, and AT were employed by Vaxxinova Japan K.K, Tokyo, Japan. SM, SK, and KO are authors of the patent-covering materials and techniques described in this manuscript (EU patent, EP15800403.6; Japanese patent, 2016-523581).

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1182930/full#supplementary-material

References

1. Sparagano OAE, George DR, Harrington DWJ, Giangaspero A. Significance and control of the poultry red mite, *Dermanyssus gallinae*. *Annu Rev Entomol.* (2014) 59:447–66. doi: 10.1146/annurev-ento-011613-162101

2. Knee W, Proctor H. Host records for *Ornithonyssus sylviarum* (Mesostigmata: Macronyssidae) from birds of North America (Canada, United States and Mexico). *J Med Entomol.* (2007) 44:709–13. doi: 10.1093/jmedent/44.4.709

3. Wood HP. *Tropical Fowl Mite in the United States: With Notes on Life History and Control*. Washington, DC: US Dept of Agriculture (1920).

4. McCulloch JB, Owen JP, Hinkle NC, Mullens BA, Busch JB. Genetic structure of northern fowl mite (Mesostigmata: Macronyssidae) populations among layer chicken flocks and local house sparrows (Passeriformes: Passeridae). *J Med Entomol.* (2020) 57:122–30. doi: 10.1093/jme/tjz136

5. Chauve C. The poultry red mite *Dermanyssus gallinae* (De Geer, 1778): current situation and future prospects for control. *Vet Parasitol.* (1998) 79:239–45. doi: 10.1016/S0304-4017(98)00167-8

6. Chen BL, Mullens BA. Temperature and humidity effects on off-host survival of the northern fowl mite (Acari: Macronyssidae) and the chicken body louse (Pthiraptera: Menoponidae). *J Econ Entomol.* (2008) 101:637–46. doi: 10.1093/jee/101.2.637

7. Sparagano O, Ho J. Parasitic mite fauna in Asian poultry farming systems. *Front Vet Sci.* (2020) 7:400. doi: 10.3389/fvets.2020.00400

8. Sigognault Flochlay A, Thomas E, Sparagano O. Poultry red mite (*Dermanyssus gallinae*) infestation: a broad impact parasitological disease that still remains a significant challenge for the egg-laying industry in Europe. *Parasit Vect.* (2017) 10:357. doi: 10.1186/s13071-017-2292-4

9. Murthy GSS, Panda R. Prevalence of *Dermanyssus* and *Ornithonyssus* species of mites in poultry farms of Vikarabad area of Hyderabad. *J Parasit Dis.* (2016) 40:1372–5. doi: 10.1007/s12639-015-0693-x

10. Jansson DS, Otman F, Lundqvist L, Höglund J, Engström A, Chirico J. Northern fowl mite (*Ornithonyssus sylviarum*) in Sweden. *Med Vet Entomol.* (2014) 28:443–6. doi: 10.1111/mve.12053

11. Soares NM, Tucci EC, Guastalli, Yajima, H. Control of *Orinthonyssus sylviarum* (Canestrini and Fanzago, 1877) (Acari: Macronyssidae) infestation in commercial laying hens by using *Azadirachta indica* extract. *Rev Bras Parasitol Vet*. (2008) 17:175–8. doi: 10.1590/S1984-29612008000400001

12. Watson CR. Human infestation with bird mites in Wollongong. *Commun Dis Intell Q Rep*. (2003) 27:259–61.

13. Marangi M, Cafiero MA, Capelli G, Camarda A, Sparagano OAE, Giangaspero A. Evaluation of the poultry red mite, Dermanyssus gallinae (Acari: Dermanyssidae) susceptibility to some acaricides in field populations from Italy. *Exp Appl Acarol.* (2009) 48:11–8. doi: 10.1007/s10493-008-9224-0

14. Kim SI Yi JH, Tak J, Ahn YJ. Acaricidal activity of plant essential oils against *Dermanyssus gallinae* (Acari: Dermanyssidae). *Vet Parasitol.* (2004) 120:297–304. doi: 10.1016/j.vetpar.2003.12.016

15. Tomer H, Blum T, Arye I, Faigenboim A, Gottlieb Y, Ment D. Activity of native and commercial strains of *Metarhizium* spp. against the poultry red mite Dermanyssus gallinae under different environmental conditions. *Vet Parasitol.* (2018) 262:20–5. doi: 10.1016/j.vetpar.2018.09.010

16. Mul M, van Niekerk T, Chirico J, Maurer V, Kilpinen O, Sparagano O, et al. Control methods for *Dermanyssus gallinae* in systems for laying hens: Results of an international seminar. *Worlds Poult Sci J.* (2009) 65:589–600. doi: 10.1017/S0043933909000403

17. Ariizumi T, Murata S, Fujisawa S, Isezaki M, Sato T, Oishi E, et al. In vitro evaluation of a cysteine protease from poultry red mites, *Demanyssus gallinae*, as a vaccine antigen for chickens. *Poult Sci.* (2021) 101:101638. doi: 10.1016/j.psj.2021.101638

18. Fujisawa S, Murata S, Isezaki M, Ariizumi T, Sato T, Oishi E, et al. Characterization of a novel cysteine protease inhibitor from poultry red mites: potential vaccine for chickens. *Vaccines.* (2021) 9:1472. doi: 10.3390/vaccines9121472

19. Fujisawa S, Murata S, Takehara M, Aoyama J, Morita A, Isezaki M, et al. *In vitro* characterization of adipocyte plasma membrane-associated protein from poultry red mites, *Dermanyssus gallinae*, as a vaccine antigen for chickens. *Vaccine.* (2021) 39:6057–66. doi: 10.1016/j.vaccine.2021.08.104

20. Fujisawa S, Murata S, Isezaki M, Ariizumi T, Sato T, Oishi E, et al. Characterization of copper transporter 1 from *Dermanyssus gallinae* as a vaccine antigen. *Parasitology.* (2022) 149:105–15. doi: 10.1017/S0031182021001608

21. Murata S, Taniguchi A, Isezaki M, Fujisawa S, Sakai E, Taneno A, et al. Characterisation of a cysteine protease from poultry red mites and its potential use as a vaccine for chickens. *Parasite.* (2021) 28:9. doi: 10.1051/parasite/2021005

22. Bartley K, Huntley JF, Wright HW, Nath M, Nisbet AJ. Assessment of cathepsin D and L-like proteinases of poultry red mite, *Dermanyssus gallinae* (De Geer), as potential vaccine antigens. *Parasitology.* (2012) 139:755–65. doi: 10.1017/S0031182011002356

23. Bartley K, Wright HW, Huntley JF, Manson ED, Inglis NF, McLean K, et al. Identification and evaluation of vaccine candidate antigens from the poultry red mite (*Dermanyssus gallinae*). *Int J Parasitol.* (2015) 45:819–30. doi: 10.1016/j.ijpara.2015.07.004

24. Price DRG, Kuster T, Qines Q, Oliver EM, Bartley K, Nunn F, et al. Evaluation of vaccine delivery systems for inducing long-lived antibody responses to *Dermanyssus gallinae* antigen in laying hens. *Avian Pathol.* (2019) 48:60–74. doi: 10.1080/03079457.2019.1612514

25. Xu X, Wang C, Liu Q, Huang Y, Sun W, Pan B. Two ferritins from Dermanyssus gallinae: characterization and in vivo assessment as protective antigens. *Pest Manag Sci.* (2022) 78:561–71. doi: 10.1002/ps.6664

26. Harrington D, Canales M, de la Fuente J, Robinson K, Guy J, Sparagano O. Immunisation with recombinant proteins subolesin and Bm86 for the control of *Dermanyssus gallinae* in poultry. *Vaccine.* (2009) 27:4056–63. doi: 10.1016/j.vaccine.2009.04.014

27. Githaka NW, Konnai S, Isezaki M, Goto S, Xavier MA, Fujisawa S, et al. Identification and functional analysis of ferritin 2 from the Taiga tick Ixodes persulcatus Schulze. *Ticks Tick Borne Dis.* (2020) 11:101547. doi: 10.1016/j.ttbdis.2020.101547

28. Arosio P, Ingrassia R, Cavadini P. Ferritins: a family of molecules for iron storage, antioxidation and more. *Biochim Biophys Acta.* (2009) 1790:589–99. doi: 10.1016/j.bbagen.2008.09.004

29. Hajdusek O, Sojka D, Kopacek P, Buresova V, Franta Z, Sauman I, et al. Knockdown of proteins involved in iron metabolism limits tick reproduction and development. *Proc Natl Acad Sci USA.* (2009) 106:1033–8. doi: 10.1073/pnas.0807961106

30. Hajdusek O, Almazán C, Loosova G, Villar M, Canales M, Grubhoffer L, et al. Characterization of ferritin 2 for the control of tick infestations. *Vaccine.* (2010) 28:2993–8. doi: 10.1016/j.vaccine.2010.02.008

31. Galay RL, Aung KM, Umemiya-Shirafuji R, Maeda H, Matsuo T, Kawaguchi H, et al. Multiple ferritins are vital to successful blood feeding and reproduction of the hard tick *Haemaphysalis longicornis*. *J Exp Biol.* (2013) 216:1905–15. doi: 10.1242/jeb.081240

32. García-García JC, Gonzalez IL, González DM, Valdés M, Méndez L, Lamberti J, et al. Sequence variations in the Boophilus microplus Bm86 locus and implications for immunoprotection in cattle vaccinated with this antigen. *Exp Appl Acarol.* (1999) 23:883–95. doi: 10.1023/A:1006270615158

33. García-García JC, Montero C, Redondo M, Vargas M, Canales M, Boue O, et al. Control of ticks resistant to immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, *Boophilus microplus*. *Vaccine.* (2000) 18:2275–87. doi: 10.1016/S0264-410X(99)00548-4

34. Parizi LF, Utiumi KU, Imamura S, Onuma M, Ohashi K, Masuda A, et al. Cross immunity with *Haemaphysalis longicornis* glutathione S transferase reduces an experimental *Rhipicephalus* (Boophilus) *microplus* infestation. *Exp Parasitol.* (2011) 127:113–8. doi: 10.1016/j.exppara.2010.07.001

35. Sabadin GA, Parizi LF, Kiio I, Xavier MA, Matos RS, Camargo-Mathias MI, et al. Effect of recombinant glutathione S-transferase as vaccine antigen against *Rhipicephalus appendiculatus* and *Rhipicephalus sanguineus* infestation. *Vaccine.* (2017) 35:6649–56. doi: 10.1016/j.vaccine.2017.10.026

36. Xavier MA, Konnai S, Parizi LF, Githaka NW, Isezaki M, Goto S, et al. Cross-species reactivity of antibodies against *Ixodes persulcatus* ferritin 2 to *Rhipicephalus microplus*. *Jpn J Vet Res.* (2021) 69:57–65. doi: 10.14943/jjvr.69.1.57

37. Kasaija PD, Contreras M, Kabi F, Mugerwa S, de la Fuente J. Vaccination with recombinant subolesin antigens provides cross-tick species protection in Bos indicus and crossbred cattle in Uganda. *Vaccines.* (2020) 18:319. doi: 10.3390/vaccines8020319

38. Parizi LF, Githaka NW, Logullo C, Konnai S, Masuda A, Ohashi K, et al. The quest for a universal vaccine against ticks: cross-immunity insights. *Vet J.* (2012) 194:158–65. doi: 10.1016/j.tvjl.2012.05.023

39. Takehara H, Murata S, Katakura K, Fujisawa S, Hmoon MM, Win SY, et al. Haematophagous mites on poultry farms in the Republic of the Union of Myanmar. *Heliyon.* (2019) 5:e01544. doi: 10.1016/j.heliyon.2019.e01544

40. Kumar S, Stecher G, Li M, Knyaz C, Tamura K, MEGA X. Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol.* (2018) 35:1547–9. doi: 10.1093/molbev/msy096

41. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant.* (2013) 48:452–8. doi: 10.1038/bmt.2012.244

42. Wright HW, Bartley K, Huntley JF, Nisbet AJ. Characterisation of tropomyosin and paramyosin as vaccine candidate molecules for the poultry red mite, *Dermanyssus gallinae*. *Parasite Vector.* (2016) 12:544. doi: 10.1186/s13071-016-1831-8

43. Oleaga A, González-Pérez S, Peréz-Sánchez R. First molecular and functional characterisation of ferritin 2 proteins from *Ornithodoros argasid* ticks. *Vet Parasitol.* (2022) 304:109684. doi: 10.1016/j.vetpar.2022.109684

44. Pham DQD, Winzerling JJ. Insect ferritins: typical or atypical? *Biochim Biophys Acta.* (2010) 1800:824–33. doi: 10.1016/j.bbagen.2010.03.004

45. Kopácek P, Zdychová J, Yoshiga T, Weise C, Rudenko N, Law JH. Molecular cloning, expression and isolation of ferritins from two tick species-*Ornithodoros moubata* and *Ixodes ricinus*. *Insect Biochem Mol Biol.* (2003) 33:103–13. doi: 10.1016/S0965-1748(02)00181-9

46. Kopáček P, Perner J, Sojka D, Šíma R, Hajdušek O. Molecular targets to impair blood meal processing in ticks. In: Meng CQ, Sluder AE, editors. *Ectoparasites: Drug Discovery Against Moving Targets*. John Wliley & Sons (2018). p. 139–65.

47. Ndawula Jr C, Sabadin GA, Parizi LF, da Silva Vaz Jr I. Constituting a glutathione S-transferase-cocktail vaccine against tick infestation. *Vaccine.* (2019) 37:1918–27. doi: 10.1016/j.vaccine.2019.02.039

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Finisher lamb growth and rumen fermentation responses to the plane of nutrition and naturally occurring coccidiosis

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The objective of the present study was to investigate the interaction of plane of nutrition and naturally occurring coccidiosis on finisher lamb growth performance, FAMACHA score, and rumen volatile fatty acid profile. The study included 30 Suffolk, Dorset or Suffolk x Dorset lambs and were divided into 2 groups based on their initial body weight and assigned to 2 feeding groups differing in dietary energy intake to create lambs representing divergent growth curves due to differing nutritional management. Lambs with naturally occurring coccidiosis and healthy lambs were present in both feeding groups making a 2 × 2 factorial arrangement of treatments, (a) high plane of nutrition (HPN) lambs with no clinical coccidiosis diagnosis (HPNH), (b) HPN lambs with clinical coccidiosis (HPNC), (c) low plane of nutrition (LPN) lambs with no clinical coccidiosis diagnosis (LPNH), (d) LPN lambs with clinical coccidiosis (LPNC). Body weight and FAMACHA scores were recorded once every 2 weeks. On d 65 of feeding, lambs were slaughtered, and rumen fluid samples were collected and analyzed for volatile fatty acid concentrations. All response variables were analyzed statistically using a linear mixed effects model with fixed effects for plane of nutrition, health status, and a random effect for initial body weight nested within the pen. The total and average weight gain were not associated with planes of nutrition, health status, or the interaction. Health status had an impact on FAMACHA© score ($P = 0.047$) and concentration of isobutyrate ($P = 0.037$) and tended to affect total VFA ($P = 0.085$) and acetate ($P = 0.071$) concentrations. The interaction between the plane of nutrition and the health status tended to affect butyrate concentration ($P = 0.058$). These data support the conclusion that coccidiosis infection impacted on rumen fermentation in a manner independent of the plane of nutrition; however, the translation of these rumen level impacts did not translate to the production responses.

KEYWORDS

average daily gain, *Eimeria* spp., FAMACHA© score, volatile fatty acid, anemia

Introduction

Per capita lamb consumption in the U.S. is at a 20-year high, reaching 0.62 kg in 2021 (1). Despite high consumption, the U.S. sheep inventory is at all-time low of ~ 5.2 million sheep and lambs in 2019 (2). This discrepancy is largely because the domestic sheep industry fulfills only 50% of the U.S. demand for lamb meat and <30% of the demand for wool (3). Failure to meet this demand has resulted in increased levels of sheep meat and wool importation in the U.S. (1). Based on these market shifts, and the fact that the sheep industry is vital to the livelihood of many farmers throughout the country (4), there is potential for expanding

the commercial sheep industry in the U.S. Additionally, sheep are used as a natural, low-cost means of managing rangelands, forests, and agricultural lands, because most of the nutrient requirements of mature sheep can be achieved through grazing, making sheep production suitable for low-input production environments.

Meeting adequate nutrition requirements of young lambs with high potential for live weight gain (4), however, requires more intensive feeding strategies. According to the NRC (4), young lambs, between 60 and 150 days of age, need diets with high protein and energy content which exceed the levels supplied in most forages used for sheep rearing. With seasonal fluctuations of forage quality most of the hay used for sheep feeding is low in crude protein and essential macro and micro minerals (5, 6). Therefore, to obtain high growth performance, diets with a high proportion of concentrate are typically used for finishing lambs. A great deal of research shows that lambs grow faster on concentrate-based diets than on forage-based diets (7–9). However, these feeding strategies often involve confinement feeding of lambs, resulting in challenges associated with maintaining flock health (2).

Maintaining a healthy flock is imperative for successful sheep operations because disease compromises overall growth rate, immunity, and reproductive performance, all leading to substantial economic losses (10, 11). The Animal and Plant Health Inspection Service of U.S. Department of Agriculture reported that internal parasites caused ∼ 16 % loss in lambs (2) and a survey conducted by the same authority reported that gastrointestinal (GI) parasites are the number one health concern among sheep stakeholders (2). A 7-year review of clinical cases at Auburn University Veterinary Medical Teaching Hospital in Alabama found that parasite infection was the primary reason that 70% of sheep and 91% of goats were examined and treated by hospital clinicians (12). Gastrointestinal infestation with parasites is a particularly challenging situation because the infestation both increases cost of energy requirements due to activation of immune responses, and also depresses rumen function and negative consequences on metabolism (13) leading to lower energy supply to the animal (14). Coccidiosis is a major parasitic infection caused by *Eimeria* spp. protozoan that commonly infects the small and large intestines of sheep (15). Symptoms of clinical coccidiosis are diarrhea, dehydration, decreased appetite, weight loss, and death (16, 17). The effects of *Eimeria* infections on animals are thought to depend on the environmental situation, immunity of the animals (18), and the plane of nutrition. Studies focused on controlling coccidiosis are abundant in literature (19–22) but these studies are limited in exploring the interaction between plane of nutrition and naturally occurring coccidia on growth performance and rumen fermentation. Most of the coccidia associated studies have focused on the lower GI tract since it is the primary site of pathological change. Nevertheless, the impairment in the gastrointestinal tract may affect the function and activity of the rumen, contributing to impaired volatile fatty acid (VFA) production and reduced energy supplies to the animal. Because VFA are the main energy source for ruminants (23) and considering the cost of energy to maintain immunity and nutrient metabolism when lambs are under infection, it is worthwhile to investigate the effect of plane of nutrition and coccidiosis on rumen VFA production. Thus, the objective of this study was to explore how dietary manipulation and naturally occurring disease interact to influence animal performance and fermentation outcomes which will help to implement better health and nutritional management protocols.

Materials and methods

Animals, diets and experimental design

All the procedures and animal use described in this study were approved by the Virginia Tech Institutional Animal Care and Use Committee (Protocol #20-175). The study included 30 commercial wethers (Suffolk, Dorset or Suffolk x Dorset) that were group fed in a standard production feedlot (330 m^2) at the Smithfield Farm, Virginia Tech, Blacksburg, VA. Prior to initiating the experiment all lambs were dewormed using fenbendazole (Panacur 10 mg/kg body weight, Merck Animal Heath, DE, USA) and Levamisole Hydrochloride (Prohibit at 8 mg/kg of body weight, AgriLabs, MO, USA), per standard industry management and veterinary recommendation. Deworming occurred 3 weeks prior to the start of the experiment and was not expected to interfere with the experimental measurements. Lambs were between 04 and 06 months of age at the start of the study. The lambs were classified by initial body weight targeting different finishing weights to cater market demands: low (28.4 ± 4.31 kg) and high (36.1 ±2.37 kg). Lambs were fed either (1) a low plane of nutrition (LPN) diet targeting 100 g/d weight gain or, (2) a high plane of nutrition (HPN) diet targeting 200 g/d weight gain. Due to market preferences, there is considerable variation in production systems regarding rates of lamb growth in the U.S (24). Including two planes of nutrition in this study allowed for generalization of results among different growth trajectories of finishing lambs. Grouping animals based on starting weights was reflective of industry settings where animals are grouped and purchased based on body weight. Body weight at the start of the study would be an emergent property of health, nutritional, genetic, and environmental factors, and is a complex phenotype that should not be conflated to reflect only differences in planes of nutrition.

Across plane of nutrition groups, lambs averaged 32.1 ± 4.5 kg body weight at the beginning of the experiment. After introduction to the feedlot environment, animals were naturally infected with *Eimeria* spp. Animals began showing signs of coccidiosis after 13 days, and both groups of animals received herd-level coccidiosis treatment with amprolium (Corid 9.6 % solution, 8 mg of amprolium per 1 kg of body weight for 5 consecutive days). Animals did not have a history of anticoccidial treatment prior to entering the feedlot.

Out of 30 lambs, 04 lambs died (1 due to coccidiosis, 2 due to pneumonia and coccidiosis, and 1 for undiagnosed reasons) during the experimental period and those lambs were excluded from the data set. From the 26 lambs that completed the trial, 9 lambs were diagnosed with clinical coccidiosis and recovered after treatment (infected lambs were individually treated following the same protocol as herd level treatment). The low nutritional group consisted of 14 lambs of which 6 lambs were diagnosed with coccidiosis. The HPN group consisted of 12 lambs, of which 3 lambs were diagnosed with coccidiosis. The resulting 4 treatment

groups were (a) HPN lambs with no clinical coccidiosis diagnosis (HPNH), (b) HPN lambs with clinical coccidiosis (HPNC), (c) LPN lambs with no clinical coccidiosis diagnosis (LPNH), and (d) LPN lambs with clinical coccidiosis (LPNC). Diets in both groups were consisted with *ad libitum* grass hay and a commercial concentrate (Cargill Animal Nutrition, Minneapolis, MN, USA) supplement and were fed twice daily at 0900 h and 1700 h. Lambs in the LPN group received 0.45 kg of concentrate per lamb and 0.90 kg of concentrate per lamb in the HPN group. Target was to provide two levels of energy to achieve two different ADG. Both groups had access to fresh, clean water and mineral supplements throughout the day.

Measurements, sampling and laboratory analysis

Body weight and FAMACHA© scores were recorded once every 2 weeks. The FAMACHA system was used to determine the lower eye mucous membrane color that correspond to different levels of anemia: 1 = red, non-anemic; 2 = red-pink, non-anemic; 3 = pink, mildly anemic; 4 = pink-white, anemic; 5 = white, severely anemic (25). Although the FAMACHA© is developed to assess anemia caused by *Haemonchus* species, the tool has been used as an anemia indicator caused by a wide range of gastrointestinal parasites including *Eimeria* species (26, 27). Previous studies by Nurzaty Ewani et al., (26) and Wang et al., (28) observed anemia and diarrhea associated with lambs infected with *Eimeria* spp. Further, if lambs have more *Haemonchus,* this likely suppresses immune system and lambs will more likely be clinical for coccidia. Lambs were slaughtered on d 65 of the experiment and rumen fluid samples were collected from each animal promptly after slaughter. Samples were collected and processed in the laboratory and stored in glass vials at −20°C until further analysis. Approximately 100 g of concentrate and grass hay were collected weekly, composited, and stored in −20°C until analysis.

Volatile fatty acid concentrations were analyzed using gas chromatography. Concentration of total VFA and individual VFA per lamb were determined using a Hewlett-Packard 5,890 series gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a glass packed column (23110-U, Sigma-Aldrich) with N_2 as a carrier gas (24 mL/min). The inlet was 150°C, the flame ionization detector was 180°C, and the oven temperature was 175°C. Each run was 18 min long to separate acetate (retention time: 1.6 min), propionate (3.0 min), isobutyrate (5.2 min), butyrate (6.8 min), pivalic acid (internal standard; 7.8 min), 2-methylbutyrate (11 min), isovalerate (12.9 min), and valerate (16.1 min). To prevent carryover effects between samples and to maintain similar conditions, between each sample distilled H_2O was injected. Feed samples were dried for 24 h at 55°C in a forced-air oven (Thermo Scientific Heratherm Advanced Protocol Ovens Model 51028115, Fisher Scientific, Waltham, MA) and ground to pass through a 1 mm screen of a Wiley mill (Model 4, Thomas Scientific, Swedesboro, NJ). Proximate analysis of feed samples was done by an external laboratory (Cumberland Valley Analytical Services, Waynesboro, PA). Analyses included DM (29, 30), N (method 990.03; Leco FP-528 Nitrogen Combustion Analyzer, Leco Corp., St. Joseph, MI), ADF [method 973.18; (31)], NDF (32), lignin (29), starch (33), ash [method 942.05; (31)], and minerals by inductively coupled plasma [method 985.01; (31)].

Statistical analysis

Statistical analyses were conducted in R version 4.1.2 (34) using the lme4 and lmerTest packages (35). Response variables included were total body weight gain, average daily gain, FAMACHA score and concentrations and molar proportions of individual VFA. The following model was fitted for all variables:

$$Yijklm = \mu + Ni + Hj + (NHij) + BWk(l) + Pl + eijklm,$$

where Yij is the dependent variable, μ is the overall mean, Ni is the fixed effect of the plane of nutrition, Hj is the fixed effect of health status, NHij is the interaction effect, BWk is the random effect of initial body weight of individual lamb nested within the pen, and eijklm is the residual error. Analysis of variance (ANOVA) was performed for each variable and estimated marginal means were calculated using the emmeans package (36). Significance was declared at $P < 0.05$ and a tendency considered when $0.05 \leq P < 0.10$. Importantly, in our statistical analysis, we used the effect of initial body weight to explore how group responses might differ due to initial differences in the animals (expressed as differences in body weight) vs. those differences based on the nutritional treatments. Due to a failure in sampling of rumen fluid, data from 2 animals from HPNH group were not used for the statistical analysis.

Results

Growth performance and FAMACHA score

The effects of the plane of nutrition and health status on growth performance and FAMACHA© score of lambs are presented in Table 1. The plane of nutrition, status of health or the interaction did not have an effect on total gain or ADG. Numerically, the highest WG and ADG were associated with HPNH group, and values in other groups were not significantly different from each other. Although the numerical differences support the idea that nutrition and health interact to support performance, the variability around individual animal performance within the study precluded identification of statistically significant relationships. Status of health had an effect on FAMACHA© scores ($P = 0.047$) and lower values were associated with healthy groups irrespective of the plane of nutrition, with numerically higher FAMACHA© scores were associated with coccidiosis conditions.

Rumen volatile fatty acid profile

Concentrations and molar proportions of individual VFA are presented in Table 2. Health status caused by the coccidiosis infection had an effect on concentration of isobutyrate ($P = 0.037$) and tended to alter the concentrations of total VFA ($P = 0.085$)

TABLE 1 Weight gain and FAMACHA score of lambs in response to the plane of nutrition and health status.

Item	HPNH	HPNC	LPNH	LPNC	SEM	*P*-value		
						Nutrition	Health	Nutrition × Health
Total WG, kg	20.3	15.90	16.7	16.8	3.83	0.738	0.482	0.463
ADG, g	87.5	68.8	72.0	72.4	16.52	0.738	0.482	0.463
FAMACHA	1.70	1.93	1.70	2.23	0.311	0.720	0.047*	0.348

HPNH, high plane of nutrition healthy; HPNC, high plane of nutrition coccidiosis infected; LPNH, low plane of nutrition healthy; LPNC, low plane of nutrition coccidiosis infected; WG, weight gain; ADG, average daily gain. Number of lambs included in each group was; HPNH = 7, HPNC = 3, LPNH = 8, LPNC = 6.
*$P \leq 0.05$.

TABLE 2 Volatile fatty acid profiles of lambs in response to the plane of nutrition and health status.

Item	HPNH	HPNC	LPNH	LPNC	SEM	*P*-value		
						Nutrition	Health	Nutrition × Health
Total VFA, mM	46.5	45.3	47.2	29.0	7.55	0.380	0.085	0.129
Acetate, mM	29.7	28.4	29.4	18.6	5.08	0.418	0.071	0.150
Propionate, mM	11.63	11.46	12.31	7.31	2.02	0.426	0.121	0.147
Butyrate, mM	2.88	3.57	3.18	1.73	0.675	0.357	0.433	0.058
Valerate, mM	0.704	0.760	0.696	0.532	0.144	0.436	0.644	0.354
Isovalerate, mM	0.482	0.397	0.576	0.286	0.131	0.950	0.120	0.376
Isobutyrate, mM	0.799	0.513	0.897	0.592	0.157	0.573	0.037*	0.938
A/P	2.50	2.52	2.50	2.59	0.182	0.484	0.744	0.324
Acetate, %	64.3	64.0	62.5	62.5	2.22	0.516	0.938	0.928
Propionate, %	25.0	25.4	25.9	25.0	1.40	1.000	0.843	0.586
Butyrate, %	5.97	6.69	6.35	6.61	0.759	0.861	0.436	0.710
Valerate, %	1.45	1.73	1.50	1.80	0.397	0.773	0.120	0.959
Isovalerate, %	0.983	0.892	1.2	1.15	0.393	0.645	0.749	0.929
Isobutyrate, %	1.67	1.17	1.84	2.12	0.701	0.558	0.697	0.196

HPNH, high plane of nutrition healthy, HPNC, high plane of nutrition coccidiosis infected, LPNH, low plane of nutrition healthy, LPNC, low plane of nutrition coccidiosis infected, WG, weight gain, ADG, average daily gain. Number of lambs included in each group was; HPNH = 7, HPNC = 3, LPNH = 8, LPNC = 6.
*$P \leq 0.05$.

and acetate ($P = 0.071$). The interaction effect of the plane of nutrition and health status tended to affect butyrate concentrations ($P = 0.058$). Molar proportions of individual VFA were not affected by the plane of nutrition, health status or the interaction. Numerically, concentrations of total VFA and individual VFA in HPNH, HPNC and LPNH groups were quite similar to one another, while the LPNC group had the lowest reported values.

Discussion

Growth performance and FAMACHA© score

According to the NRC (4) guidelines for sheep nutrient requirements, 4–7 month old finisher lambs should achieve an ADG of range of 205 to 295 g. In the present study, irrespective of the plane of nutrition and health status neither group reached the recommended ADG, partially because diets were formulated to target lower rates of gain than suggested in the NRC (4). Despite the highest ADG of 87.5 g associated with the HPNH group; it is still <50% of the target used in formulation. Possible explanations for the limited growth rates observed in the present study could be poor feed intake and forage quality. Based on the chemical analysis of the forage and concentrates (Table 3) fed to the lambs, we observed that grass hay was lower in protein supply of 7.7% and higher NDF of 69%. This forage was quite mature and likely had poor digestibility. Another possible explanation for lower than the standard ADG we observed in the HPNH group is the negative impact of whole herd coccidiosis treatment and subclinical coccidiosis. Coccidiosis treatment is known to depress feed and water intake, thus may have negatively impacted productivity of animals. Further, the effect of coccidiosis on weight gain and feed efficiency has been inconsistent in studies and it is even more challenging to ascertain the influence of subclinical infections (37). Nevertheless, subclinical coccidiosis may contribute to low weight gain, reduced feed intake and feed utilization (38, 39). However, the lower ADG observed in the present study closely follows the ADG values reported in several previous studies (40, 41). In Atti and Mahouachi (40) they observed the highest ADG of 108 g/day for the high nutrition group and 61 g/day for the low nutrition

TABLE 3 Chemical composition of grass hay and commercial concentrate fed to lambs during the experiment.

Item	Grass hay	Commercial concentrate*
Dry matter, %	93.9	91.6
Composition, % of DM		
Crude protein	7.70	15.7
NDF	69.0	12.3
ADF	39.1	6.70
Lignin	4.80	0.92
Starch	3.10	49.5
Ash	5.74	8.03
Calcium	0.39	1.09
Phosphorus	0.32	0.52
Magnesium	0.27	0.36
Potassium	1.83	0.85

*Ingredients of the commercial concentrate: Processes grain by-products, grain products, roughage products, plant protein products, cane molasses (with propionic acid, sodium benzoate, potassium sorbate as preservatives), calcium carbonate salt, ammonium chloride, lignin sulfonate, soybean oil, sodium selenite, potassium sulfate, vitamin E supplement, magnesium sulfate, vitamin A supplement, vitamin D3 supplement, zinc oxide, manganous oxide, ferrous sulfate, calcium iodate, cobalt carbonate.
Lambs in 2 nutritional planes (HPN and LPN) received the diet in this table consisted of grass hay (*ad libitum*) and different levels of commercial concentrate (0.90 kg/animal/day for HPN group and 0.45 kg/animal/day for LPN group).

group while Bhatt and Sahoo (41) reported 99 to 140 g/day ADG values. In contrast to present results which did not show an effect of nutritional plane on total weight gain, numerous previous studies have linked plane of nutrition and growth performance. For example, a previous study (42) reported that the plane of nutrition had a dramatic effect on lamb live weight, with low and high lambs differing in weight by 9.1 kg ($P < 0.001$) at weaning and by 14.9 kg at slaughter. Indeed, standard understanding of energetics also supports the expectation that lambs fed greater energy content diets should grow more rapidly. Although we observed numerically higher total weight gains and ADG in the HPN group compared with the LPN group, the variability induced by the added health challenge, and the small number of animals used in each group may help explain why these numerical differences did not approach statistical significance. Indeed, our numerical differences are sensible given the basic understanding of nutritional energetics and concur well with other previous work (43, 44) reporting positive gain responses in feedlot lambs with dietary concentrate.

A major limitation of implementing a selective treatment approach for parasitic infections has been the lack of an efficient and economical means of identifying those animals' requiring treatment. To address this issue, FAMACHA score has been utilized successfully in African countries (45, 46) and the United States (47, 48). As shown in Table 1, FAMACHA© score did not differ between groups in response to the plane of nutrition but was affected by the health status ($P = 0.047$). The highest FAMACHA© score was observed in the LPNC group and is indicative of anemia due to the impaired ability of host to absorb nutrients caused by coccidiosis infection. In agreement with our results, the severity of coccidiosis in previous studies showed a significant correlation ($r = 0.48$, $P < 0.01$) with FAMACHA© score (26, 27). In these studies, they observed FAMACHA© score ranged between 2 and 3 which is slightly higher than the observed FAMACHA scores in the present study, which ranged between 1.43 (HPN, healthy) to 2.43 (LPN, infected). Another rational explanation for the anemic conditions we observed in infected groups may have caused by the occurrence of *H. contortus* even though it was not determined in the present study. *H. contortus* is the most pathogenic blood-sucking gastrointestinal nematode in ruminants and it causes anemic condition and also contributes to the severity of coccidia. This explanation is supported by a study where they reported anemic conditions in lambs and goat kids infected with both *Eimeria* spp. and *H. contortus* (49).

Rumen VFA

Rumen VFA profile is known to be altered in response to the different ratios of forage to concentrate due to the changes in nutrient supply. However, other than isobutyrate, no differences of VFA concentrations or molar proportions in response to the plane of nutrition and health status were observed in this study. This is not particularly surprising because animals were fed similar diets, differing only by the mass of concentrate allocated daily. The lack of dietary influence on VFA might reflect the ability of the rumen and the animal to adapt to appropriate dietary concentrate: forage ratios through the self-adjustment of forage intake under *ad libitum* access to forage. In agreement with our results, previous studies reported no changes in total VFA of Tibetian sheep fed with different ratios of forage and concentrate (50). In ruminants, total VFA concentrations may be as low as 30 mM or be in excess of 200 mM but is typically between 70 and 130 mM (51). In our data we observed total VFA concentrations across groups were closer to the lower end. These data suggest that altering the plane of nutrition, within the bounds of this study, while maintaining *ad libitum* access to hay, supported fairly consistent rumen VFA conditions. Importantly, this should not be conflated with similar energy supplied by VFA from the different planes of nutrition, because rumen VFA concentrations do not take into account production, absorption, and interconversion of VFA, and are considerably influenced by rumen fluid pool size and dynamics. As such, these data are best interpreted to support a consistent form of fermentation among the two planes of nutrition.

Effect of coccidiosis on rumen fermentation and VFA profile is not extensively studied mainly because the negative impacts localized in the small and large intestines (16, 52). Coccidiosis from the *Eimeria spp*., results in destruction of the epithelial cells of the intestine hence the coccidia infection strongly interacts with the digestive microflora. A previous study reported a significant change in digestive microflora in goat kids where they observed progressive reduction of the Gram-positive population from 84% pre-infection to 24.3% after the onset of diarrhea. On the other hand, the Gram-negative population was conversely increased from 16% pre-infection to 75.7% after diarrhea (53). Therefore, we can assume that infection of coccidiosis also has the capacity to alter rumen microflora of lambs. This mechanism supports the lower concentrations of total VFA and individual VFA associated

with coccidiosis infected groups. Lower concentrations may also reflect dysregulated fluid dynamics, which are consistent with GI infection.

We did not observe differences in molar proportions of individual VFA across groups, but the low butyrate molar proportions in all groups is noteworthy. Moreover, the interaction effect of nutrition and coccidiosis infection had a tendency toward altering butyrate concentration ($P = 0.058$) resulting lowest concentration in LPNC group, and both observations suggest modifications to butyrate in the rumen. Alterations in butyrate production make sense given its role in functional development of rumen epithelium (54, 55) and also in lower GI tract (56, 57). Studies have reported that increased concentration of butyrate is highly correlated with the enlargement of the ruminal epithelium absorptive surface area (58). We can speculate that the interactive effect of health and nutrition across all the groups suppressed the activity of certain rumen microorganisms which are responsible for producing butyrate hence low concentration and molar proportion of butyrate. It is possible that these shifts may also confer under-development and reduced functionality of the rumen and intestinal epithelium cells. This hurdle of poor rumen functionality possibly lowered feed utilization by lambs leading to the low weight gains observed. One can argue that the above concept can be reverse engineered; impaired health directly suppressed the development and functionality of the rumen epithelium and limited the utilization of butyrate by the cell wall hence accumulating more butyrate in the rumen. The observed numerically higher butyrate molar proportions in coccidiosis infected groups support the possible importance of this feedback in driving animal physiology. The same explanation of acid accumulation and hence slowing down the synthesis of VFA can be applied to the other VFA concentration data as well (59). Nevertheless, the lack of confirming measurements and prevailing lack of statistically significant differences among molar proportions precludes definitive discussion on these linkages. Future work should further explore the role of butyrate dynamics and epithelial function during GI parasite infection to more thoroughly explore these concepts.

Conclusion

This study sought to investigate the effect of plane of nutrition and coccidiosis infection on finisher lamb growth performance, FAMACHA score, and rumen volatile fatty acid profile. Plane of nutrition did not have an impact on any of the response variables considered. Health status showed a significant effect on FAMACHA score and concentrations of isobutyrate and tendency toward total VFA, acetate, and butyrate suggesting both or either, alteration in rumen microbial profile or rumen epithelium as explanations. An interaction between the plane of nutrition and health status was identified for butyrate concentrations. Overall, this result leads us to assume that changes in nutritional plane could have an impact on growth performance but in our study the effect of health status were more prominent suggesting that it is challenging to overcome the effect of suppressed immunity through nutritional strategies.

In future studies, it would be beneficial to focus on the effect of coccidiosis infection during rumen and overall gut development (preferably lambs below 3 months old age) and consequently how it will impact overall rumen function later in life. This will be helpful to develop clinical and nutritional interventions to support animals that have been exposed to coccidiosis and other related infections.

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 Virginia Tech Institutional Animal Care and Use Committee (Protocol #20-175).

Author contributions

SS and RW: conceptualization and design of the study. SS and BR: performed the experiment. SS, BR, ME, and EH: collection of samples. SS: sample preparation, formal analysis, manuscript writing, and editing. RW: statistical analysis and editing the manuscript. HS: animal care and animal health consulting. All authors contributed to the editing of the final version of the manuscript.

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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. American Sheep Industry Association. *Sheep Industry Review*. (2021).

2. USDA NAHMS. *Sheep Needs Assesment*. (2021).

3. Purcell W. *Analysis of Lamb Demand in the United States*. (2007)

4. National Research Council. *Committee on Nutrient Requirements of Small, Council National Research, Ruminants Committee on the Nutrient Requirements of Small, Agriculture Board on, Earth Division on, Studies Life, Nutrient requirements of small ruminants: sheep, goats, cervids, and new world camelids* (2007).

5. Griggs TC, Drake DR, Parkinson SC. "Production and quality of warm-season annual forage grasses," in *Proceedings, Idaho Alfalfa and Forage Conference* (2008).

6. McCartney D, Fraser J, Ohama A. Potential of warm-season annual forages and Brassica crops for grazing: a Canadian review. *Can J Anim Sci.* (2009) 89:431–40. doi: 10.4141/CJAS09002

7. Fimbres H, Hernández-Vidal G, Picón-Rubio JF. Kawas JR, Lu CD. Productive performance and carcass characteristics of lambs fed finishing ration containing various forage levels. *Small Rumin Res.* (2002) 43:283–88. doi: 10.1016/S0921-4488(02)00014-7

8. Demirel G, Ozpinar H, Nazli B, Keser O. Fatty acids of lamb meat from two breeds fed different forage: concentrate ratio. *Meat Sci.* (2006) 72:229–35. doi: 10.1016/j.meatsci.2005.07.006

9. Archimède H, Pellonde P, Despois P, Etienne T, Alexandre G. Growth performances and carcass traits of Ovin Martinik lambs fed various ratios of tropical forage to concentrate under intensive conditions. *Small Rumin Res.* (2008) 75:162–70. doi: 10.1016/j.smallrumres.2007.10.001

10. Odden A, Enemark HL, Robertson LJ, Ruiz A, Hektoen L, Stuen S. Treatment against coccidiosis in Norwegian lambs and potential risk factors for development of anticoccidial resistance—a questionnaire-based study. *Parasitol Res.* (2017) 116:1237–45. doi: 10.1007/s00436-017-5400-7

11. Dudko P, Junkuszew A, Bojar W, Milerski M, Szczepaniak K, Le Scouarnec J. et al. Effect of dietary supplementation with preparation comprising the blend of essential oil from *Origanum vulgare* (lamiaceae) and Citrus spp(citraceae) on coccidia invasion and lamb growth. *Ital J Anim Sci.* (2018) 17:57–65. doi: 10.1080/1828051X.2017.1346965

12. Pugh DG, Navarre CB. Internal parasite control strategies. *Vet Clin North Am Food Anim Pract.* (2001) 17:231–44. doi: 10.1016/S0749-0720(15)30026-8

13. Mavrot F, Hertzberg H, Torgerson P. Effect of gastro-intestinal nematode infection on sheep performance: a systematic review and meta-analysis. *Parasit Vectors.* (2015) 8:1–11. doi: 10.1186/s13071-015-1164-z

14. Jacobson C, Larsen JWA, Besier RB, Lloyd JB, Kahn LP. Diarrhoea associated with gastrointestinal parasites in grazing sheep. *Vet Parasitol.* (2020) 282:109139. doi: 10.1016/j.vetpar.2020.109139

15. Raue K, Heuer L, Böhm C, Wolken S, Epe C, Strube C. 10-year parasitological examination results (2003 to 2012) of faecal samples from horses, ruminants, pigs, dogs, cats, rabbits and hedgehogs. *Parasitol Res.* (2017) 116:3315–30. doi: 10.1007/s00436-017-5646-0

16. Chartier C, Paraud C. Coccidiosis due to Eimeria in sheep and goats, a review. *Small Rumin Res.* (2012) 103:84–92. doi: 10.1016/j.smallrumres.2011.10.022

17. Andrews AH. Some aspects of coccidiosis in sheep and goats. *Small Rumin Res.* (2013) 110:93–5. doi: 10.1016/j.smallrumres.2012.11.011

18. Gregory MW. Catchpole Janet, Joyner LP, Parker BNJ. Observations on the epidemiology of coccidial infections in sheep under varying conditions of intensive husbandry including chemoprophylaxis with monensin. *Parasitol.* (1983) 87:421–27. doi: 10.1017/S0031182000082949

19. Fitzgerald PR, Mansfield ME. Ovine coccidiosis: effect of the antibiotic monensin against Eimeria ninakohlyakimovae and other naturally occurring coccidia of sheep. *Am J Vet Res.* (1978) 39:7–10.

20. Taylor MA, Catchpole J, Marshall J, Marshall RN, Hoeben D. Histopathological observations on the activity of diclazuril (Vecoxan®) against the endogenous stages of Eimeria crandallis in sheep. *Vet Parasitol.* (2003) 116:305–14. doi: 10.1016/S0304-4017(03)00256-5

21. Saratsis A, Voutzourakis N, Theodosiou T, Stefanakis A, Sotiraki S. The effect of sainfoin *(Onobrychis viciifolia*) and carob pods (*Ceratonia siliqua*) feeding regimes on the control of lamb coccidiosis. *Parasitol Res.* (2016) 115:2233–42. doi: 10.1007/s00436-016-4966-9

22. de Souza Rodrigues F, Cezar AS, de Menezes FR, Sangioni LA, Vogel FSF, de Avila BS. Effect of toltrazuril at single dose against *Eimeria* spp. in lambs kept at pasture in condition favorable to coccidiosis. *Small Rumin Res.* (2020) 192:106211. doi: 10.1016/j.smallrumres.2020.106211

23. Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol Rev.* (1990) 70:567–90. doi: 10.1152/physrev.1990.70.2.567

24. Thorne JW, Murdoch BM, Freking BA, Redden RR, Murphy Thomas W, Taylor JB, et al. Evolution of the sheep industry and genetic research in the United States: opportunities for convergence in the twenty-first century. *Anim Genet.* (2021) 52:395–408. doi: 10.1111/age.13067

25. Van Wyk JA, Bath GF. The FAMACHA system for managing haemonchosis in sheep and goats by clinically identifying individual animals for treatment. *Vet Res.* (2002) 33:509–29. doi: 10.1051/vetres:2002036

26. Nurzaty Ewani AH, Ariff OM, Sani RA, Rasedee A. Relationship between coccidiosis infection and hematological profile, body weight and famacha scores in Dorper sheep. *Mal J Anim Sci.* (2014) 17:103–10.

27. Meradi S, Bentounsi B. Lamb's Eimeria infections raised in a steppic region and their impacts on clinical indicators (FAMACHA© and Disco). *J Parasit Dis.* (2021) 45:599–605. doi: 10.1007/s12639-020-01336-z

28. Wang CR, Xiao JY, Chen AH, Chen J, Wang Y, Gao JF, et al. Prevalence of coccidial infection in sheep and goats in northeastern China. *Vet Parasitol.* (2010) 174:213–17. doi: 10.1016/j.vetpar.2010.08.026

29. Goering HK, Van Soest PJ. *Forage fiber analyses (apparatus, reagents, procedures, and some applications)*. US Agricultural Research Service. (1970).

30. Shreve B, Thiex N, Wolf M. *NFTA Method 2.1. 4—Dry matter by oven drying for 3 hr at 105C*. National Forage Testing Association, Lincoln, NE. (2006).

31. Horwitz W, Latimer GW. *Association of Official Analytical chemists*. Gaithersburg: MD, USA. (2000).

32. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci.* (1991) 74:3583–97. doi: 10.3168/jds.S0022-0302(91)78551-2

33. Hall MB. Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method recommended for AOAC collaborative study. *J AOAC Int.* (2009) 92:42–9. doi: 10.1093/jaoac/92.1.42

34. R Core Team. *R: A Language and Environment For Statistical Computing*. Published online (2021).

35. Bates D, Mächler M, Bolker B, Walker S. *Fitting Linear Mixed-Effects Models Using lme4*. arXiv preprint arXiv:1406.5823 (2014). doi: 10.18637/jss.v067.i01

36. Lenth R, Singmann H, Love J, Buerkner P, Herve M. *Package 'emmeans'* (2019).

37. De la Fuente C, Cuquerella M, Carrera L, Alunda JM. Effect of subclinical coccidiosis in kids on subsequent trichostrongylid infection after weaning. *Vet Parasitol.* (1993) 45:177–83. doi: 10.1016/0304-4017(93)90072-U

38. Gauly M, Reeg J, Bauer C, Erhardt G. Influence of production systems in lambs on the Eimeria oocyst output and weight gain. *Small Rumin Res.* (2004) 55:159–67. doi: 10.1016/j.smallrumres.2004.02.001

39. Kaya G. Prevalence of Eimeria species in lambs in Antakya province. *Turk J Vet Anim Sci.* (2004) 28:687–92. Available online at: journals.tubitak.gov.tr/veterinary/vol28/iss4/9

40. Atti N, Mahouachi M. The effects of diet, slaughter weight and docking on growth, carcass composition and meat quality of fat-tailed Barbarine lambs. *A review Trop Anim Health Prod.* (2011) 43:1371–78. doi: 10.1007/s11250-011-9865-6

41. Bhatt RS, Sahoo A. Effect of adding formaldehyde treated protein alone and with Saccharomyces cerevisiae in diet on plane of nutrition, growth performance, rumen fermentation and microbial protein synthesis of finisher lambs. *Small Rumin Res.* (2019) 171:42–8. doi: 10.1016/j.smallrumres.2018.12.005

42. Hegarty RS, Shands C, Marchant R, Hopkins DL, Ball AJ, Harden S. Effects of available nutrition and sire breeding values for growth and muscling on the development of crossbred lambs. 1: Growth and carcass characteristics. *Aus J Agric Res.* (2006) 57:593–603. doi: 10.1071/AR04275

43. Fimbres H, Kawas JR, Hernández-Vidal G, Picón-Rubio JF, Lu CD. Nutrient intake, digestibility, mastication and ruminal fermentation of lambs fed finishing ration with various forage levels. *Small Rumin Res.* (2002) 43:275–81. doi: 10.1016/S0921-4488(02)00013-5

44. Papi N, Mostafa-Tehrani A, Amanlou H, Memarian M. Effects of dietary forage-to-concentrate ratios on performance and carcass characteristics of growing fat-tailed lambs. *Anim Feed Sc Technol.* (2011) 163:93–8. doi: 10.1016/j.anifeedsci.2010.10.010

45. Ejlertsen M, Githigia SM, Otieno RO, Thamsborg SM. Accuracy of an anaemia scoring chart applied on goats in sub-humid Kenya and its potential for control of Haemonchus contortus infections. *Vet Parasitol.* (2006) 141:291–301. doi: 10.1016/j.vetpar.2006.05.020

46. Sissay MM, Uggla A, Waller PJ. Epidemiology and seasonal dynamics of gastrointestinal nematode infections of sheep in a semi-arid region of eastern Ethiopia. *Vet Parasitol.* (2007) 143:311–21. doi: 10.1016/j.vetpar.2006.08.026

47. Kaplan RM, Burke JM, Terrill TH, Miller JE, Getz WR, Mobini S, et al. Validation of the FAMACHA© eye color chart for detecting clinical anemia in sheep

and goats on farms in the southern United States. *Vet Parasitol.* (2004) 123:105–20. doi: 10.1016/j.vetpar.2004.06.005

48. Burke JM, Kaplan RM, Miller JE, Terrill TH, Getz WR, Mobini S, et al. Accuracy of the FAMACHA system for on-farm use by sheep and goat producers in the southeastern United States. *Vet Parasitol.* (2007) 147:89–95. doi: 10.1016/j.vetpar.2007.03.033

49. Acharya Mohan, Burke Joan M, Miller James E, Terrill Thomas H, Wood Erin L, Muir James P. Quebracho tannins aid in the control of *Eimeria* spp. and gastrointestinal nematodes in lambs and goat kids. *Vet Parasitol.* (2020) 288:109295. doi: 10.1016/j.vetpar.2020.109295

50. Liu Hongjin, Xu Tianwei, Xu Shixiao, Ma Li, Han Xueping, Wang Xungang, et al. Effect of dietary concentrate to forage ratio on growth performance, rumen fermentation and bacterial diversity of Tibetan sheep under barn feeding on the Qinghai-Tibetan plateau. *PeerJ.* (2019) 7:e7462. doi: 10.7717/peerj.7462

51. Dijkstra J, Forbes JM, France J. *Quantitative Aspects of Ruminant Digestion and Metabolism*. Cambridge: CABI Pub. (2005). doi: 10.1079/9780851998145.0000

52. Etsay K. Megbey S, Yohannes H. Prevalence of sheep and goat coccidiosis in different districts of Tigray region, Ethiopia Niger. *J Anim Sci.* (2020) 22:61–9.

53. Mohammed RA, Idris OA, El Sanousi SM, Abdelsalam EB. The effect of coccidian infection on the gut microflora of Nubian goat kids. *DTW Deutsche Tierarztliche Wochenschrift.* (2000) 107:414–16.

54. Lin L, Xie F, Sun D, Liu J, Zhu W, Mao S. Ruminal microbiome-host crosstalk stimulates the development of the ruminal epithelium in a lamb model. *Microbiome.* (2019) 7:1–16. doi: 10.1186/s40168-019-0701-y

55. Liu L, Sun D, Mao S, Zhu W, Liu J. Infusion of sodium butyrate promotes rumen papillae growth and enhances expression of genes related to rumen epithelial VFA uptake and metabolism in neonatal twin lambs. *J Anim Sci.* (2019) 97:909–21. doi: 10.1093/jas/sky459

56. Górka P, Kowalski ZM, Pietrzak P. Kotunia Anna, Jagusiak W, Holst JJ, et al. Effect of method of delivery of sodium butyrate on rumen development in newborn calves. *J Dairy Sci.* (2011) 94:5578–88. doi: 10.3168/jds.2011-4166

57. Kowalski ZM, Górka P, Flaga J, Barteczko A, Burakowska K, Oprzadek J, et al. Effect of microencapsulated sodium butyrate in the close-up diet on performance of dairy cows in the early lactation period. *J Dairy Sci.* (2015) 98:3284–91. doi: 10.3168/jds.2014-8688

58. Malhi M, Gui H, Yao L, Aschenbach JR, Gäbel G, Shen Z. Increased papillae growth and enhanced short-chain fatty acid absorption in the rumen of goats are associated with transient increases in cyclin D1 expression after ruminal butyrate infusion. *J Dairy Sci.* (2013) 96:7603–16. doi: 10.3168/jds.2013-6700

59. Monteiro HF, Faciola AP. Ruminal acidosis, bacterial changes, and lipopolysaccharides. *J Animal Sci.* (2020) 98:skaa248. doi: 10.1093/jas/skaa248

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A century of "anticoccidial drugs": bibliometric analysis

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Publications are an important measure of scientific and technological progress. The quantitative examination of the number of publications in a certain research topic is known as bibliometrics. Bibliographic studies are widely used to analyse the condition of research, future potential, and current growth patterns in a certain topic. It can serve as a basis for making decisions and implementing strategies to achieve long-term development goals. To our knowledge, no research has been conducted in these domains; so, this work aims to employ bibliometric analysis to provide comprehensive data on publications related to anticoccidial drugs. As a result, the current study uses bibliometric analysis to track the evolution of anticoccidial drugs and its consequences in the academic and public worlds via a survey of relevant scientific and popular publications. The Dimensions database was used to retrieve the bibliographical statistics, which were then cleaned and analyzed. The data was also loaded into the VOS viewer, which generated a network visualization of the authors with the most joint articles. The investigation discovered three stages of publications and citations since the first article on anticoccidial drugs in 1949. The first stage, which ran from 1920 to 1968, was characterized by a scarcity of research articles on anticoccidial drugs. From 1969 to 2000, the second stage was marked by a stable and marginally increased number of articles. The scientific field was characterized by an increasing trend in the number of publications and their citations from 2002 to 2021. The study gave a complete list of the top anticoccidial drugs funding agents, countries, research institutes, most cited publications, and important co-authorship and partnerships. The outcomes of the study will help veterinary practitioners and researchers understand the trends and best sources of knowledge in the field of anticoccidial medications.

KEYWORDS

anticoccidial, bibliometric, research, publications, analysis

1. Introduction

Over the past several decades, numerous attempts have been conducted to unpack and dissect anticoccidial drugs' development, efficacy, and challenges. Examples of anticoccidial drug subgroups include Ionophores, Sulphonamides, Vitamin Antagonists, Nicarbazin, Quinolones, and others (1). Anticoccidial drugs are used to treat infectious diseases in the intestinal tract along with the new development in coccidiosis vaccines (2, 3). An ideal anticoccidial drug has been found to have high efficacy in performing a broad-spectrum activity (4). In addition, an ideal drug must have large therapeutic index (TI). A drug with a high therapeutic index is considered safer than one with a low therapeutic index because it can be administered at a wider range of doses before adverse effects occur. The drug is also should be cost-effective, making it adequately affordable to the market (5). Besides, some past researchers have observed that most drug subgroups do not affect organoleptic criteria in determining the meat and carcass quality. Ideally, most anticoccidial drugs can be metabolized and excreted without toxic residuals (6).

Coccidiostat failure has also been observed in several publications. Some of the causes identified include more than average oocysts exposure in the host. Besides, some of the drug subgroups may not be effective when treating or preventing all the Eimeria species (7–9). Besides, poor management, especially in poultry production, has also been identified as a cause of wet litter, increasing the risk of Coccidiostat failure (10). Prolonged drug use and intercurrent diseases can lead to drug resistance (7, 11). The efficacy of the anticoccidial drugs has been discussed widely and in-depth, with numerous publications being conducted since 1955 when Nicarbazin, the first broad-spectrum anticoccidial drug was approved (2, 12–14).

The study will employ prior literature as datasets holding information such as authors, titles of publications, years of publication, funding agents, the source institutes and countries. As a result, these data be used to establish a quantitative intellectual framework for anticoccidial medication research. However, due to the enormous number of publications on the subject, manually gathering and compiling all data is impractical, thanks to internet databases and software-based techniques that can easily enable effective data accessibility. As a result, the study seeks to give a bibliometric and visual analysis of anticoccidial drugs by studying the intellectual domain and the time evolution of anticoccidial drug research.

The objectives of this study are 1) Analyze the trend in anticoccidial drugs publications in terms of the volume of documentation and citations in the field, 2) Analyze the most frequently cited research to contribute to the knowledge in the field of anticoccidial drugs, 3) Create awareness of the topmost recognized publishers, the most active country, the most active institutions and the top most funding agents in the field of anticoccidial drugs, and 4) Highlight the topmost authors with the highest number of jointly authored publications on anticoccidial drugs. To the best of our knowledge, no research has been done on these areas of thought, hence the goal of this paper is to use bibliometric analysis to give full data on anticoccidial drug publications.

2. Materials and methods

2.1. Research questions

The questions raised in this study include 1) What is the trend in the volume of publications and documentation citation of the anticoccidial drugs over the past century? 2) What are the topmost countries that researchers prefer for their works on anticoccidial drugs? 3) What are the topmost researchers, research institutes, and frequent funding agents in the field of anticoccidial drugs? 4) What is the intellectual structure of the joint authorship on anticoccidial drug publications?

2.2. Bibliometric approach

The research employs bibliometric analysis and science mapping to perform the quantitative and visual analysis of anticoccidial drugs. Bibliometrics was first created by Otlet in 1934 (15). Later, Broadus defined the term as the quantitative study of bibliographic units or published units (16). In 1969, Pritchard coined Bibliometrics to replace the statistical bibliography (17). The research approach has become a valuable tool for evaluating the research outputs and dealing with ever-increasing information. Besides, databases and software have made it possible and practical to obtain and analyze massive and complex bibliometric data (18). Currently, several tools exist to analyze the trends of publications, visualize the citation network, and identify new topics and trend patterns for the scientific disciplines. These tools can conduct a science mapping using the bibliometric approach to produce a spatial representation of networks. The information from the publications, such as the publication's year, title, author(s), and citations, are crucial in providing these visual representations (19). Besides, the visual maps allow one to view the linkages and development of knowledge that would not be possible without statistics.

For this study, data collection involves collecting relevant literature published and collected in different databases for further analysis. The data collection was achieved by first identifying the databases and choosing appropriate search strategy techniques, data retrieval techniques, and cleaning the data before feeding them into different tools for analysis and visualization.

2.3. Retrieval of data

Nowadays, several databases exist that offer bibliometric data. Examples of such databases include Web of Science, Scopus, PubMed, Cochrane Central Register of Controlled Trials (CENTRAL), Google Scholar, Dimensions, CORE, BASE, Science.gov, Crossref API, Microsoft Academic Search, and JSTOR Data for Research. For this research, Dimensions[1] will be used to retrieve the bibliometric data because it is open software, and the data will be easily integrated into the VOS viewer. The study will use VOS viewer as the software tool to provide the network visualizations for the bibliometric data on anticoccidial drugs. VOS is a free online computer program for scientific mapping, offering a cluster display of complex networks (20).

In this study, the "dimensions database" was used to retrieve bibliometric data owing to 1) Dimensions covers a wider range of research outputs beyond scholarly articles and conference proceedings, including grants, datasets, clinical trials, patents, and policy documents, 2) citation analysis tools that allow users to identify highly cited articles and authors, track citation trends over time, and measure the impact of research, and 3) Dimensions has a more modern and

1 https://www.dimensions.ai/

intuitive user interface that allows users to filter and sort search results more easily. It also offers more visualization tools and interactive features, such as citation maps and co-author networks.

2.4. Search strategy, exclusion criteria and limitations

The study used "anticoccidial drug" as the keyword to search relevant publications. The data retrieved comprised all the publications with the term "anticoccidial drug" in either their title, abstract or the main document. The study did not exclude any of the parameters provided in the dimensions database since the study intended to explore all the publications stored on anticoccidial drugs. The documents retrieved included articles, book chapters, editorial materials, and reviews. The search strategy retrieved a total of 5,000 publications. However, using a single dataset was limiting since some critical publications may not have been included in the Dimensions Database. Besides, the database also does not provide some bibliometric features such as keywords and references compared to other Databases. Since only English words were employed to search for relevant publications, the search may have left out some critical articles published in other languages such as French, Spanish, and Chinese. Therefore, the results may not be generalized to other researchers published in non-English speaking countries. Although the analysis may not include all the crucial publications on anticoccidial drugs, this study's results offer a reliable insight into the trends and patterns in the publications that have been made on anticoccidial drugs.

2.5. Preferred reporting items for systematic reviews and meta-analysis (PRISMA)

The study employed PRISMA in identifying, screening, and selecting the studies to be included in various analyses. The criteria were inclusive of all the years since the trend analysis was crucial in determining the progress made in terms of publications. Although the criteria did not specify a particular type of study, the database selection generated articles, chapters, edited books, and reprinted publications only. Upon retrieving the publications' data, the data was cleaned by removing duplicates. The study also excluded publications that were not relevant to the study. Only the publications with the most recent citations were included in the risk of bias assessment. Details of the screening process and selection of the studies are shown in Figure 1.

2.6. Quality assessment and risk of bias

Two independent researchers assessed the selected publications to determine their eligibility for the study. The majority of the selected articles were considered to be of high quality in terms of the methodology used and the relevance of the abstract to the study's issue. During the screening stage for each study, the researchers were keen to determine the method of study participant selection, the strategy to reporting used, the methodologies used to measure the outcomes, the presence of detection bias, and attrition bias. Depending on the information given in the publications, each component was classified as uncertain, low, or high. Summary of quality assessment outcomes is provided in Table 1.

3. Results

3.1. Bibliometric analysis of the publication trends in anticoccidial drugs

The bibliometric study on anticoccidial drugs from 1920 to 2021 revealed three stages of development based on the total publication in each of the years, as shown in Figure 2. The first stage, identified from 1920 to 1968, was marked by a low number of research publications related to anticoccidial drugs. This was likely because the research was not fully developed in that period. The second stage, which runs from 1969 to 2000, was marked by constant and slightly significant publications until 2001 when there was a surge in the number of publications, almost double from the previous year. The third stage began in 2002 to 2021, where the research field was marked by an ever-constant increasing trend in the number of publications and their citations. The number of citations in the two last years has decreased because the recent publications did not have the advantage of the time to allow awareness among the researchers, pending inclusion into the database and the closure due to COVID-19.

3.2. Contribution to the anticoccidial field by country

The study revealed that publications on anticoccidial drugs originated from 102 countries. The United States was identified as the leading country of publications, followed by China and the United Kingdom. Figures 3, 4 show the overall distribution of the countries and the countries with over 100 publications, respectively. The full list of per-country publications is provided in Supplementary Table S1.

3.3. The leading institutions

The publications on anticoccidial drugs were investigated by the contribution of research institutes. The leading research institutions with more than 30 publications evaluating the anticoccidial drug are provided in Figure 5. The full list of per-institute publications is provided in Supplementary Table S2. These institutions contributed to the previously described country's rank with the top four countries in anticoccidial publications, United States, United Kingdom, China, and Egypt.

3.4. The most frequent funding agents

The most frequent funding agents are displayed in Figure 6 and Supplementary Table S3. The National Natural Science Foundation of China is the leading funding institution in this field, having funded 312 research. Among the top funding agents was the European commission, US dept. of Agriculture, Japan JSPS and the Australian research council.

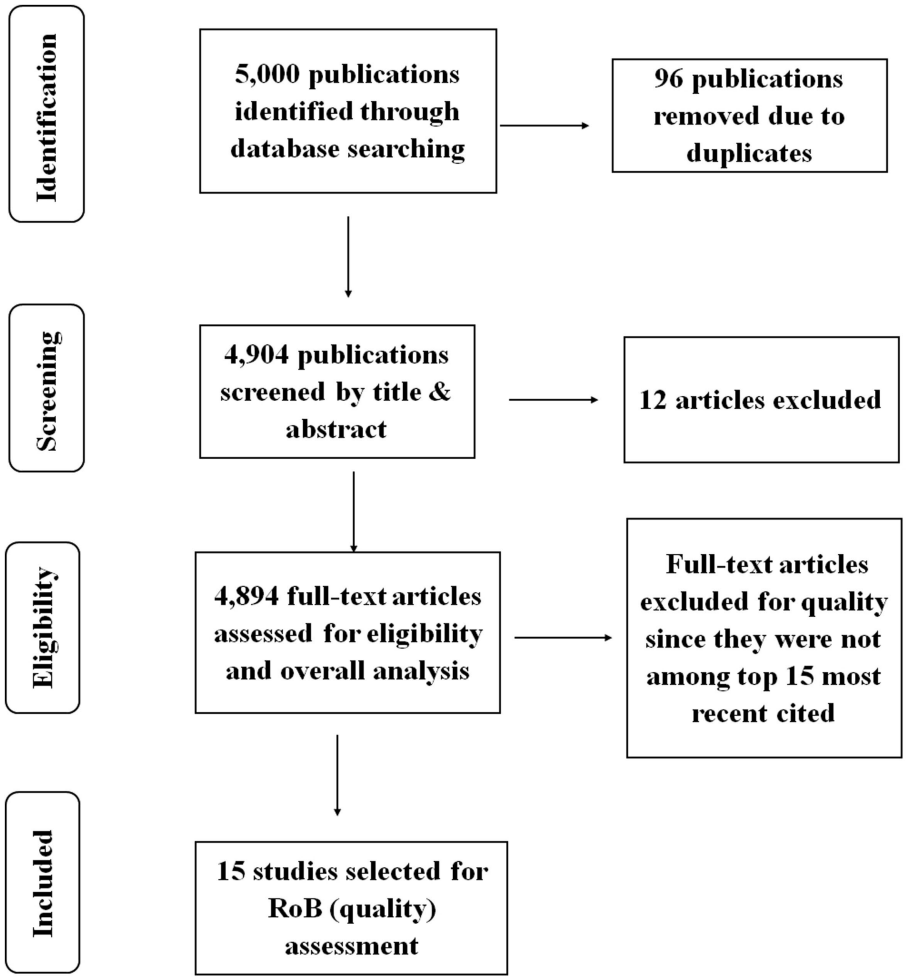

FIGURE 1
PRISMA flow chart for bibliometric analysis of anticoccidial drugs.

3.5. The recently cited publications on anticoccidial drugs

In terms of the most widely recently cited publications on anticoccidial drugs, Johnson & Reid (21) lead both in the total citations as well as the most recently cited works despite having made the publication in 1970. The experiments on chicken lesion scoring seem to have remained constant throughout the years and are widely recognized by researchers in this field. A study on gastrointestinal microbiome among broiler chickens published just recently in 2017 (22) was in the second rank with 149 recent citations despite having been published less than 50 years ago. Among the 15 selected research on anticoccidial drugs, all of them have more than 50 recent citations, revealing that they have received widespread interest in this field (Table 2).

3.6. Topmost authors with joint publications

The study used the VOS viewer to visualize the authors with the highest collaboration in anticoccidial drugs. Based on the network visualization provided in Figure 7, Li Xiangrui, Zhao Qiping, Suo Xun and Lillehoj Hyun were among the highest authors in anticoccidial drugs with the most joint publications. The network helps identify the authors with the highest collaborations and shows that authors can jointly publish with other authors. The different colours identify the various topics discussed by the authors related to anticoccidial drugs.

3.7. Eimeria species

The fraction of publications citing Eimeria species was traced in the publications. The traced species comprise *Eimeria acervulina, Eimeria brunetti, Eimeria maxima, Eimeria mitis, Eimeria necatrix, Eimeria praecox,* and *Eimeria tenella*. Each species of Eimeria affects a different part of the chicken's digestive tract, and can cause a range of symptoms including diarrhea, decreased appetite, weight loss, and even death (32). The search comprised the inclusion of species name in the title or abstract of the publication. The outcomes of the search revealed 1,073 publications for *E. acervulina,* 3,854 for *E. brunetti,* 10,964 for *E. maxima,* 3,189 for *E. mitis,* 5,840 for *E. necatrix,* 3,414 for *E. praecox,* and 18,971 *E. tenella*.

In comparison with the chicken Eimeria species, the bovine and ovine Eimeria also received a great bulk of scientific contributions. The

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TABLE 1 Quality assessment for risk of systematic bias for the articles included in the systematic review on the efficacy of anticoccidial drugs (categorized as low, high, and unclear).

Study (author and year)		Selection of participants (Selection bias)	Selective outcome reporting	Measurement of exposure (measurement exposure)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)
1	Johnson and Reid (21)	unclear	low	high	unclear	low
2	Clavijo and Flórez (22)	high	low	unclear	unclear	low
3	Dalloul and Lillehoj (3)	low	low	low	high	low
4	Blake and Tomley (8)	unclear	low	high	low	low
5	Williams (23)	high	low	low	unclear	low
6	Danzeisen et al. (24)	low	high	low	unclear	unclear
7	Chapman (25)	unclear	unclear	low	unclear	low
8	Peek and Landman (26)	low	unclear	high	low	low
9	Blake et al. (27)	high	low	unclear	low	unclear
10	Chapman et al. (28)	low	high	unclear	unclear	low
11	Noack et al. (2)	high	high	high	unclear	low
12	Quiroz-Castañeda and Dantán-González (29)	low	unclear	unclear	low	high
13	Williams (30)	high	low	low	unclear	unclear
14	Collier et al. (9)	unclear	low	high	high	unclear
15	Chapman et al. (31)	unclear	low	high	low	low

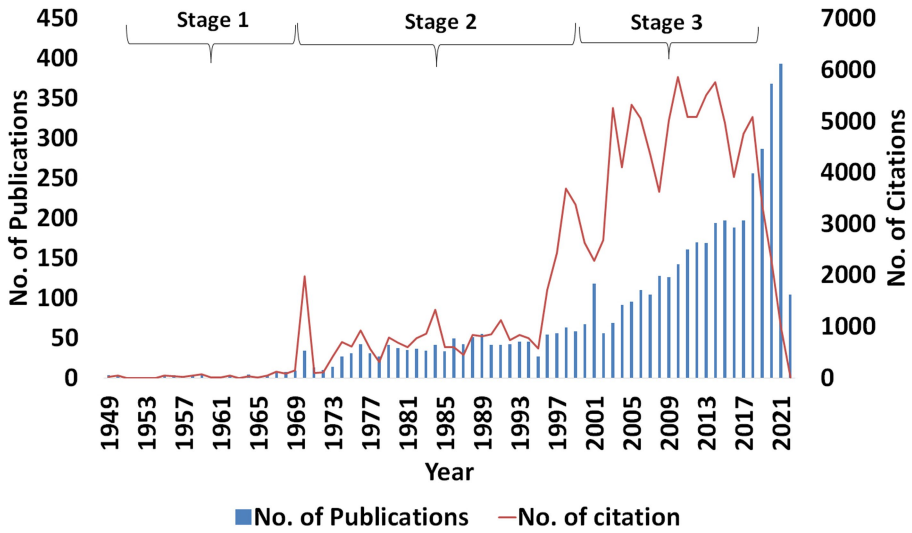

FIGURE 2
Annual total output of publications and citations on anticoccidial drugs. The data was retrieved from 1920 to 2021.

retrieved numbers were 12,090 for *E. bovis*, 3,226 for *E. ellipsoidalis*, 3,236 for *E. cylindrica* and 3,611 for E. zuernii. There was 2,761 articles for *E. ovinoidalis* in sheep and 2,810 for *E. ninakohlyakimovae* in goats.

4. Discussion

Based on the data retrieved, the first publication on anticoccidial drugs was first published in 1949 by Waletzky, Hughes & Brandt on the anticoccidial activity of Nitrophenide (31, 33). Since then, publications on anticoccidial drugs showed progressive advancements. This study's objective was to evaluate the trends in this field and acknowledge the authors, publications, funding agents, research network in anticoccidial drugs and broad topics that researchers have cited. Based on the performance analysis of publications on anticoccidial drugs, the research established a strong growth in the number of publications and citations over the years. In 2021, there were 393 publications on anticoccidial drugs,

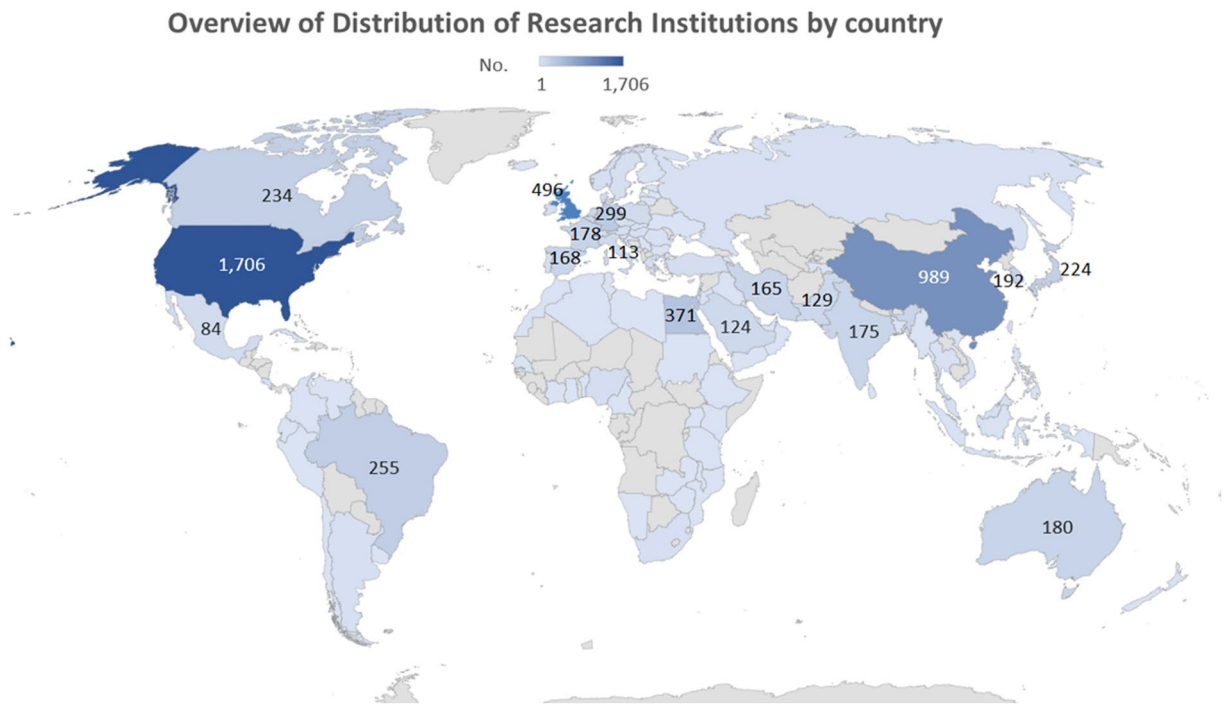

FIGURE 3
Overview of Distribution of Countries with more than 100 publications on anticoccidial drugs.

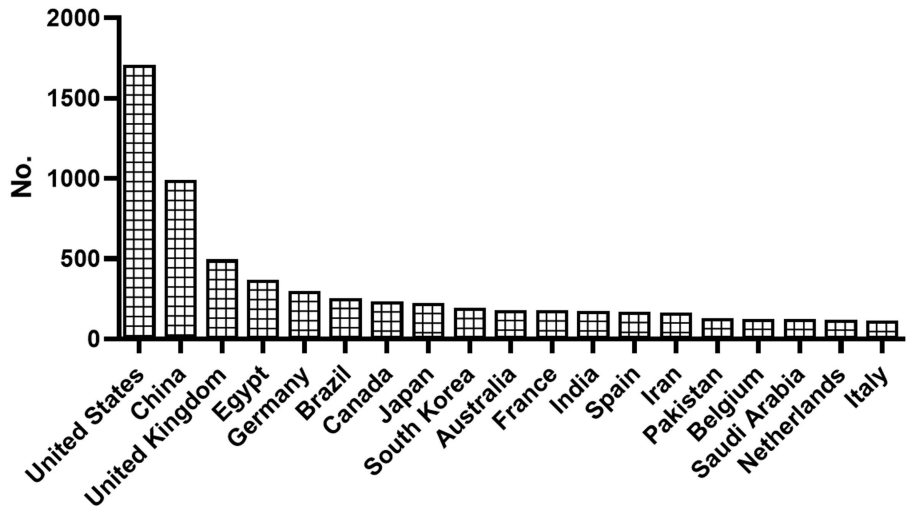

FIGURE 4
The rank of countries with more than 100 publications in the field of anticoccidial drugs.

which reveals that more and more researchers have continued to gain interest in anticoccidial chemotherapy. Besides, some researchers have found the drug's efficiency in reducing disease resistance necessitating increased research in the field to unfold the causes and, most importantly, come up with mitigative measures and advanced medicines to fight the pathogens (3, 8, 22). The number of citations in each year has also increased, with a declined trend observed in the last 3 years. This decline can be explained by limited time exposure of the publications to a broad group of researchers and might be due to the closure during COVID-19 outbreak. However, it is highly expected that the total number of citations will increase as more researchers return to pre-COVID-19 circumstances.

The bibliometric analysis identified that despite the anticoccidial drugs made for several animals in the livestock industry, such as dogs, cattle, and rabbits, to name a few, most of the publications were done

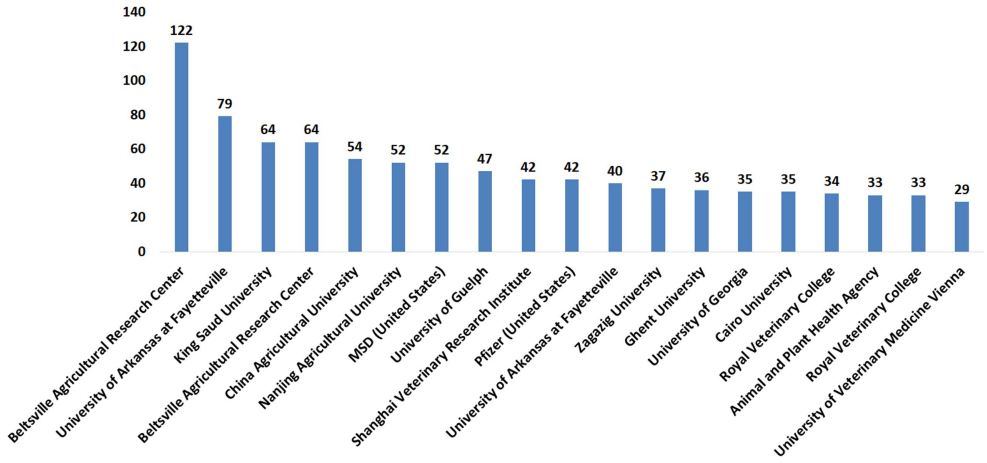

FIGURE 5
The Research Institutions with over 30 publications on anticoccidial drugs.

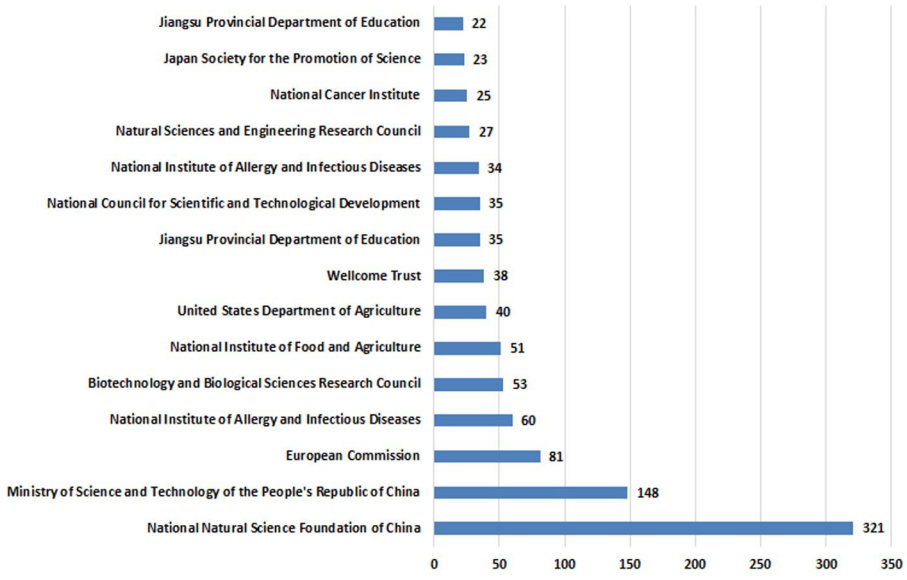

FIGURE 6
The Most Frequent Funding Agents for anticoccidial drugs.

on chicken (2, 26). Research shows that coccidiosis is ranked among the most costly disease in chickens, explaining the increased studies in the area (27). Besides, the intestinal disease is mainly spread by contact. Among the livestock, poultry is among the most crowded type of farming since several chickens can be reared in a small area (29, 30).

The bibliometric study on anticoccidial drugs is relevant, especially to both veterinary practitioners and scholars, in giving guidance on the trends in the field. Besides, they can gain awareness of the appropriate places to search for relevant information in the field. The co-author network provided, along with other science of mapping visualization techniques, can offer guidance on some of the authors widely known in the field. Besides, other databases can offer more information, such as the most widely used keywords, publications references, and citation networks. Hence, one of the limitations of this study is the use of one database. However, combining several databases may likely lead to biased data since some of the information may be easily duplicated.

The bibliometric analysis supports the importance of pathogenic Eimeria species. According to the estimated publications count, *E. tenella* followed by *E. maxima* and *E. necatrix*. The highly pathogenic chicken Eimeria as *E. tenella* and gut-impacting *E. maxima* are attracting the bulk of research related to coccidiosis. Similarly, *E. bovis* research constituted the major trend in large animals coccidiosis research.

TABLE 2 Most recently cited papers in anticoccidial drugs.

R	Total citation	Title	Authors	Year	Recent citations	Reference
1	876	Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens	Johnson and Reid	1970	159	(21)
2	190	The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review	Clavijo and Flórez	2017	142	(22)
3	364	Poultry coccidiosis: recent advancements in control measures and vaccine development	Dalloul and Lillehoj	2006	98	(3)
4	230	Securing poultry production from the ever-present Eimeria challenge	Blake and Tomley	2013	98	(8)
5	319	Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity	Williams	2005	96	(23)
6	244	Modulations of the Chicken Cecal Microbiome and Metagenome in Response to Anticoccidial and Growth Promoter Treatment	Danzeisen et al.	2011	82	(24)
7	167	Milestones in avian coccidiosis research: A review	Chapman	2014	82	(25)
8	183	Coccidiosis in poultry: anticoccidial products, vaccines, and other prevention strategies	Peek and Landman	2011	79	(26)
9	67	Re-calculating the cost of coccidiosis in chickens	Blake et al.	2020	67	(27)
10	177	Forty years of monensin for the control of coccidiosis in poultry	Chapman et al.	2010	63	(28)
11	63	Anticoccidial drugs of the livestock industry	Noack et al.	2019	61	(2)
12	96	Control of Avian Coccidiosis: Future and Present Natural Alternatives	Quiroz-Castañeda and Dantán-González	2015	60	(29)
13	298	A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry	Williams	1999	59	(30)
14	197	Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth	Collier et al.	2007	55	(9)
15	140	A Selective Review of Advances in Coccidiosis Research	Chapman et al.	2013	51	(31)

5. Conclusion

The number of publications in a field can be taken as a good indicator of its relative level of development. Bibliometrics is the quantitative analysis of the number of scholarly works in a specific field. The existing state of research, its potential for expansion, and the rates at which new publications appear in a field are all things that bibliographic studies are commonly used to assess. As far as we are aware, no studies have been undertaken in these areas; therefore, the purpose of this work is to use bibliometric analysis to compile a large database of articles about anticoccidial medications. Therefore, the current work employs bibliometric analysis to examine key scientific and popular literature in order to trace the development of anticoccidial medications and its effects in the academic and public spheres. Since the first article on anticoccidial medicines appeared in 1949, the research found that there have been three distinct waves of publication and citation activity. During the first phase, which lasted from 1920 to 1968, there were hardly any published studies on anticoccidial medications. The second phase, which lasted from 1969 to 2000, was characterized by a rather stable and slightly elevated number of papers. Between 2002 and 2021, the scientific discipline saw a rising tide of publications and citations. The study detailed the main funding agencies, countries, research institutions, most referenced papers, and significant co-authorship and collaborations involved in the development of anticoccidial medicines. Researchers and veterinarians will benefit from the study's findings since they will have a better idea of where to look for information about anticoccidial drugs.

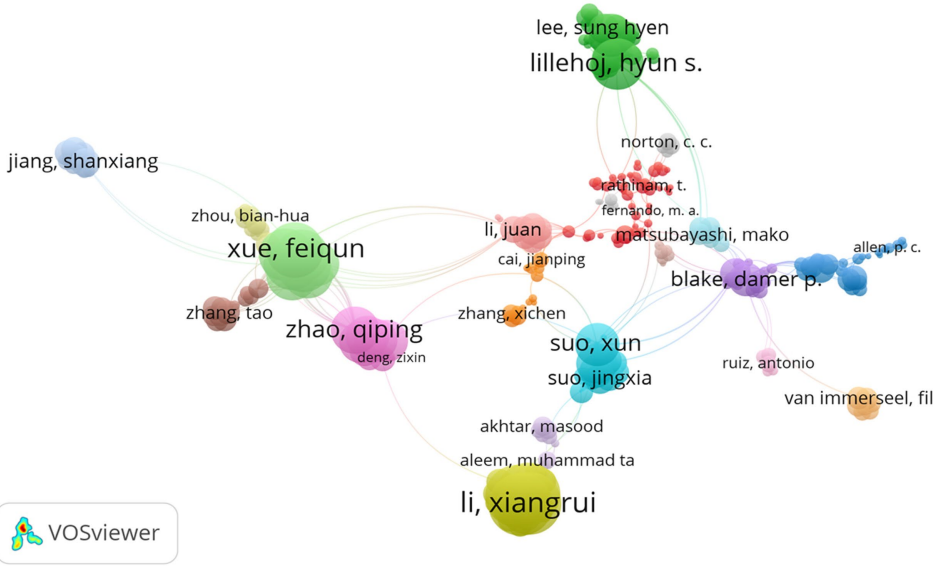

FIGURE 7
Intellectual structure of the topmost authors with joint publications.

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.

Author contributions

MK, MAM, HA, MM, MI, MA-R, and HE-B planned the study design, contributed to data analysis, and wrote and revised the manuscript. MK, IA, and KV contributed to data extraction and analysis. All authors contributed to the article and approved the submitted version.

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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.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1157683/full#supplementary-material

References

1. Chapman HD, Rathinam T. Focused review: the role of drug combinations for the control of coccidiosis in commercially reared chickens. *Int J Parasitol Drugs Drug Resist*. (2022) 18:32–42. doi: 10.1016/j.ijpddr.2022.01.001

2. Noack S, Chapman HD, Selzer PM. Anticoccidial drugs of the livestock industry. *Parasitol Res*. (2019) 118:2009–26. doi: 10.1007/s00436-019-06343-5

3. Dalloul RA, Lillehoj HS. Poultry coccidiosis: recent advancements in control measures and vaccine development. *Expert Rev Vaccines*. (2006) 5:143–63. doi: 10.1586/14760584.5.1.143

4. Verma PK, Bais I, Parmar MS, Gopal A, Gupta V. Anticoccidial drugs used in the poultry: an overview. *Sci Int*. (2013) 1:261–5. doi: 10.17311/sciintl.2013.261.265

5. Silpa L, Niepceron A, Laurent F, Brossier F, Pénichon M, Enguehard-Gueiffier C, et al. Synthesis and evaluation of the anticoccidial activity of trifluoropyrido [1, 2-a] pyrimidin-2-one derivatives. *Bioorg Med Chem Lett*. (2016) 26:114–20. doi: 10.1016/j.bmcl.2015.11.018

6. Mooney D, Richards KG, Danaher M, Grant J, Gill L, Mellander P-E, et al. An investigation of anticoccidial veterinary drugs as emerging organic contaminants in groundwater. *Sci Total Environ*. (2020) 746:141116. doi: 10.1016/j.scitotenv.2020.141116

7. Rybicki MJ. Coccidiostats in treating coccidiosis. *ŻYWNOŚĆ Nauka Technologia Jakość*. (2020) 125:127–37. doi: 10.15193/zntj/2020/125/364

8. Blake DP, Tomley FM. Securing poultry production from the ever-present Eimeria challenge. *Trends Parasitol*. (2014) 30:12–9. doi: 10.1016/j.pt.2013.10.003

9. Collier C, Hofacre C, Payne A, Anderson D, Kaiser P, Mackie R, et al. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth. *Vet Immunol Immunopathol*. (2008) 122:104–15. doi: 10.1016/j.vetimm.2007.10.014

10. Chapman HD. Applied strategies for the control of coccidiosis in poultry. *CABI Rev*. (2018) 13:1–11. doi: 10.1079/PAVSNNR201813026

11. Martins RR, Silva LJ, Pereira AM, Esteves A, Duarte SC, Pena A. Coccidiostats and poultry: a comprehensive review and current legislation. *Foods*. (2022) 11:2738. doi: 10.3390/foods11182738

12. Kandeel M. Efficacy of amprolium and toltrazuril in chicken with subclinical infection of cecal coccidiosis. *Indian J Pharmacol*. (2011) 43:741–3. doi: 10.4103/0253-7613.89845

13. Chapman H. Evaluation of the efficacy of anticoccidial drugs against Eimeria species in the fowl. *Int J Parasitol*. (1998) 28:1141–4. doi: 10.1016/S0020-7519(98)00024-1

14. Awad AM, El-Nahas AF, Abu-Akkada SS. Evaluation of the protective efficacy of the anticoccidial vaccine Coccivac-B in broilers, when challenged with Egyptian field isolates of E. tenella. *Parasitol Res*. (2013) 112:113–21. doi: 10.1007/s00436-012-3112-6

15. Rousseau R. Forgotten founder of bibliometrics. *Nature*. (2014) 510:218. doi: 10.1038/510218e

16. Broadus RN. Toward a definition of "bibliometrics". *Scientometrics*. (1987) 12:373–9. doi: 10.1007/BF02016680

17. Pritchard A. Statistical bibliography or bibliometrics. *J Doc*. (1969) 25:348–9.

18. Chen C. CiteSpace II: detecting and visualizing emerging trends and transient patterns in scientific literature. *J Am Soc Inf Sci Technol*. (2006) 57:359–77. doi: 10.1002/asi.20317

19. Cobo MJ, López-Herrera AG, Herrera-Viedma E, Herrera F. Science mapping software tools: review, analysis, and cooperative study among tools. *J Am Soc Inf Sci Technol*. (2011) 62:1382–402. doi: 10.1002/asi.21525

20. van Eck NJ, Waltman L. Software survey: VOSviewer, a computer program for bibliometric mapping. *Scientometrics*. (2010) 84:523–38. doi: 10.1007/s11192-009-0146-3

21. Johnson J, Reid WM. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. *Exp Parasitol*. (1970) 28:30–6. doi: 10.1016/0014-4894(70)90063-9

22. Clavijo V, Flórez MJV. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: a review. *Poult Sci*. (2018) 97:1006–21. doi: 10.3382/ps/pex359

23. Williams RB. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. *Avian Pathol*. (2005) 34:159–80. doi: 10.1080/03079450500112195

24. Danzeisen JL, Kim HB, Isaacson RE, Tu ZJ, Johnson TJ. Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PLoS One*. (2011) 6:e27949. doi: 10.1371/journal.pone.0027949

25. Chapman HD. Milestones in avian coccidiosis research: a review. *Poult Sci*. (2014) 93:501–11. doi: 10.3382/ps.2013-03634

26. Peek H, Landman W. Coccidiosis in poultry: anticoccidial products, vaccines and other prevention strategies. *Vet Q*. (2011) 31:143–61. doi: 10.1080/01652176.2011.605247

27. Blake DP, Knox J, Dehaeck B, Huntington B, Rathinam T, Ravipati V, et al. Re-calculating the cost of coccidiosis in chickens. *Vet Res*. (2020) 51:115. doi: 10.1186/s13567-020-00837-2

28. Chapman HD, Jeffers TK, Williams RB. Forty years of monensin for the control of coccidiosis in poultry. *Poult Sci*. (2010) 89:1788–801. doi: 10.3382/ps.2010-00931

29. Quiroz-Castañeda RE, Dantán-González E. Control of avian coccidiosis: future and present natural alternatives. *Biomed Res Int*. (2015) 2015:430610. doi: 10.1155/2015/430610

30. Williams R. A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. *Int J Parasitol*. (1999) 29:1209–29. doi: 10.1016/S0020-7519(99)00086-7

31. Chapman HD, Barta JR, Blake D, Gruber A, Jenkins M, Smith NC, et al. A selective review of advances in coccidiosis research. *Adv Parasitol*. (2013) 83:93–171. doi: 10.1016/B978-0-12-407705-8.00002-1

32. Rialch A. Poultry coccidiosis: a continuous economic threat to poultry industry. *INDiAN FARmER* (2003); 455:378.

33. Waletzky E, Hughes C, Brandt M. The anticoccidial activity of nitrophenide. *Ann N Y Acad Sci*. (1949) 52:543–57. doi: 10.1111/j.1749-6632.1949.tb53942.x

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Impact of *Eimeria meleagrimitis* and intermittent amprolium treatment on performance and the gut microbiome composition of Turkey poults

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Introduction: Drug-sensitive live coccidiosis vaccines have been used to control coccidiosis and renew drug sensitivity in commercial chicken operations. However, only limited species coverage vaccines have been available for commercial turkey producers. This study aimed to assess the effect of an *E. meleagrimitis* vaccine candidate, with and without amprolium intervention, on performance and oocyst shedding. Additionally, the effect of vaccination, amprolium treatment, and *E. meleagrimitis* challenge on intestinal integrity and microbiome composition was evaluated.

Methods: Experimental groups included: (1) NC (non-vaccinated, non-challenged control); (2) PC (non-vaccinated, challenged control); (3) VX + Amprol (*E. meleagrimitis* candidate vaccine + amprolium); and 4) VX (*E. meleagrimitis* candidate vaccine). For VX groups, 50% of the direct poults were orally vaccinated at DOH with 50 sporulated *E. meleagrimitis* oocysts and were comingled with contact or non-vaccinated poults for the duration of the study. From d10-14, VX + Amprol group received amprolium (0.024%) in the drinking water. All groups except NC were orally challenged with 95K *E. meleagrimitis* sporulated oocysts/mL/poult at d23. At d29, ileal and cecal contents were collected for 16S rRNA gene-based microbiome analysis.

Results and Discussion: VX did not affect performance during the pre-challenge period. At d23-29 (post-challenge), VX groups had significantly ($P < 0.05$) higher BWG than the PC group. Contacts and directs of VX groups in LS had significantly reduced compared to PC. As anticipated, amprolium treatment markedly reduced fecal and litter OPG for the VX + Amprol group compared to the VX group which did not receive amprolium. The ileal and cecal content results showed that the PC group had different bacterial diversity and structure, including alpha and beta diversity, compared to NC. Linear discriminant analysis Effect Size (LEfSe) identified that *Lactobacillus salivarius* (ASV2) was enriched in PC's ileal and cecal content. Compared to NC and PC, the vaccinated groups showed no distinct clusters, but there were similarities in the ileal and cecal communities based on Bray-Curtis

and Jaccard distances. In conclusion, these results indicate that vaccination with this strain of *E. meleagrimitis*, with or without amprolium intervention, caused a very mild infection that induced protective immunity and challenge markedly affected both the ileal and cecal microbiome.

KEYWORDS

***Eimeria*, coccidiosis, vaccination, turkey, microbiome, amprolium**

1. Introduction

Eimeria, a genus of obligate intracellular protozoa, cause intestinal coccidiosis in many vertebrate hosts including poultry. Host intestinal epithelial cells are invaded and destroyed by these enteric parasites, impacting gut homeostasis and performance (1). For nearly a century, chemoprophylaxis has been employed to control coccidiosis in commercial poultry (2). Nevertheless, *Eimeria* spp. have been shown to develop resistance to anticoccidial drugs (3). Anticoccidial rotation and shuttle programs have extended the period of use for some drugs by delaying anticoccidial resistance (4). Live vaccination with drug-susceptible *Eimeria* spp. possibly displaces drug-resistant wild-type *Eimeria* strains in the barn environment (4). Application of live coccidiosis vaccine followed by delayed anticoccidial intervention in the feed or drinking water, a program called bioshuttle, permits the development of immunity and improves performance compared to ionophore treatment alone (5). Thus, an efficacious coccidiosis control program would incorporate judicious use of anticoccidial drugs and vaccination to prevent selection of drug-resistant phenotypes in the field.

There are seven *Eimeria* species that infect domestic turkeys (*Meleagris gallopavo* [var. domesticus]) that have been characterized (6). Of those, *E. meleagrimitis, E. adenoeides, E. gallopavonis,* and *E. dispersa* have been shown to cause clinical disease in commercial turkeys (7). Infection with multiple *Eimeria* spp. makes it challenging to predict the impact a single species has at the flock level. A live coccidiosis vaccine containing only two species, *E. meleagrimitis* and *E. adenoeides*, is the only vaccine approved for use in turkeys. There is no evident cross-protection between *Eimeria* species infecting turkeys (8). As a result, an optimal vaccine formulation to displace the drug-resistant wild-type *Eimeria* spp. would consist of those currently affecting the farm. Vaccination with drug-sensitive strains would be the most cost-effective option available to shift the population of *Eimeria* oocysts from pan-resistant to pan-sensitive in a flock. Tailoring a vaccine to contain only the species relevant to a particular complex would be ideal to avoid introducing non-relevant strains. Since *Eimeria* spp. are also prevalent in wild turkey populations (9) and the probability of exposure to anticoccidials is low, *Eimeria* spp. recovered from wild turkeys should be evaluated as potential vaccine candidates. Live vaccination can negatively affect performance and amprolium has been briefly applied to the drinking water to reduce vaccine-related effects without disrupting immune development (7). However, it appears that the timing of application post-vaccination should be selected based on oocyst cycling to not impede immunity development (10).

At present, there are no reports on the impact of live coccidiosis vaccination and/or intermittent amprolium intervention on the intestinal microbiome or gut integrity in turkeys. The complex interactions between the host and the gut microbiome affect digestion and the overall health of the host (11). Consequently, the replication of *Eimeria* spp. within the host may shift the microbiome's composition and increase intestinal permeability. These effects may be directly or indirectly related. A serum biomarker, fluorescein isothiocyanate-dextran, also known as FITC-d, has been used to assess gastrointestinal permeability in necrotic enteritis and coccidiosis models in chickens (12, 13). The relationship between live coccidiosis vaccination with and without amprolium intervention and its effect on intestinal integrity and the gut microbiome post-challenge with *E. meleagrimitis* has not been evaluated.

In a previous study, our group collected wild turkey fecal samples and generated single oocyst-derived stocks for five of the major *Eimeria* spp. relevant to commercial turkeys: *E. meleagrimitis, E. dispersa, E. meleagridis, E. gallopavonis,* and *E. adenoeides*. *Eimeria meleagrimitis,* one of the more pathogenic species that infect turkeys (14). *Eimeria meleagrimitis* was also the most prevalent species detected in commercial turkey flocks in the midwestern United States (15). Although not all turkey *Eimeria* spp. induce clinical disease, flock performance can be severely impacted and must be considered when vaccinating commercial turkey flocks, especially if additional *Eimeria* spp. have been detected in previous flocks. This current investigation aimed to assess the protective efficacy of a wild turkey-derived, anticoccidial-sensitive (monensin, zoalene, and amprolium) *E. meleagrimitis* vaccine candidate obtained from wild turkeys against homologous challenge. Furthermore, the effect of amprolium intervention, vaccination, and/or challenge on the gut microbiome and intestinal permeability was assessed.

2. Materials and methods

2.1. *Eimeria meleagrimitis*

Previously, a drug sensitive strain of *E. meleagrimitis* was recovered from wild turkey feces collected in Maine, United States in 2019. Methods used to isolate, speciate, and characterize the *E. meleagrimitis* used in the current study have been described (16). A single oocyst-derived stock was generated, identity confirmed (PCR and sequencing), and used for vaccination and challenge in the present study. The stock was stored at 4°C in a 2.5% potassium dichromate (PDC; Sigma-Aldrich Co.) solution for >3 months before use.

2.2. Preparation of vaccine and challenge stocks

Stocks used for vaccination or challenge were prepared 24 h prior to use. Freshly sporulated *E. meleagrimitis* oocysts were centrifuged at

1300×g for 10 m to remove the PDC solution. The supernatant was discarded, and the pelleted oocysts were resuspended in 0.9% sterile saline. A McMaster chamber was used to determine the concentration of the stock solution or sporulated oocysts/mL (17). For vaccination, oocysts were prepared to achieve a final concentration of ~50 sporulated *E. meleagrimitis* oocysts/0.25 mL/poult and a challenge concentration of ~95,000 sporulated *E. meleagrimitis* oocysts/1 mL/turkey.

2.3. Amprolium

Amprolium (Amprol® 9.6% Oral Solution: Huvepharma), a synthetic anticoccidial, was administered in the drinking water at a concentration of 0.024% from d10-d14 per manufacturer's guidelines. Only the VX + Amprol received amprolium treatment.

2.4. Experimental design

The experimental design is presented at the top in Figure 1. A total of 280 day-of-hatch (DOH) female turkey poults (Nicholas genetics) were obtained from a commercial hatchery. Poults were individually neck-tagged and randomly allocated into the following treatment groups: (1) NC (non-vaccinated, non-challenged control), (2) PC (non-vaccinated, challenged control), (3) VX + Amprol (*E. meleagrimitis* candidate vaccine + amprolium), and (4) VX (*E. meleagrimitis* candidate vaccine). For the vaccinated groups, 50% of the poults (referred to as directs) received 50 sporulated *E. meleagrimitis* (VX) oocysts/0.25 mL/poult *via* oral gavage immediately prior to placement. At placement, the directs were commingled with non-vaccinated (contacts) poults for the duration of the study. The NC, PC, and contacts of the vaccinated group did not receive any treatment before placement. At d23, turkeys in all groups, excluding the NC group, were challenged with ~95,000 sporulated *E. meleagrimitis* oocysts/1 mL/turkey by oral gavage. Individual body weights (BW) were recorded at DOH, d8, d23, and d29 (termination) to determine average body weight gain (BWG).

Each treatment group was housed in a single 7x7ft floor pen with fresh pine shavings ($n = 70$ poults/pen). Poults were housed in a section of the pen from DOH-d10 to simulate commercial brooding density (0.475 sq. ft./poult). A standard cardboard barrier was used to segregate the poults from the entirety of the pen during the first 10 days. At d10, the barrier was removed, and poults were reared at a density of 0.817 sq. ft./poult from d10-d29 (termination). Litter moisture was maintained in each pen using a generic garden pump sprayer every morning from d2-d7. No additional modifications were made to the litter for the duration of the study. All turkey poults were provided feed and water *ad libitum* throughout all experiments. The lighting program followed management guidelines for commercial turkey hens (18). All animal handling procedures complied with the Institutional Animal Care and Use Committee (IACUC protocol #21117) of the University of Arkansas.

2.5. Fecal and litter OPG

Fecal and litter samples were collected from d5-d28 post-vaccination to assess oocyst shedding. Individual fecal samples were collected from a subset of the directs and contacts in the vaccinated groups ($n = 10$ individual samples from d5-d6; $n = 1$ pooled sample at d7; $n = 5$ individual samples at d8; and $n = 3$ individual samples from d9-d28). The difference in the number of individual fecal samples collected throughout the duration of the experiment was due to the sheer amount of time it took to collect the samples. Initially, the goal was to collect $n = 10$ individual samples per group and vaccination level. However, $n = 3$ individual samples were a more feasible number to collect. Pooled fecal samples were collected for the NC and PC group ($n = 3$) from d5–d29. For each treatment group, pooled litter samples were collected from random locations in the pen ($n = 3$). To determine fecal and litter oocysts per gram of feces (OPG), all fecal and litter samples were collected in 5 mL microcentrifuge tubes or 50 mL polypropylene centrifuge tubes, respectively, and then weighed and suspended in 2.5% PDC at a final concentration of 1:2 (w/v). Fecal or litter samples were processed to determine OPG counts using a

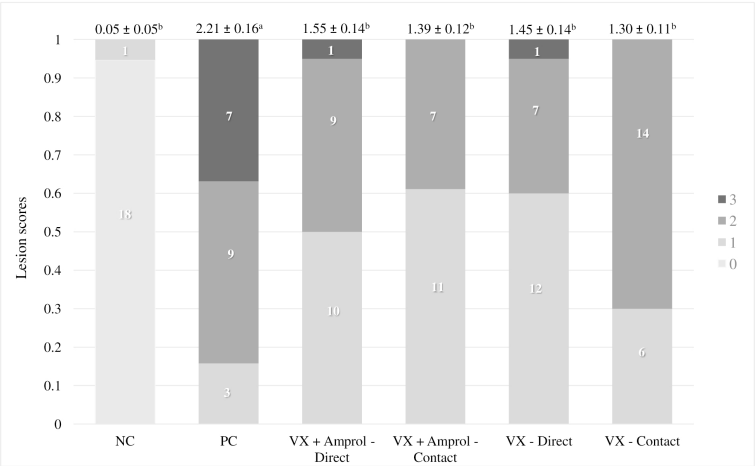

FIGURE 1
Graphical representation of the experimental timeline and the fluorescein isothiocyanate dextran (FITC-d) administration and general overview of FITC-d assay methodology (Created with BioRender.com).

McMaster counting chamber using a standard formula that includes the initial weight of the sample, volume of saturated NaCl solution, and any additional dilutions that were required (17, 19, 20).

2.6. Lesion scores

At d29, or 6d post-homologous challenge, the duodenum to the lower intestine was evaluated post-mortem for each group and vaccination level ($n = 18–20$) to evaluate macroscopic lesions using methods similar to El-Sherry et al. (21) and Gadde et al. (22). Lesions were scored from 0 to 4: "0" represents a healthy organ, whereas a score of "4" represents severe coccidiosis.

2.7. Serum fluorescein isothiocyanate dextran

FITC-d (ng/mL) was used as a biomarker to evaluate intestinal permeability as described by Baxter et al. (23) and the methodology has been presented at the bottom in Figure 1. At the end of the trial, turkey poults ($n = 18–20$ for NC and PC; $n = 13–15$ for vaccinated groups) were orally gavaged with 8.32 mg of FITC-d per kg of body weight (FITC-d, MW 3–5 KDa; Sigma-Aldrich Co). One hour after FITC-d administration, turkeys were euthanized by CO_2 inhalation. Blood samples were collected from the femoral vein and centrifuged (1,000 × g for 10 m at 4°C) to separate the serum.

2.8. Microbiome

Ileal and cecal contents were collected from 29-day-old turkey poult hens ($n = 6$/treatment). Samples were stored at-20°C in an RNA/DNA (Zymo Research, Irvine, CA, United States) shield until DNA extraction was performed. Total genomic DNA of ileal and cecal content samples was extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germantown, MD, United States) according to the manufacturer's protocol. The concentration of DNA was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Madison, WI, United States) and diluted to 10 ng/μL with DNase/RNase-free water. The V4 region of the 16S rRNA gene was amplified using primer sequences (forward: 5′-GTGCC AGCMGCCGCGGTAA-3′ and reverse: 5′-GGACTACHVGGG TWTCTA AT-3′) attached with gene-specific Illumina adapters for each sample (24). PCR amplification was performed using a T100 thermal cycler (Bio-Rad, Hercules, CA, United States). All 16S PCRs conditions consisted of a 30s initial denaturation at 95°C: 30 cycles at 95°C for 10s, annealing at 55°C for 30s, extension at 72°C for 60s, and a final extension at 72°C for 10 m. The PCR products were determined on a 1% agarose gel and normalized using a SequalPrepTM Normalization Plate Kit (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's recommendations. All purified PCR amplicons were pooled to generate a sequencing library (25). After concentration, the quality of the library was confirmed by KAPA Illumina Library Quantification Kits (Roche, Indianapolis, IN, United States) *via* a quantitative PCR (qPCR, Eppendorf, Westbury, NY, United States) assay and an Agilent 2,100 Bioanalyzer System (Agilent, Santa Clara, CA, United States).

The library was sequenced on a MiSeq sequencer (MiSeq Reagent Kit v2, 500 cycles; Illumina, San Diego, CA, United States). To detect any contamination, a mock community (ZymoBIOMICS™ Microbial Community Standard; Zymo, Irvine, CA, United States), a negative control for DNA, and a negative control for PCR amplification were included in sequencing. Sequencing files obtained from the Illumina sequencer were pre-processed, quality filtered ($Q > 30$), and analyzed using the QIIME2 (2021.4 release) software (26). Deblur algorithm was used for sequence trimming, denoising, chimera removal, and features binning at the amplicon sequence variants (ASV) level (27). Naïve Bayes classifier was employed for the assignment of all sequences into bacterial taxonomy using the Greengenes (v13_8 clustered at 99% identity) reference database. The raw data are available in the NCBI SRA database with the BioProject ID PRJNA.

2.9. Statistical analysis

All data were subjected to analysis of variance (ANOVA) as a completely randomized design using JMP Pro 14 software. Significant differences among the means were determined by Tukey's multiple comparison test for BW, BWG, and serum FITC-d, where statistically significant differences between the means were set at $p < 0.05$. The LS data were determined using Proc Mixed Analysis by SAS 9.4 at $p < 0.0001$. Oocysts per gram of feces (OPG) and litter OPG data were expressed as mean using JMP Pro 14 software.

Alpha diversity, including the Shannon Index and the number of Observed ASVs, was compared using a two-tailed Wilcoxon signed-rank test between two groups ($p < 0.10$). Beta diversity based on Bray-Curtis and Jaccard distances was tested using an analysis of similarity (ANOSIM). The outputs of diversity were visualized using the "ggplot2" package in *R* (v4.1.2). The linear discriminant analysis (LDA) effect size (LefSe), an analytical tool for discovering and interpreting biomarkers of high-dimensional data, was used to identify the signature bacteria associated with the growth stages and intestinal segments. LDA score>2 was used as a criterion for judging the significant effect size (28). The signature bacteria were visualized in a heat map using the "pheatmap" function in R.

3. Results

3.1. Performance

For average BW, there were no significant differences between all groups at DOH or d8 (Table 1). However, there were significant ($p < 0.05$) differences in BW at d23 only between PC and VX + Amprol – contact, with the VX group having the lower BW at d23. The BW at d29 showed that VX – contact had a markedly higher value than PC with significant ($p < 0.05$) differences; in contrast, the other groups evaluated had no significant differences.

There were no significant differences in average BWG from DOH-d8 across all groups. The DOH-d23 BWG (pre-challenge BWG) was significantly ($p < 0.05$) higher in PC compared to contacts in VX + Amprol group. However, the post-challenge (d23-d29) BWG of the PC group was significantly ($p < 0.05$) reduced compared to NC and vaccinated groups. Although d23-d29 BWG was considerably ($p < 0.05$) increased for vaccinated groups compared to PC and NC,

TABLE 1 Effect of *Eimeria meleagrimitis* vaccination, with and without amprolium intervention, and/or *E. meleagrimitis* challenge on average body weight (BW), body weight gain (BWG), lesion scores (LS), and serum FITC-d in turkey poults.

Treatment	NC	PC	VX+Amprol direct	VX+Amprol contact	VX direct	VX contact
BW (g)[1]						
DOH	57.96 ± 0.61	58.31 ± 0.57	57.63 ± 0.77	59.23 ± 0.79	57.00 ± 0.62	57.29 ± 0.68
d8	154.86 ± 2.41	158.28 ± 2.12	163.11 ± 3.66	157.50 ± 3.37	159.00 ± 2.54	155.31 ± 4.84
d23	535.08 ± 7.71^{ab}	559.81 ± 8.83^{a}	524.70 ± 11.62^{ab}	515.33 ± 12.23^{b}	533.43 ± 11.58^{ab}	536.40 ± 9.70^{ab}
d29	730.60 ± 11.92^{ab}	706.60 ± 11.90^{b}	750.83 ± 20.55^{ab}	736.47 ± 18.44^{ab}	764.73 ± 16.63^{ab}	783.73 ± 13.67^{a}
BWG (g)[1]						
DOH-d8	96.84 ± 2.27	99.97 ± 2.02	105.49 ± 3.47	98.68 ± 3.19	102.00 ± 2.38	98.03 ± 2.46
DOH-d23	477.00 ± 7.56^{ab}	501.17 ± 8.75^{a}	467.07 ± 11.44^{ab}	456.47 ± 12.05^{b}	476.33 ± 11.58^{ab}	478.80 ± 9.53^{ab}
DOH-d29	672.52 ± 11.74^{ab}	648.00 ± 11.78^{b}	693.20 ± 16.11^{ab}	677.60 ± 14.79^{ab}	707.63 ± 12.88^{ab}	726.13 ± 10.20^{a}
d23–d29	195.52 ± 5.40^{b}	145.05 ± 5.10^{c}	226.13 ± 9.85^{a}	221.13 ± 7.01^{a}	231.30 ± 566^{a}	247.33 ± 5.24^{a}
LS[2]	0.05 ± 0.05^{c}	2.21 ± 0.16^{a}	1.39 ± 0.12^{b}	1.55 ± 0.14^{b}	1.30 ± 0.11^{b}	1.45 ± 0.14^{b}
FITC-d [ng/mL][1]	141.37 ± 29.78^{abc}	269.74 ± 25.25^{a}	206.61 ± 11.69^{abc}	250.11 ± 32.87^{ab}	74.72 ± 29.89^{bc}	65.38 ± 58.77^{c}

Data are expressed as the mean ± standard error.
[a]Different superscripts indicate significant differences between the treatments at $p \leq 0.05$.
[b]Different superscripts indicate significant differences between the treatments at $p \leq 0.05$.
[c]Different superscripts indicate significant differences between the treatments at $p \leq 0.05$.
[1]Statistical evaluation using ANOVA followed by *post hoc* Tukey's range test.
[2]Statistical differences between lesion scores detected using SAS proc mixed analysis.
BW: body weight; BWG: body weight gain; LS: lesion scores; FITC-d: fluorescein isothiocyanate dextran.

DOH-d29 BWG was only significantly ($p < 0.05$) different between the PC and VX – contact groups.

3.2. Lesion scores and serum FITC-d

At d23, all turkeys, except for the NC, were orally challenged with *E. meleagrimitis* (95,000 sporulated oocysts/mL). After 6d post-challenge, intestinal lesion scores were evaluated using methods similar to El-Sherry et al. (21) and Gadde et al. (22). No scores of 4 were observed in the current study. Lesion scores were significantly ($p < 0.0001$) reduced in direct and contact of VX and VX + Amprol than in PC (Table 1; Figure 2). The average of lesion scores in the vaccinated level group did not have significant differences. The distribution of lesion scores at d29 has been presented in Figure 2. The vaccinated and NC groups had less severe lesion scores than the PC group. Serum FITC-d levels in the PC group were significantly ($p \leq 0.05$) higher than VX – direct and contact (Table 1). Additionally, serum FITC-d levels for the VX + Amprol – contact group were significantly ($p < 0.05$) higher compared to the VX – contact group.

3.3. Fecal and litter OPG

There was a sharp increase in fecal OPG at d6 for turkeys that received 50 *E. meleagrimitis* oocysts at DOH compared to those that did not directly receive the vaccination (Figure 3). From d10-14, the VX + Amprol group received amprolium in the drinking water which reduced fecal OPG for both the direct and contact poults for this period (Figure 3). Alternatively, the naïve contact poults that did not receive any drug intervention to attenuate oocyst cycling had a sharp increase in fecal OPG from d11-d15 compared to all other groups. The importance of proper coccidiosis vaccination methods was represented by the difference in fecal OPG when comparing the trend in the contact of VX group demonstrated that delayed exposure to the vaccine leads to much higher fecal OPG compared to those directly vaccinated at hatch, and those that were treated with amprolium of the directs and contacts. The greatest peak in fecal OPG for the PC was at d24, which was ~24 h post-challenge with *E. meleagrimitis* (95,000 sporulated oocysts/mL). Oocysts were detected in the feces and litter of the NC group at d24 and d25. This was unexpected and suggested that there was low-level cross-contamination likely prior to the challenge period.

For litter OPG (Figure 4), there were differences in peaks between vaccinated groups associated with the administration of amprolium from d10-d14. For instance, the group that did not get drug intervention to attenuate oocyst cycling (VX group) had multiple spikes in litter OPG after d14, whereas the VX + Amprol group had more uniform litter OPGs with only a single sharp increase between d6-d9 (Figure 4).

3.4. Microbiome

Figure 5 shows the phylum and genus composition in the ileal and cecal content by group. At the phylum level, Firmicutes (89.3–95.9%) was the most dominant taxa, followed by Proteobacteria (2.7–10.0%) for both ileal and cecal contents for all groups assessed (Figure 5). Actinobacteria (0.4–2.2%) was enriched in ileal contents, and Tenericutes (0.8–3.1%) was enriched in cecal contents for all groups.

Lactobacillus was the predominant genus in the ileum for all groups except for the VX – Amprol contact group which had a higher abundance of *Streptococcus* compared to all treatment groups (Figure 5). The group with the highest abundance of *Lactobacillus* in the ileum was the PC group (54.2%). For the VX groups, the proportion of *Lactobacillus* was higher in the VX group (37.1% for

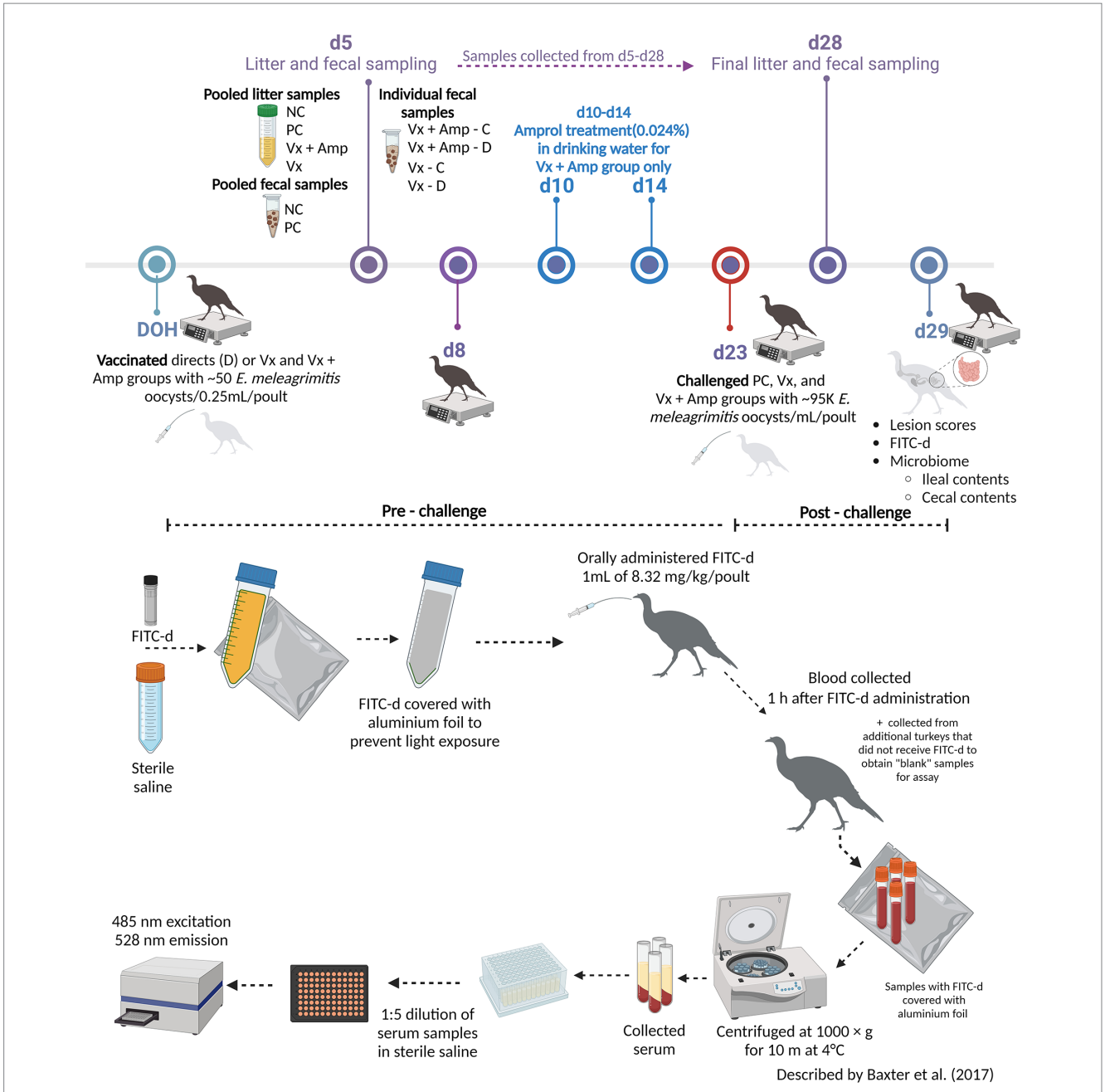

FIGURE 2
Cumulative lesion scores 6days post-challenge. At day 23, all poults, except for the NC, were orally challenged with *E. meleagrimitis* (95,000 sporulated oocysts/mL). Six days post-challenge (day 29), a subset of the poults from each group and vaccination level (n=18-20/group) were lesion scored. A lesion score of "0" represents a healthy intestinal tract whereas a score of "4" represents severe coccidiosis. No lesion scores of 4 were observed. Numbers within columns indicate the number of poults evaluated for each lesion score (0–3). Mean lesion score±standard error presented above columns. Means further separated using Proc Mixed Analysis (SAS 9.4). $^{a-c}$Different superscripts between treatment groups indicate means differ significantly (p< 0.05) (Created with Biorender.com).

directs and 26.6% for contacts) than in the VX + Amprol group (17.0% for directs and 9.5% for contacts) in ileal contents. Additionally, a higher abundance of *Clostridium* was observed in the VX group (15.4% for direct and 18.8% for contact) compared to the VX + Amprol group (10% for direct and 10% for contact). The VX + Amprol group was dominated by *Turicibacter* in the ileum compared to all other groups (24.6% for directs and contacts).

The dominant genera in the cecal contents at the genus level were *Faecalibacterium*, *X. Ruminococcus*, and *Lactobacillus* (Figure 5). The highest abundance of *Faecalibacterium* was in the VX + Amprol group (14.7% for directs and 15.8% for contacts) followed by the NC (10.9%) and VX contacts (11.0%) in cecal contents. However, X. *Ruminococcus* abundance was elevated for the VX contact group (11.5%) and VX + Amprol – contact group (10.2%) compared to the VX direct group (8.3%) and VX + Amprol – direct group (7.8%). *Clostridium* abundance in the cecal contents was highest in the VX – contact group (4.1%) compared to all treatment groups.

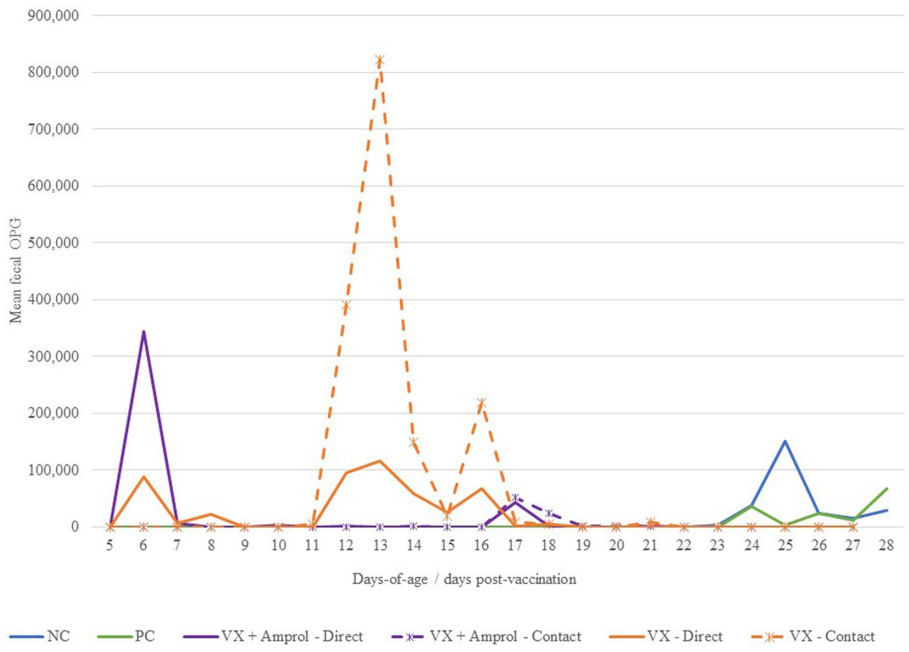

FIGURE 3
Effect of *E. meleagrimitis* vaccination and/or challenge with and without amprolium intervention on mean fecal oocyst per gram (OPG). For fecal OPG, individual fecal samples were collected from the direct and contact poults (n=3–10 individual fecal samples/group/vaccination level/day) and pooled fecal samples were collected for NC and PC. At DOH, 50% of the poults in the vaccinated groups orally received 50 sporulated *E. meleagrimitis* (VX) oocysts immediately prior to placement. The NC, PC, and contacts did not receive any treatment before placement. VX+Amprol group received amprolium in the drinking water from d10-d14 at 0.024%. At d23, turkeys were orally challenged with *E. meleagrimitis* (95,000 oocysts/mL) except for negative control (NC).

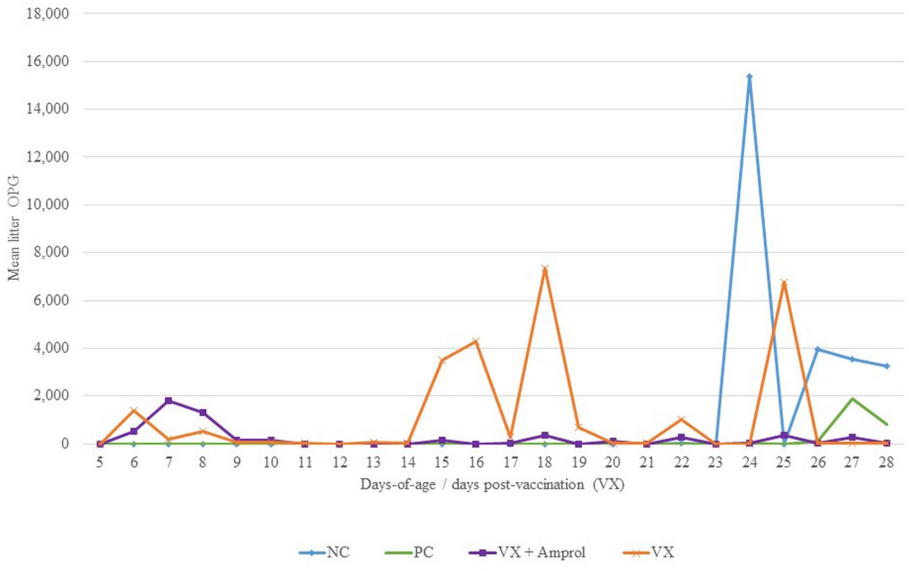

FIGURE 4
Effect of *E. meleagrimitis* vaccination and/or challenge with and without amprolium intervention on mean litter OPG. Pooled litter samples were collected for each treatment group (n=3). At DOH, 50% of the poults in the vaccinated groups orally received 50 sporulated *E. meleagrimitis* (VX) oocysts immediately prior to placement. The NC, PC, and contacts did not receive any treatment before placement. VX+Amprol group received amprolium in the drinking water from d10-d14 at 0.024%. At d23, turkeys were orally challenged with *E. meleagrimitis* (95,000 oocysts/mL) except for negative control (NC).

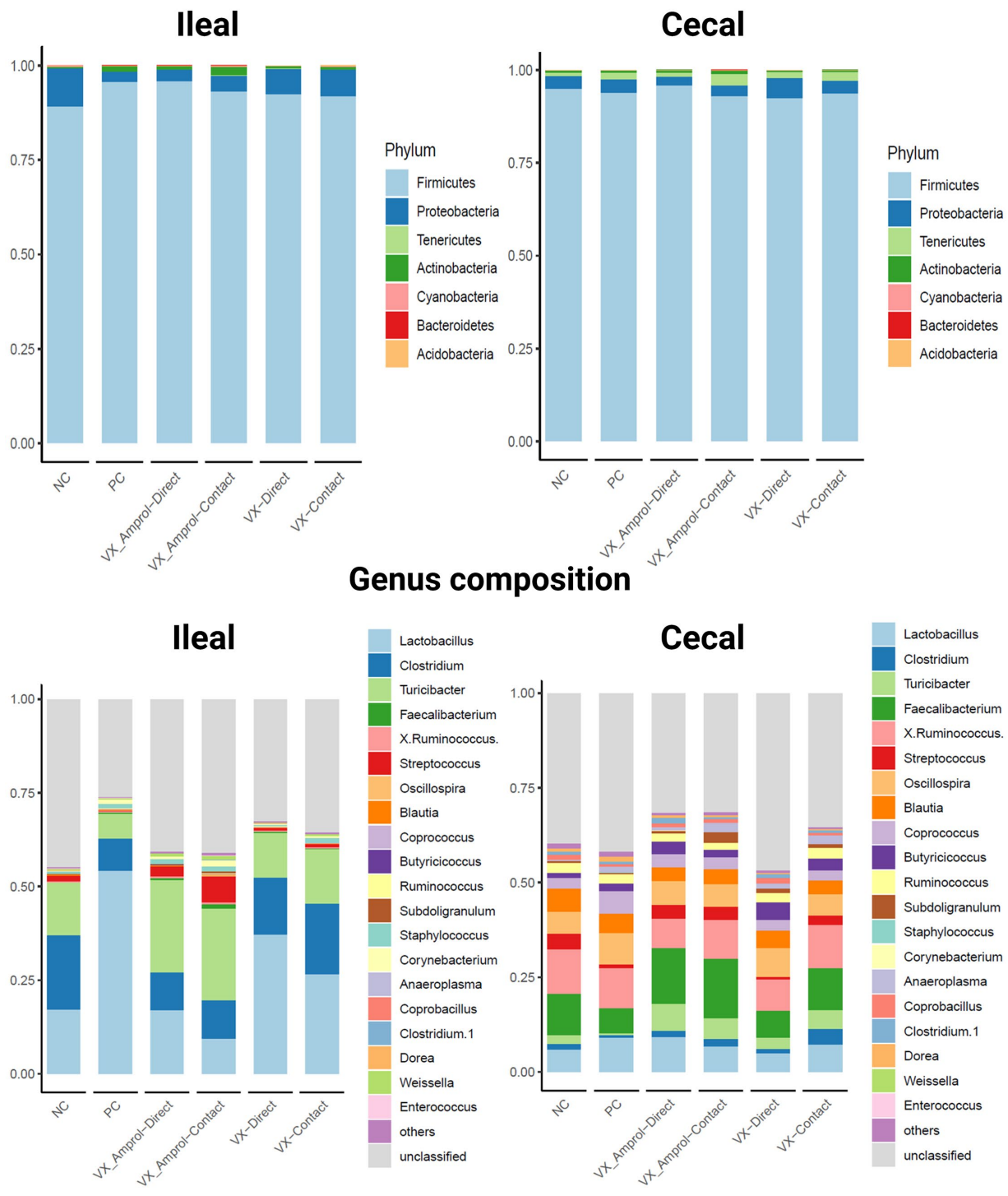

FIGURE 5
Effect post-challenge of *Eimeria meleagrimitis* vaccination with and without amprolium intervention on phylum and genus composition in the ileum and cecal contents. All groups, except the NC group, were challenged with 95,000 sporulated *E. meleagrimitis* sporulated oocysts at d23. At d29, or 6d post-challenge, ileal and cecal contents were collected from 29-day-old turkey poult hens, *n*=6/treatment/vaccination level (Created with BioRender.com).

The results showed that ileal and cecal contents in the PC group did not exhibit a different bacterial diversity and structure, including alpha and beta diversity, when compared to NC (Figures 6, 7). Alpha diversity was measured using the Shannon Index and the number of observed ASVs. There were no significant ($p > 0.10$) differences for alpha diversity in ileal or cecal contents

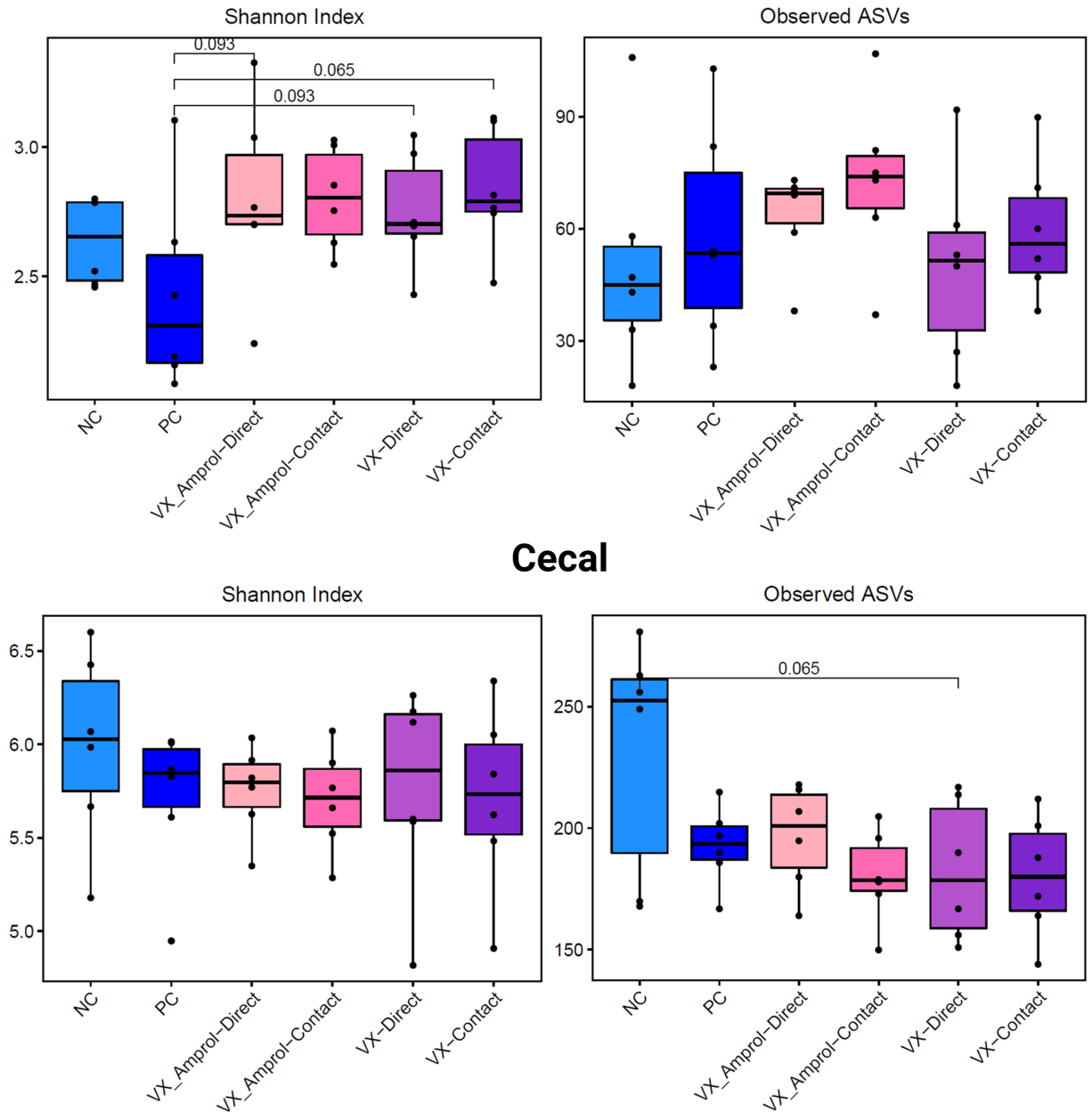

FIGURE 6
Alpha diversity of ileal and cecal contents collected at d29 (6d post-challenge). Alpha diversity was measured using Shannon Index (left) and number of Observed ASVs (right). Statistical comparison was made using the two-tailed Wilcoxon signed-rank test between two groups (p<0.10) (Created with BioRender.com).

across vaccinated groups (Figure 6). However, the PC group had significantly ($p < 0.10$) lower alpha diversity (Shannon Index) in the ileal contents compared to all vaccinated groups, except for the VX + Amprol contact group. In the ceca, the NC group had significantly ($p = 0.065$) higher observed ASVs compared only to the VX – direct group. No distinct clusters were observed between the cecum and ileum-based Bray-Curtis and Jaccard distances across all groups (Figure 7).

Linear discriminant analysis Effect Size (LEfSe) was employed to identify bacterial biomarkers for each group. In the PC group, *Lactobacillus salivarius* (ASV2) was enriched in ileal and cecal content (Figures 8, 9). *Faecalibacterium prausnitzii* (ASV15 in ileal and ASV7 in cecal) was enriched in the cecal and ileal community of the VX + Amprol – contact group, while *Turicibacter* (ASV4) was overrepresented in VX + Amprol – direct group in ileal and cecal contents (Figures 8, 9). *Pepetostreptococcaceae* (ASV65) was only enriched in VX – contact group ileal contents (Figure 8), while *Ruminococcaceae* (ASV25) and *Lachnospiraceae_Ruminococcus* (ASV23) were greater in VX – direct and VX – contact group cecal contents, respectively (Figure 9).

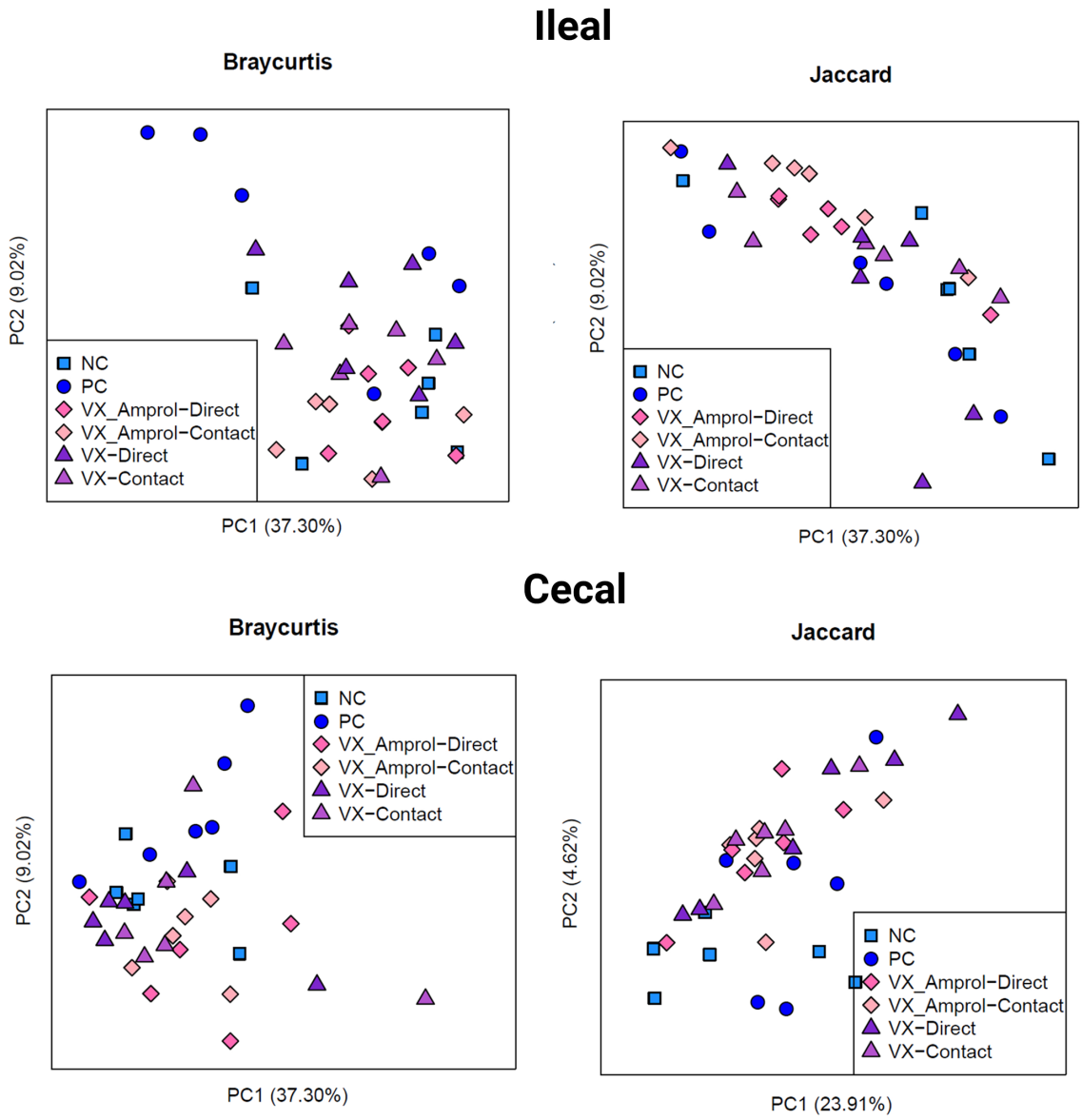

FIGURE 7
Beta diversity of ileal and cecal contents collected at d29 (6d post-challenge). Beta diversity was evaluated using Bray Curtis (left) and Jaccard (right) distances. The outputs of diversity were visualized using the "ggplot2" package in R (v4.1.2). Analysis was conducted using an analysis of similarity (ANOSIM) (Created with BioRender.com).

4. Discussion

The importance of proper uptake of a live *E. meleagrimitis* vaccine candidate at hatch by turkey poults was demonstrated in the present study by comparing the difference in fecal OPG between contacts and directs of the VX group and between contacts of the VX and VX + Amprol group. Directs in the VX groups had attenuated shedding compared to the contact counterparts. Only numerical differences in BW or BWG between the vaccinated treatment groups were observed suggesting that this strain of *E. meleagrimitis* is relatively non-pathogenic, especially considering the "contact" poults were not directly vaccinated and the number of oocysts that were ingested was not controlled. Since susceptibility to *Eimeria* spp. infection increases with age, and the severity is associated with the number of oocysts ingested, a negative impact on performance was anticipated for contact poults, especially those that did not receive intermittent amprolium treatment.

It is important to note that there was apparent cross-contamination that occurred in the NC group which was reflected by litter and fecal OPG late in the study. Sporulated oocysts are incredibly resilient and although steps were taken to prevent cross contamination in the facility, dust and dander may have been a factor (29). Husbandry for both control groups was conducted before the vaccinated groups with showers being required between each check. Coccidiostat inclusion will be considered for the NC group in future studies.

Interestingly, the contact and directs in the VX group had improved gut integrity 6d post-challenge compared to all other treatments and were also numerically heavier than those that received

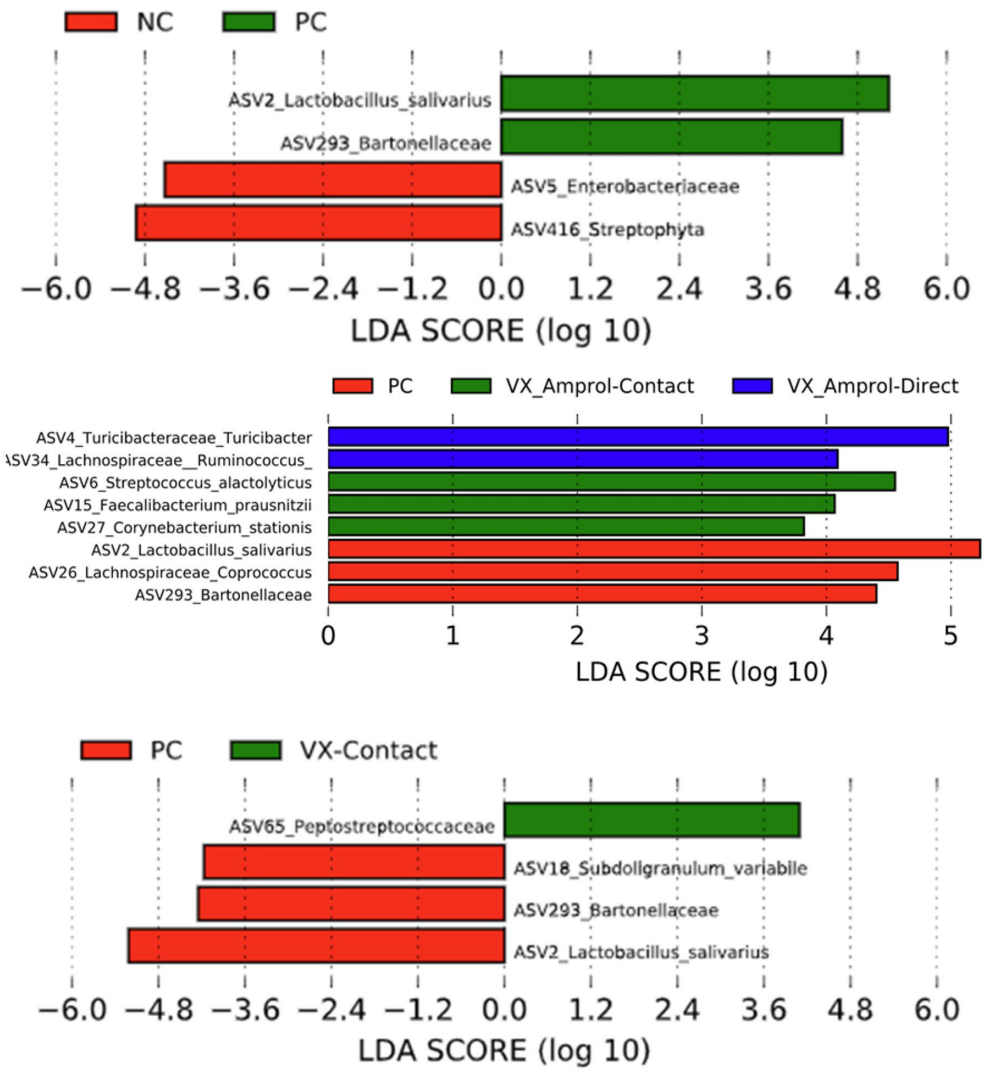

FIGURE 8
Linear discriminant analysis effect size (LEfSe) for negative control (NC) and positive control (PC); PC, VX+Amprol-Contact, and VX+Amprol-Direct; and PC and VX-Contact in ileal contents at the genus level. LEfSe was used to identify the signature bacteria associated with the ileal contents. LDA score>2 was used as a criterion for judging the significant effect size (Created with BioRender.com).

amprolium in the drinking water from d0-14. Even though there were no differences in lesion scores between VX groups, the serum FITC-d levels of the VX + Amprol group were similar to the PC groups, which may suggest the timing of amprolium administration from d10-d14 was potentially too early. In the field, amprolium is generally administered in the diet ~d16 to mitigate performance losses associated with live coccidiosis vaccination at hatch. However, this practice is not accepted by all countries including many in Europe (30). Nevertheless, we hypothesize that arresting *E. meleagrimitis* development shortly after initiation of the second cycle by administering amprolium in the drinking water would have beneficial effects overall. A more comprehensive study is currently underway to validate these results.

This present study provides an initial evaluation of the effects of *E. meleagrimitis* and amprolium on the gut microbiome, specifically the ileal and cecal microbiome of turkey poults. *Eimeria* spp. infection impedes digestion and absorption of nutrients by impairing the intestinal barrier function, causing bacterial translocation, and disrupting gut homeostasis (31). The gut microbiome influences host performance and resistance to enteric pathogens (32), and the effects of the gut microbiome on the overall performance and health of chickens have been previously described (33–35). However, the microbiome of chickens and turkeys is only 16–19% similar at the genus level, indicating distinct variations between the two avian species (35). Several investigators have assessed the impact of coccidiosis on the gut microbiome of chickens (36, 37). In contrast, research evaluating the effect of live coccidiosis vaccination and anticoccidial drugs on the turkey gut microbiome is lacking.

In the current study, there was increased heterogeneity in the microbiome composition of cecal contents compared to the ileal

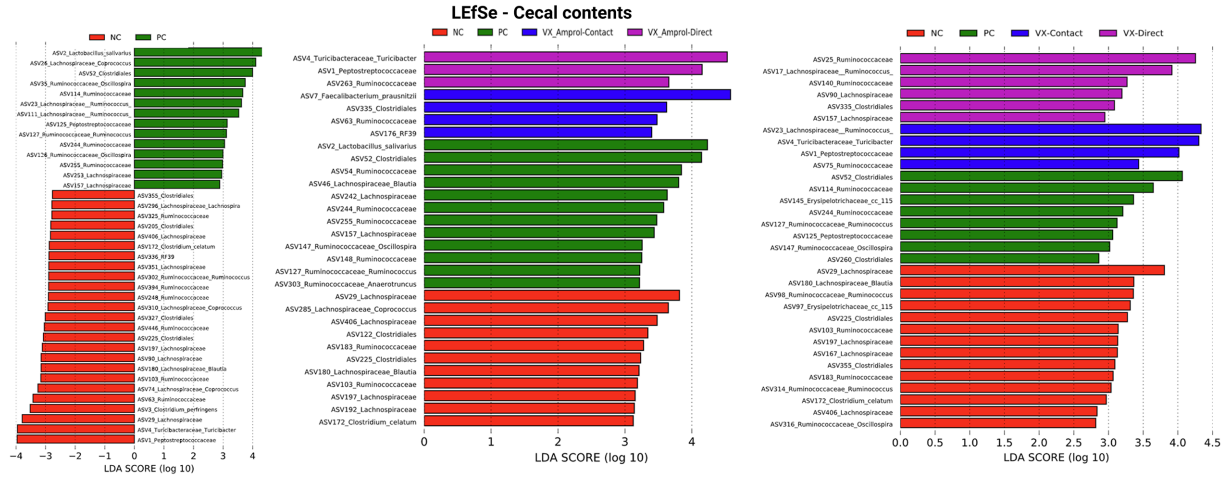

FIGURE 9
Linear discriminant analysis effect size (LEfSe) on effect post-challenge of *Eimeria meleagrimitis* vaccination with and without Amprol intervention in turkey cecal bacterial populations at the genus level for negative control (NC) and positive control (PC); PC, VX+Amprol-Contact, and VX+Amprol-Direct; and PC, VX-Contact and VX-Direct. LEfSe was used to identify the signature bacteria associated with the growth stages and intestinal segments. LDA score>2 was used as a criterion for judging the significant effect size (Created with BioRender.com).

contents. These results aligned with previous findings described by D'Andreano et al. (38), who assessed the gut microbiome of different gut sections of hemorrhagic enteritis-infected turkeys. Furthermore, the lack of significant differences in alpha and beta diversity across treatment groups is similar to a report published by Macdonald et al. (39), who demonstrated that live coccidiosis vaccination did not affect alpha diversity in the ceca of broiler chickens. In the present study, there was an apparent shift in the microbiome composition at both the phylum and genus levels. For example, the *E. meleagrimitis* challenge at d23 increased the abundance of *Lactobacillus salivarius* in the ileum of the PC group compared to the NC and the vaccinated groups, which was unexpected. Interestingly, Latorre et al. (12) observed the same phenomenon in necrotic enteritis-challenged chickens. Bacteria in the small intestine compete with the host to acquire and utilize amino acids, whereas the bacteria in the ceca capitalize on those amino acids or nutrients that bypass the small intestine (40). Perhaps the abundance of *Lactobacillus* is associated with the over-proliferation of lactobacilli due to the malabsorption of nutrients by the host associated with the *E. meleagrimitis* challenge. In contrast, amprolium administration was associated with a reduction in *Lactobacillus* in the ileum but an increase in *Turicibacter*, a known butyric acid producer associated with a normal/healthy gut in chickens (12). Although synthetic anticoccidials do not have antimicrobial effects, these drugs may indirectly affect the host's gut microbiome since they affect parasite metabolism after intracellular invasion. The complexity of the host-microbiota-protozoa interaction and its effects on host immune development and performance requires further investigation.

Based on the results from the present study, vaccination with a non-attenuated strain of *E. meleagrimitis* obtained from wild turkey feces induced a mild infection providing protective immunity with and without amprolium intervention, which affected gut integrity and shifted the ileal and cecal luminal microbiome in turkey poults. The impact of a bioshuttle program on the intestinal microbiome requires further investigation.

5. Conclusion

Vaccination with *E. meleagrimitis* obtained from wild turkey feces induced a mild infection providing protective immunity based on lesion scores and performance. Results indicated that amprolium intervention could be used to attenuate *E. meleagrimitis* oocyst shedding but additional studies are required to determine the effect of a bioshuttle on gut permeability and the microbiome in turkey poults.

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 Animal care and handling procedures complied with the University of Arkansas Institutional Animal Care and Use Committee (Animal Use Protocol #21026).

Author contributions

CT-P, DG, GT-I, LB, and JB conceptualized the study. JL, JC, RS-C, AF, and MC handled the methodology. MC and JZ were in charge of the software. LB, JB, BH, and GT-I validated the study. CT-P and DG

performed the formal analysis. CT-P, DG, and GT-I prepared and wrote the original draft. XH-V, JB, DG, and GT-I contributed to the writing, reviewing, and editing of the final manuscript. DG, BH, and GT-I oversaw the project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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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. Shivaramaiah C, Barta JR, Hernandez-Velasco X, Téllez G, Hargis BM. Coccidiosis: recent advancements in the immunobiology of Eimeria species, preventive measures, and the importance of vaccination as a control tool against these apicomplexan parasites. *Vet Med Res Rep*. (2014) 28:23–34. doi: 10.2147/VMRR.S57839

2. Chapman HD. Milestones in avian coccidiosis research: a review. *Poult Sci*. (2014) 93:501–11. doi: 10.3382/ps.2013-03634

3. Chapman HD. Biochemical, genetic and applied aspects of drug resistance in Eimeria parasites of the fowl. *Avian Pathol*. (1997) 26:221–4. doi: 10.1080/03079459708419208

4. Chapman HD, Jeffers TK. Vaccination of chickens against coccidiosis ameliorates drug resistance in commercial poultry production. *Int J Parasitol: Drug*. (2014) 4:214–7. doi: 10.1016/j.ijpddr.2014.10.002

5. Montoya A, Quiroz M. Field experience in the USA of a concurrent use of coccidiosis vaccine in combination with ionophores in the feed of broiler chickens (2013). Available at: https://www.novusint.com/Portals/0/Documents/3628%20Field%20Experience.pdf (Accessed October 11, 2022).

6. Chapman HD. Coccidiosis in the Turkey. *Avian Pathol*. (2008) 37:205–3. doi: 10.1080/03079450802050689

7. Lund EE, Farr MM. Coccidiosis of the Turkey In: HE Biester and LH Schwarte, editors. *Diseases of poultry*. Ames Iowa: Iowa State University Press (1965). 1088–93.

8. Imai RK. (2018). Diversity and cross-immunity of Eimeria species infecting turkeys in commercial flocks in Canada. [master's thesis] Guelp: University of Guelph.

9. Ruff MD, Schorr L, Davidson WR, Nettles VF. Prevalence and identity of coccidia in pen-raised wild turkeys. *J Wildl*. (1988) 24:711–4. doi: 10.7589/0090-3558-24.4.711

10. Pohl SK. (2012). Effects of the anticoccidial drug amprolium on broiler breeder performance and enteric health following coccidiosis vaccination. [master's thesis] Texas: Texas A&M University.

11. Salzman NH. Microbiota–immune system interaction: an uneasy alliance. *Curr Opin Microbiol*. (2011) 14:99–105. doi: 10.1016/j.mib.2010.09.018

12. Latorre JD, Adhikari B, Park SH, Teague KD, Graham LE, Mahaffey BD, et al. Evaluation of the epithelial barrier function and ileal microbiome in an established necrotic enteritis challenge model in broiler chickens. *Front Vet Sci*. (2018) 5:9. doi: 10.3389/fvets.2018.00199

13. Bortoluzzi C, Lumpkins B, Mathis GF, França M, King WD, Graugnard DE, et al. Zinc source modulates intestinal inflammation and intestinal integrity of broiler chickens challenged with coccidia and *Clostridium perfringens*. *Poult Sci*. (2019) 98:2211–9. doi: 10.3382/ps/pey587

14. Imai RK, Barta JR. Distribution and abundance of Eimeria species in commercial Turkey flocks across Canada. *Can Vet J*. (2019) 60:153–9.

15. Duff AF, Briggs WN, Bielke JC, McGovern KE, Trombetta M, Abdullah H, et al. PCR identification and prevalence of Eimeria species in commercial Turkey flocks of the Midwestern United States. *Poult Sci*. (2022) 101:101995. doi: 10.1016/j.psj.2022.101995

16. Trujillo Peralta MC. (2022) Isolation of drug-sensitive Eimeria species from wild Turkey feces and development of a model bioshuttle program for Eimeria meleagrimitis for domestic turkeys. [master's thesis] Fayetteville, (AR): University of Arkansas.

17. Conway DP, McKenzie ME. *Poultry coccidiosis: diagnostic and testing procedures*. Ames, Iowa: Blackwell Publishing (2007).

18. Aviagen Turkeys Inc. Management Guidelines, Raising Commercial Turkeys (2021). Available at: https://www.aviagenturkeys.us/uploads/2021/08/20/Aviagen%20Commercial%20Guide.pdf (accessed September 15, 2022).

19. Hodgson JN. Coccidiosis: oocyst counting technique for coccidiostat evaluation. *Exp Parasitol*. (1970) 28:99–2. doi: 10.1016/0014-4894(70)90073-1

20. Long PL, Rowell JG. Sampling broiler house litter for coccidial oocysts. *Br Poult Sci*. (1975) 16:583–2. doi: 10.1080/00071667508416233

21. El-Sherry S, Rathinam T, Hafeez MA. Biological re-description of a genetically typed, single oocyst line of the Turkey coccidium, Eimeria meleagrimitis Tyzzer 1929. *Parasitol Res*. (2014) 113:1135–46. doi: 10.1007/s00436-014-3751-x

22. Gadde UD, Rathinam T, Finklin MN, Chapman HD. Pathology caused by three species of Eimeria that infect the Turkey with a description of a scoring system for intestinal lesions. *Avian Pathol*. (2020) 49:80–6. doi: 10.1080/03079457.2019.1669767

23. Baxter MFA, Merino-Guzman R, Latorre JD, Mahaffey BD, Yang Y, Teague KD, et al. Optimizing fluorescein isothiocyanate dextran measurement as a biomarker in a 24-h feed restriction model to induce gut permeability in broiler chickens. *Front Vet Sci*. (2017) 4:56. doi: 10.3389/fvets.2017.00056

24. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA*. (2011) 108:4516–22. doi: 10.1073/pnas.1000080107

25. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol*. (2013) 79:5112–20. doi: 10.1128/AEM.01043-13

26. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. (2019) 37:852–7. doi: 10.1038/s41587-019-0209-9

27. Amir A, McDonald D, Navas-Molina JA, Kopylova E, Morton JT, Zech XZ, et al. Deblur rapidly resolves single-nucleotide community sequence patterns. *mSystems*. (2017) 2:e00191–16. doi: 10.1128/mSystems.00191-16

28. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol*. (2011) 12:R60. doi: 10.1186/gb-2011-12-6-r60

29. Price KR. Use of live vaccines for coccidiosis control in replacement layer pullets. *J Appl Poult Res*. (2012) 21:679–2. doi: 10.3382/japr.2011-00486

30. Koutsoumanis K, Allende A, Alvarez-Ordóñez A, Bolton D, Bover-Cid S, Chemaly M, et al. Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 12: Tetracyclines: tetracycline, chlortetracycline, oxytetracycline, and doxycycline. *EFSA J*. (2021) 19:e06864. doi: 10.2903/j.efsa.2021.6864,

31. Shang Y, Kumar S, Oakley B, Kim WK. Chicken gut microbiota: importance and detection technology. *Front Vet Sci*. (2018) 5:254. doi: 10.3389/fvets.2018.00254

32. Madlala T, Okpeku M, Adeleke MA. Understanding the interactions between Eimeria infection and gut microbiota, towards the control of chicken coccidiosis: a review. *Parasite*. (2021) 28:48. doi: 10.1051/parasite/2021047

33. Yeoman CJ, ChiaN JP, Sipos M, Goldenfeld ND, White BA. The microbiome of the chicken gastrointestinal tract. *Anim Health Res Rev*. (2012) 13:89–99. doi: 10.1017/S1466252312000138

34. Danzeisen JL, Calvert AJ, Noll SL, McComb B, Sherwood JS, Logue CM, et al. Succession of the Turkey gastrointestinal bacterial microbiome related to weight gain. *PeerJ*. (2013) 1:e237. doi: 10.7717/peerj.237

35. Wei S, Morrison M, Yu Z. Bacterial census of poultry intestinal microbiome. *Poult Sc*. (2013) 92:671–3. doi: 10.3382/ps.2012-02822

36. Wu SB, Stanley D, Rodgers N, Swick RA, Moore RJ. Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria infection, induce large changes in the caecal microbiota of broiler chickens. *Vet Microbiol*. (2014) 169:188–7. doi: 10.1016/j.vetmic.2014.01.007

37. Orso C, Stefanello TB, Franceschi CH, Mann MB, Varela APM, Castro IMS, et al. Changes in the ceca microbiota of broilers vaccinated for coccidiosis or supplemented with salinomycin. *Poult Sci*. (2020) 100:100969. doi: 10.1016/j.psj.2020.12.066

38. D'Andreano S, Sànchez Bonastre A, Francino O, Cuscó Martí A, Lecchi C, et al. The gastrointestinal microbial population of Turkey (*Meleagris gallopavo*) affected by hemorrhagic enteritis virus. *Poult Sci*. (2017) 96:3550–8. doi: 10.3382/ps/pex139

39. Macdonald SE, Nolan MJ, Harman K, Boulton K, Hume DA, Tomley FM, et al. Effects of Eimeria tenella infection on chicken caecal microbiome diversity, exploring variation associated with severity of pathology. *PLoS One*. (2017) 12:e0184890. doi: 10.1371/journal.pone.0184890

40. Apajalahti J, Vienola K. Interaction between chicken intestinal microbiota and protein digestion. *Anim Feed Sci Technol*. (2016) 221:323–0. doi: 10.1016/j.anifeedsci.2016.05.004

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Molecular characterization of *Fasciola hepatica* in endemic regions of Colombia

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Fasciola hepatica is a zoonotic trematode that affects a wide range of hosts, including cattle, sheep, and goats. The economic impact of the parasite on the cattle industry is significant, with high losses reported worldwide. While its impact on human health was previously underestimated, recent years have seen a rise in fascioliasis cases, leading to increased interest among researchers globally. To characterize the genetic diversity and intraspecific variation of this parasite in South America, specifically in Colombia, we collected 105 adult parasites from cattle bile ducts in seven Colombian departments (Antioquia, Boyacá, Santander, Cauca, Cundinamarca, Nariño, Norte de Santander, and Santander) to assess the parasite's phenotypic analyses, genetic diversity, and population structure. A computer image analysis system (CIAS) was applied based on standardized morphological measurements. Liver-fluke size was studied by principal component analysis (PCA). DNA sequences were obtained for nuclear markers such as the 28S, β-tubulin 3, ITS1, ITS2, and the mitochondrial marker Cytochrome Oxidase I (COI). Multiple statistical tests were performed, and the parasite's population structure was analyzed. Maximum Likelihood (ML) phylogenetic reconstructions were carried out using the sequences obtained herein and sequences available in GenBank. Morphological results revealed that all the obtained individuals matched *F. hepatica*'s morphology. There was no evidence of high genetic diversity, and the absence of genetic structure at the country-level was notable, possibly caused by a demographic expansion of this trematode in Colombia or the low resolution of the molecular markers employed. Future studies are still needed to unveil the genetic population structure of *F. hepatica* across the country.

KEYWORDS

***Fasciola hepatica*, phylogeneitc tree, Colombia, genetic diversity, population structure**

Introduction

Fasciola hepatica (1) a hepatic trematode, is a pathogen that affects both cattle and humans, causing a parasitic disease called fascioliasis (2). Its high pathogenicity during the disease's invasive or acute phase, and during bile or chronic phase in cattle, sheep, and goats, causes huge economic losses of approximately $200 million USD per year. This is due to the confiscation of livers in slaughterhouses, weight gain reduction, and milk production (3–7). Recent research highlights the importance of the disease in human health, with the World Health Organization including it in their roadmap of neglected tropical diseases for 2030, and promoting the use of One Health strategies as a transversal approach (8, 9).

The global distribution of fascioliasis is wide, resulting from both the historical movement of Old-World animals during colonization and the geographic distribution of Limneidae snails, which act as intermediate hosts for the parasite (10). As a result, the prevalence of the disease in cattle varies widely worldwide. In Africa, prevalence ranges from 1.2 to 91%, while in the Americas, it varies between 24.5 and 100%. In Asian countries, prevalence values fluctuate from 0.71 to 69.2%, while in Papua New Guinea and Australia, the values range from 26.5 to 81%. In Europe, the prevalence varies between 0.12 and 86% (11).

The distinctive biology of *Fasciola hepatica* can affect its genetic diversity and structure (12). Within these biological parasites clonal expansion occurs inside the intermediate host (13), hence there is a possibility of the coexistence of multiple metacercariae sharing origin and genotype, and consequently, parasites sharing multilocus genotypes between definitive hosts (14). Additionally, as a hermaphrodite, the parasite has the potential to induce changes in the allele frequency of a population, and clonal expansion could involve a founder effect, resulting in changes in population structure (15).

Considering the complexity of *Fasciola* characterization through morphological examination (16), molecular approaches have been recently used to identify this parasite with higher accuracy. A variety of molecular markers, such as mitochondrial cytochrome oxidase I (COX1) and NADH dehydrogenase subunit 1 (NAD1), nuclear (28S rRNA) genes, and ribosomal internal transcribed spacers (ITS1 and ITS2), have proven useful for detecting hybrid forms of *Fasciola* (17). While molecular strategies have facilitated the identification of morphologically similar parasites (10), it is not yet the standard, and the distribution of some parasitic species is still unknown.

Fasciola hepatica is a significant economic burden in Colombia, causing losses of around $479,962 USD (18). The parasite is endemic in four recognized regions: Nariño, Cundiboyacense highlands, Santander, Norte de Santander highlands, and highlands of the west of Antioquia (19). The prevalence of *F. hepatica* varies across these areas, with values ranging from 9.5 to 30.9% (20–25). However, knowledge of the parasite's genetic diversity and intrapopulation structure in the country is limited. Thus, this study aims to genetically characterize *F. hepatica* infecting cattle and analyze its population structure in seven departments of Colombia (Antioquia, Boyacá, Santander, Cauca, Cundinamarca, Nariño, Norte de Santander, and Santander), located in endemic biogeographic regions of the parasite.

Methods

Sample collection

This study was conducted in seven departments of Colombia from 2021 to 2022: Antioquia, Boyacá, Santander, Cauca, Cundinamarca, Nariño, Norte de Santander, and Santander (Figure 1; Supplementary Table S1). During liver inspection of sacrificed animals, 15 *F. hepatica* adult samples were selected from the bile duct of 15 different cattle in each department (1 adult parasite per cattle). The flukes were washed with saline solution to remove bile residues and blood remains adhered to the parasite (26). Samples were preserved in 70% ethanol and refrigerated at 4°C to conduct the phenotypic analysis and then subjected to DNA extraction. Epidemiological cards were designed to allow data collection for each animal. This information was obtained from the Sanitary guides for the internal movement of animals (GSMI; Guías Sanitarias de Movilización Interna de Animales) issued by the Instituto Colombiano Agropecuario (Supplementary Table S2).

Ethical statement

The current study was approved by the ethics committee of the Universidad Pedagógica y Tecnológica de Colombia with the title "Caracterización molecular y análisis de la estructura genética poblacional de *Fasciola* spp. en cinco departamentos de Colombia," under report number 007/2019.

Phenotypic analyses

Standardized measurements of *Fasciola* samples were made according to the methods proposed by Valero et al. (27) and Periago et al. (16, 28). The following lineal biometric characters were measured: body length (BL), maximum body width (BW), maximum diameter of oral sucker (OSmax), maximum diameter of ventral sucker (VSmax), distance between the anterior end of the body and the ventral sucker (A-*VS*), and distance between the ventral sucker and the posterior end of the body (*VS*-P). Additionally, areas were measured, including body area (BA), oral sucker area (OSA), and ventral sucker area (VSA), and the ratio of oral sucker area over ventral sucker area (OSA/VSA) was calculated.

Measurements were taken using a microscope and captured with a digital camera (Zeiss Primotech, Germany) and analyzed with image analysis software (Zeiss Zen 3.1 Blue Edition, Jena, Germany). Univariate morphometric comparisons were applied to calculate phenotypic variations among Fasciolid adults from each department and between departments to compare them with previous reports, excluding the effect of ontogenetic variations within the group (29). Reference values of Altiplano Bolivia, Cajamarca (Peru) and San Juan (Ecuador), Valencia (Spain), Córcega (France) and Bobo Dioulasso (Burkina Faso) for *F. gigantica* (16), are shown in Supplementary Table S3. Principal component analysis (PCA) was used to summarize the majority of the variations in a multivariate data set in a few dimensions (30). Results were considered highly significant when $p < 0.01$. Non-redundant measures (measures not included in another one) used were BL, BW, OSmax, VSmax, A-*VS*, and *VS*-P.

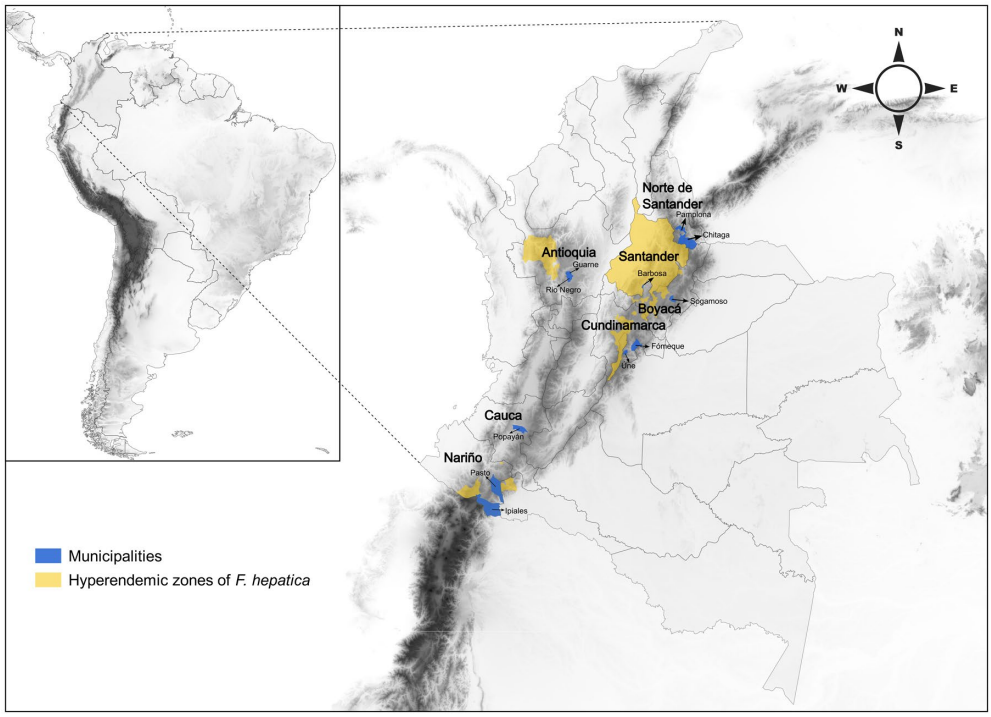

FIGURE 1
Map showing the municipalities where the collection of *Fasciola hepatica* adults was carried out, and the endemic zones for Colombia.

Extraction, amplification, and alignment of DNA data

Genomic DNA was extracted from 105 adult *F. hepatica* parasites using the Invisorb® Spin Universal Kit (Statec Molecular) following the manufacturer's protocol. The concentration of the extracted DNA was assessed using a NanoDrop ND-1000 spectrophotometer, while quality and integrity assessments were performed using electrophoresis with a 1% agarose gel. Minimum quality and integrity parameters were established to select the samples for further analysis, including DNA concentrations of at least 200 ng/μl and quality ratios between 260/280 of 1.7–2.

Molecular markers included in this study were amplified by PCR: *28S rRNA* (FAS-28sFwd-FAS-28sRV) (31), *β-tubulin 3* (FAS-BtubFwd-FAS-BtubRV) (32), Internal Transcribed Spacer 1-*ITS1* (FAS-ITS1Fwd-FAS-ITS1RV), Internal Transcribed Spacer 2-*ITS2* (FAS-ITS2Fwd-FAS-ITS2RV) (33), and Cytochrome Oxidase Subunit 1-*COI* (FAS-COIFwd-FAS-COIRV) (34). The sequences of the primers are shown in Supplementary Table S4, where fragments of 520, 836, 498, 364, and 438 bp were generated, respectively. Each PCR reaction consisted of a mixture of GoTaq Green Master Mix (Promega, Madison, WI, United States) at 1× concentration (400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP, and 3 mM MgCl2), 2.5 μM of each primer, 3 μL of total DNA, and 4.5 μL of molecular biology-grade water to complete a final volume of 25 μl.

PCR cycles were conducted with the following thermal profiles: (i) *28S*: starting denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, then 30 cycles of annealing at 60°C for 30 s, 30 cycles of extension at 72°C for 60 s, and a final extension at 72°C for 5 min; (ii) *β-tubulin 3*: starting denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 60 s, then 35 cycles of annealing at 55°C for 60 s, 35 cycles of extension at 72°C for 60 s, and a final extension at 72°C for 10 min; (iii) *ITS1*: starting denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, then 30 cycles of annealing at 55°C for 30 s, 30 cycles of extension at 72°C for 2 min, and a final extension at 72°C for 10 min; (iv) *ITS2*: starting denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 93°C for 60 s, then 35 cycles of annealing at 55°C for 60 s, 35 cycles of extension at 72°C for 60 s, and a final extension at 72°C for 2 min; (v) *COI*: starting denaturation at 94°C for 90 s, followed by 30 cycles of denaturation at 94°C for 90 s, then 30 cycles of annealing at 55°C for 90 s, 30 cycles of extension at 72°C for 2 min, and a final extension at 72°C for 10 min. Amplicons were visualized using a 1.5% agarose gel.

Purification of PCR products was performed using ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, Foster City, CA, United States) following the manufacturer's protocol, including quality and integrity DNA control. The purified products were then sequenced using Sanger sequencing. The resulting raw sequences were analyzed and contigs were assembled, verified, and edited in DNAStar Lasergene V7.1.0 (DNAStar, Inc., Madison, WI). The alignment of locus sequences, visual inspection, and manual correction of alignments were performed using Mesquite (35).

Molecular phylogenetic and population genetics analyses

To characterize the genetic variability of *F. hepatica*, we estimated the genetic divergences and calculated the number of haplotypes (h),

haplotype diversity (hd), nucleotide diversity (π) and number of segregating sites (S) using only the Colombian sequences (Supplementary Table S1) for each marker in DNASP v6.12.03 (36). We reconstructed phylogenetic relationships within the multiple *F. hepatica* samples only for markers that were informative according to the estimated genetic divergence calculations, using maximum likelihood (ML) inference on IQ-Tree 2 (37). The best substitution models for each locus were selected using ModelFinder (38), included in IQ-Tree 2, and considering the Bayesian Information Criterion for the final selection (BIC) (39). Therefore, the resulting substitution models for each locus were F81 + F for Cytochrome oxidase I (*COI*) and K2P + G4 for *β-tubulin 3*. We used UltraFast Bootstrap (40), aBayes (41), and SH-aLRT (42) to assess node support, performing each reconstruction with 1,000 pseudoreplicates. For these reconstructions, we included *Schistosoma turkestanicum* sequences obtained from GeneBank as the outgroup (Supplementary Table S5). TCS haplotype networks were constructed for both markers using PopArt v1.7 (43). As little to no intraspecific diversity was evident and no phylogeographic signal was detected in the first round of the reconstructions, we decided to include additional *F. hepatica* sequences from different countries retrieved from GenBank (Table 1) and re-run the reconstructions under the same parameters described above. The aim of the inclusion of new sequences was to determine if our sequences would cluster among themselves at a different geographic scale, indicating hypothetically that there is intraspecific diversity in *F. hepatica* at a larger geographic scale and not at the regional scale as we expected. We constructed a TCS haplotype network (47) for the *COI* and *β-tubulin 3* markers using PopArt v1.7 (43), including the new *F. hepatica* sequences from GenBank. Finally, we performed a principal coordinate analysis PCoA using the *COI* and *β-tubulin 3* alignments. To do this, we obtained a "dist" file that contained the Euclidean distances of these data sets. We then used the gl.pcoa function from the dartR package to conduct the analysis. To create the graphs, we utilized the colorplot function of the adegenet package.

Results

Morphometric analysis

Table 2 presents the morphometric values of *F. hepatica*, including extreme values, mean ± standard deviation by department, from Antioquia, Boyacá, Cauca, Cundinamarca, Nariño, Norte de Santander, and Santander. The data obtained from comparative morphometric analysis shows that there are no significant differences between *Fasciola* measurements from different departments ($p > 0.01$). Therefore, the samples do not exhibit any morphometric variation between departments (Table 2). The values of *F. hepatica* for the assessed departments, with measures of morphological traits considered useful to distinguish between *F. hepatica* and *F. gigantica*, demonstrate that none of the evaluated characteristics overlap with *F. gigantica*.

In the dispersion graph of principal components (Supplementary Figure S1), the populations from the seven departments of this study were grouped in the same cluster, which was well separated from Burkina Faso's *F. gigantica* but remarkably close to Bolivia's *F. hepatica*. This suggests that the sizes of the populations from our study and those from the Bolivian highlands are similar. Additionally, the proximity between European and Peruvian populations was observed, while Ecuadorian populations appeared to be distant from the rest of the *F. hepatica* populations analyzed (Figure 2).

Phylogenetic analysis

The analysis of genetic divergence showed that there was not a significant genetic variability in the *ITS1, ITS2*, and *28S* sequences of the Colombian samples. Although an attempt was made to concatenate the ribosomal markers, the genetic divergence calculations indicated an absence of genetic diversity. However, the *COI* and *β-tubulin 3* sequences showed a signal of genetic variability, with the *COI* sequences exhibiting less genetic variability compared to *β-tubulin 3* sequences (h 3; hd 0.648; π 0.00182 and h 10; hd 0.945; π 0.00843, respectively; Table 3).

To assess genetic divergence, only *COI* and *β-tubulin 3* markers were found to be informative, leading to the decision to perform phylogenetic analyses solely for these markers. A preliminary analysis of haplotype networks and phylogenetic trees were conducted on Colombian samples, but due to the low genetic variability of *Fasciola* in Colombia, it was not possible to detect any genetic structure among the analyzed departments (Supplementary Figures S2, S3). The resulting topologies were not consistent, and there was no grouping between departments. An external sequence analysis was subsequently performed to determine if the Colombian sequences would cluster with themselves against others on a different geographical scale. The resulting topologies for both *COI* and *β-tubulin 3* show that the Colombian sequences form paraphyletic clades, intermingled with external sequences from GenBank included in the analysis, suggesting low genetic diversity in *Fasciola* at a continental scale and corroborating the results of the genetic diversity calculations. In both reconstructions, the small distance between the terminal branches and their corresponding nodes and the presence of single clades composed of identical sequences, likely separated from the rest of the sequences by one or two SNPs, indicate this low genetic diversity.

The *COI* haplotype network revealed a new haplotype in Boyaca, Nariño, and Santander, while two haplotypes previously reported in South America, and one of them also found in Asia. On the other hand, the *β-tubulin 3* haplotype network showed that new haplotypes were found in Cauca and Nariño; Antioquia, Cauca, and Santander; Cundinamarca, and Norte de Santander. Both haplotype networks, along with the phylogenetic reconstructions and genetic diversity calculations, demonstrated a low genetic diversity between the samples, with only a few mutational steps separating the different haplotypes detected. Furthermore, external sequences grouped with Colombian sequences in both haplotype networks, corroborating the results of the topologies obtained (Figures 3, 4). However, intraspecific diversity was higher in *β-tubulin 3* sequences than *COI* sequences (Supplementary Table S6). This was evident in the phylogenetic reconstructions and haplotype networks, where multiple clades and haplotypes were composed of a single sequence separated by a small distance or a small number of mutational steps. The *COI* dataset showed genetic structuring, which was not confirmed by the *β-tubulin 3* dataset, possibly due to differences in the genetic variability detected for the two markers.

TABLE 1 Accession numbers of *Fasciola hepatica* sequences used for phylogenetic and haplotype analyses.

Marker	N°	Accession number	Country	Reference
28S	1	MN970007	Australia	Le et al., Unpublished
	2	MF678654	Australia	Calvani et al. (17)
	3	HM369302	Bulgaria	Teofanova et al. (32)
	4	KF791538	Egypt	Mohammad-Gobbah et al., Unpublished
	5	HM369311	Poland	Teofanova et al. (32)
	6	HM369358	Poland	Teofanova et al. (32)
β tub	1	HM535803	Bulgaria	Teofanova et al. (32)
	2	HM535813	Greece	Teofanova et al. (32)
	3	HM535962	Greece	Teofanova et al. (32)
	4	HM535806	Greece	Teofanova et al. (32)
	5	HM535842	Poland	Teofanova et al. (32)
ITS1	1	MN559388	Algeria	Amor et al. (48)
	2	AJ243016	Bolivia	Bargues et al. (49)
	3	MF991101	Iran	Heydarian et al., Unpublished
	4	EF612469	Iran	Lotfy et al. (50)
	5	MG569976	Mexico	Valero et al. (44)
	6	KJ689334	Peru	Reyna and Sanabria (51)
	7	KJ689322	Peru	Reyna and Sanabria (51)
	8	KJ689321	Peru	Reyna and Sanabria (51)
	9	KJ689320	Peru	Reyna and Sanabria (51)
	10	GQ231547	Tunisia	Farjallah et al. (52)
	11	GQ231546	Tunisia	Farjallah et al. (52)
ITS2	1	MG569985	Bolivia	Valero et al. (44)
	2	MT423007	Egypt	Khalafalla (53)
	3	KT033698	Iran	Shahbakhsh et al. (54)
	4	MG569976	Mexico	Valero et al. (44)
	5	MG569983	Mexico	Valero et al. (44)
	6	KJ852770	Peru	Reyna and Sanabria (51)
	7	MG569981	Spain	Valero et al. (44)
	8	MG569986	Spain	Valero et al. (44)
COI	1	MK838687	Brazil	Schwantes et al. (34)
	2	MK838686	Brazil	Schwantes et al. (34)
	3	MW867317	Ecuador	Bargues et al. (45)
	4	MN527599	Iran	Khazan et al. (55)
	5	MK447982	Iran	Javanmard et al., Unpublished
	6	MK447972	Iran	Javanmard et al., Unpublished
	7	MF788106	Iran	Heydarian et al., Unpublished
	8	KR422386	Poland	Norbury et al., unpublished
	9	MW867326	Uruguay	Bargues et al. (46)
	10	GU112483	United States	Ai et al. (56)

The principal coordinates analysis graph shows that the sequences from the seven Colombian departments analyzed in this study were grouped together in the same clusters, for both *COI* and *β-tubulin 3* markers, and were clearly distinct from sequences from Asia, Europe, and South America (refer to Supplementary Figures S4, S5). This finding is consistent with the results of the previously described morphometric analyses. Together, the data from *COI* and *β-tubulin* 3 markers confirm the low genetic diversity observed in the morphological analyses at both the country and continental levels, and provide new insights into the low molecular diversity of

TABLE 2 Comparative morphometric data (extreme values, mean±standard deviation) of *Fasciola hepatica* studied: Antioquia, Boyacá, Cauca, Cundinamarca, Nariño, Norte de Santander, and Santander (Colombia).

Adult measurements	Antioquia	Boyacá	Cauca	Cundinamarca	Nariño	Norte de Santander	Santander	*p*
Body area, BA	70.77–214.77	70.24–219.34	86.03–210.81	61.18–215.41	64.62–218.08	57.71–193.56	61.72–218.02	
	142.07 ± 52.48	154.88 ± 57.64	150 ± 41.32	127.77 ± 50.92	132.77 ± 52.32	119.27 ± 42.8	140.77 ± 52.42	0.480
Body length, BL	10.41–23.41	10.88–23.99	12.33–23.72	10.18–23.83	10.67–22.56	10.39–23.57	10.41–22.46	
	16.12 ± 4.14	18.64 ± 4.6	17.93 ± 4.25	16.39 ± 4.8	17.36 ± 4.03	16.82 ± 4.05	16.74 ± 3.91	0.684
Body width, BW	6.43–10.7	5.03–11	5–10.94	5.32–10.86	5.03–10.18	5.23–10.81	5.01–11	
	8.72 ± 1.44	7.46 ± 1.97	8.26 ± 2.18	8.26 ± 2.03	6.9 ± 1.5	7.79 ± 1.77	7.15 ± 1.86	0.085
BL/BW ratio	1.04–2.77	1.05–2.87	1.09–3	1.15–2.89	1.02–2.96	1–2.81	1.1–2.98	
	1.98 ± 0.6	1.94 ± 0.6	2.19 ± 0.69	2.13 ± 0.59	2.18 ± 0.49	1.68 ± 0.58	2.21 ± 0.59	0.168
Oral sucker area, OSA	0.24–0.5	0.2–0.49	0.2–0.49	0.2–0.45	0.2–0.5	0.2–0.49	0.24–0.48	
	0.4 ± 0.08	0.31 ± 0.09	0.33 ± 0.08	0.3 ± 0.07	0.33 ± 0.09	0.38 ± 0.08	0.34 ± 0.08	0.068
Maximum diameter of the oral sucker, OSmax	0.5–1	0.58–1	0.5–0.98	0.52–0.93	0.5–1	0.59–0.97	0.5–0.94	
	0.7 ± 0.16	0.78 ± 0.13	0.72 ± 0.17	0.77 ± 0.1	0.77 ± 0.15	0.79 ± 0.1	0.73 ± 0.16	0.577
Ventral sucker area, VSA	0.52–1.4	0.53–1.38	0.63–1.4	0.51–1.32	0.55–1.21	0.65–1.38	0.5–1.4	
	1 ± 0.29	0.93 ± 0.3	1.06 ± 0.24	0.87 ± 0.23	0.84 ± 0.24	1.01 ± 0.27	0.86 ± 0.3	0.204
Maximum diameter of the ventral sucker, VSmax	0.76–1.36	0.8–1.4	0.71–1.4	0.71–1.31	0.71–1.39	0.74–1.4	0.82–1.39	
	1.16 ± 0.16	1.08 ± 0.22	1.02 ± 0.25	1 ± 0.19	1.01 ± 0.21	1.05 ± 0.18	1.08 ± 0.19	0.366
OSA/VSA ratio	0.25–0.78	0.28–0.78	0.31–0.75	0.26–0.8	0.28–0.78	0.27–0.79	0.26–0.74	
	0.52 ± 0.16	0.6 ± 0.13	0.53 ± 0.14	0.55 ± 0.2	0.55 ± 0.15	0.48 ± 0.15	0.56 ± 0.14	0.572
Distance between the anterior end of the body and the ventral sucker, A-*VS*	1.21–2.76	1.16–2.74	1.15–2.78	1.25–2.71	1.14–2.78	1.11–2.8	1.12–2.57	
	2.01 ± 0.55	1.92 ± 0.47	2.08 ± 0.58	1.84 ± 0.45	1.73 ± 0.53	1.99 ± 0.51	1.96 ± 0.45	0.587
Distance between the ventral sucker and the posterior end of the body, *VS*-P	12.28–25.73	13.49–25.15	13.24–25.99	12.22–21.92	14.54–24.89	13.16–23.88	12.26–25.39	
	18.79 ± 5.03	19.82 ± 3.83	18.17 ± 4.58	16.78 ± 3.55	19.94 ± 3.48	18.86 ± 3.66	17.04 ± 4.44	0.240

Lineal biometric characters in mm, areas in mm^2 and ratios without units.

10.3389/fvets.2023.1171147

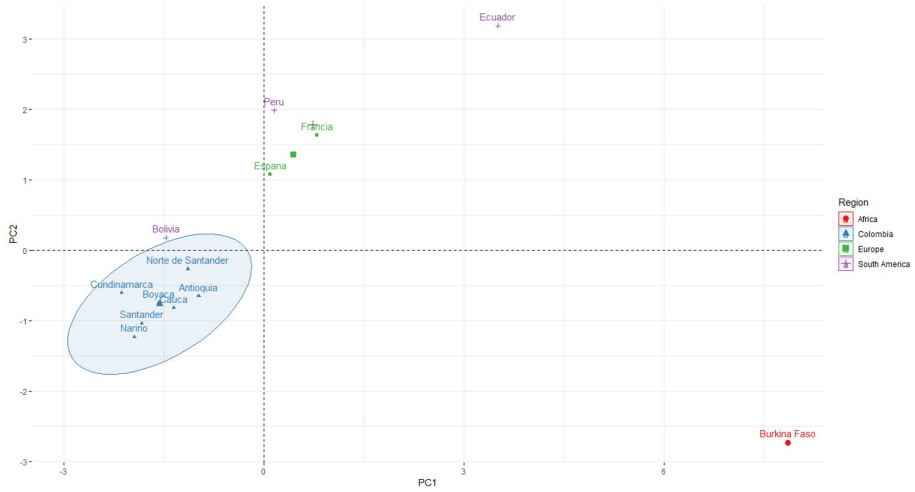

FIGURE 2
Plot for the comparison of *F. hepatica* specimens from Antioquia, Boyacá, Cauca, Cundinamarca, Nariño, Norte de Santander, and Santander (Colombia), with *F. hepatica* specimens from Altiplano Bolivia, Cajamarca (Peru), San Juan (Ecuador), Valencia (Spain), Corsica (France) and *F. gigantica* from Bobo Dioulasso (Burkina Faso). The samples are projected onto the first (PC1, 66.6%) and second (PC2, 21%) principal components.

TABLE 3 Population genetics summary statistics for each marker.

Marker	Statistic			
	h	hd	π	S
28S	2	0.143	0.00025	1
COI	3	0.648	0.00182	2
ITS1	1	0	0	0
ITS2	1	0	0	0
β tub 3	10	0.945	0.00843	25
28S + ITS1 + ITS2	1	0	0	0

h, number of haplotypes; hd, haplotype diversity; π, nucleotide diversity; S, number of segregating sites.

Colombian *F. hepatica* samples. These results suggest that *F. hepatica* has low genetic diversity at the global scale.

Discussion

This study represents the first comprehensive analysis of both the phenotype and genotype of *F. hepatica* in Colombia. Our morphological analysis of adult parasites revealed values consistent with those previously reported for *F. hepatica* in other regions of Europe and the Americas (Supplementary Table S3) (16, 44, 45), indicating the absence of *F. gigantica*. Despite being collected from different geographical areas characterized by highland environments with permanent transmission patterns, we did not observe any significant phenotypic differences between the parasites analyzed in this study. However, previous research has shown that intraspecific variability in *Fasciola* spp. can be linked to changes in altitude, as reported in studies from different regions (3, 8, 10, 57).

Valero et al. (44) found that in regions with high altitude, reduced oxygen levels induce hypoxia in hosts, affecting egg production, uterus development, and the size of the trematode body. As a result, egg production, uterus development, and the overall size of the parasite are significantly reduced in high-altitude regions, such as the Bolivian highlands where *F. hepatica* size is smaller than in Europe and other American regions (16, 46). Our study's parasitic worms showed similar sizes to those reported in the Bolivian highlands (Table 2; Figure 2; Supplementary Table S3), indicating that the samples were collected from mountain ranges at altitudes between 2,050 and 2,569 meters above sea level (masl). Bargues et al. (45) mention that there is no apparent relationship between adult trematode shape and altitude or geographic location, but phenotypical changes are linked to the definitive host, with low persistence of morphological characteristics in subsequent infections.

Bargues et al. (45) suggested that there is no significant relationship between the shape of adult *F. hepatica* and altitude or location. However, our assessments of natural populations of *F. hepatica* allowed us to distinguish two phenotypic patterns: the valley pattern and the highlands pattern. Our findings indicate that populations of Andean valleys and European populations display phenotypic homogeneity, unlike highlands populations, which exhibit a wide size range with low values. This suggests that smaller sizes are sufficient to achieve gravidity in the uterus (58), resulting in reduced egg production compared to populations described in Mexico, Ecuador, and Europe (44, 45, 59, 60). Our study collected *F. hepatica* from highland zones, and our results align with Valero et al. (44) proposal, which observed smaller *F. hepatica* sizes in Antioquia, Boyacá, Cauca, Cundinamarca, Nariño, Norte de Santander, and Santander in relation to European samples. This is consistent with the transmission patterns and epidemiology of fascioliasis in various geographical regions. For instance, in the northern highlands of Bolivia, the transmission of the disease is permanent due to stable temperatures throughout the year and the constant presence of water puddles (61). In this context, the permanent elimination of eggs becomes a priority to facilitate transmission throughout the year, as in the zones where our study was conducted. In contrast, in some Mexican regions, transmission of the trematode is seasonal (62), as in

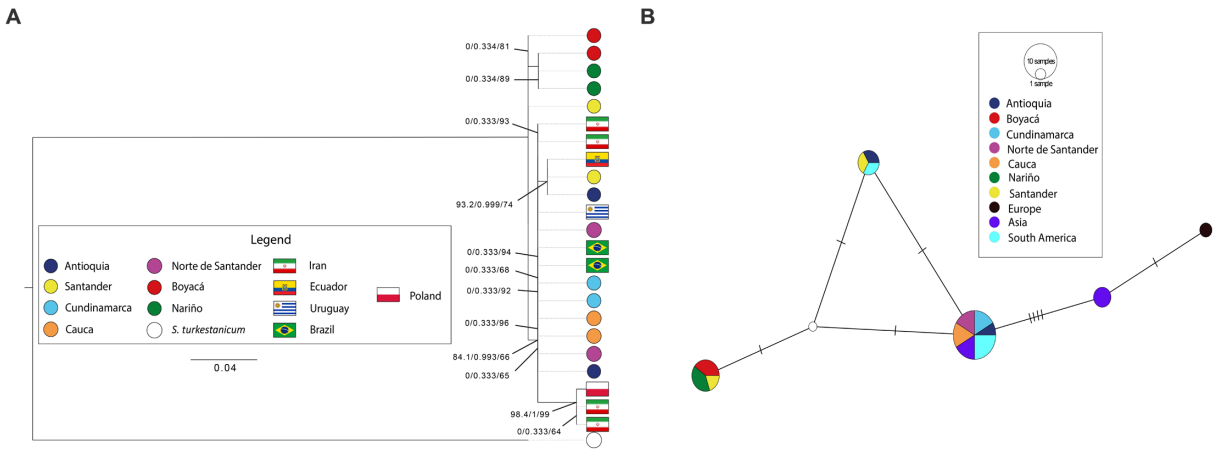

FIGURE 3
(A) Phylogenetic reconstruction with the ML algorithm based on the molecular marker *COI*. Flags represent external sequences included in this analysis. Bootstrap values on the internal nodes are shown in the following order: SH-aLRT/aBayes/UFBootstrap support. Only nodes with bootstrap values higher than 60 are shown. Black lines indicate a node's bootstrap values. **(B)** *COI* TCS haplotype network. European haplotypes includes Poland's sequence, Asian haplotypes includes Iran's sequences and South American haplotypes include sequences from Brazil, Uruguay and Ecuador. Intermediate haplotypes shown as white circles.

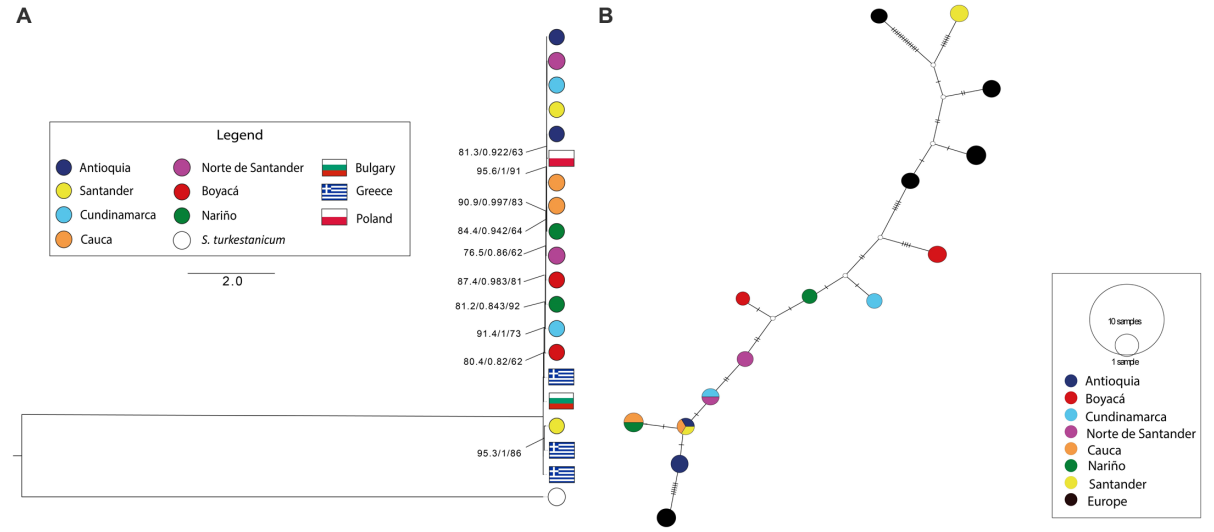

FIGURE 4
(A) Phylogenetic reconstruction with the ML algorithm based on the molecular marker *β-tubulin 3*. Flags represent the origin of external sequences included in this analysis. Bootstrap values on the internal nodes are shown in the following order: SH-aLRT/aBayes/UFBootstrap support. Only nodes with bootstrap values higher than 60 are shown. Black lines indicate a node's bootstrap values. **(B)** *β-tubulin 3* TCS haplotype network. European haplotypes include sequences from Bulgary, Greece and Poland. Intermediate haplotypes are shown as white circles.

low altitude regions in Europe, where a larger uterus can store eggs during unfavorable seasons (60).

In our study, we found that the *28S*, *ITS1*, and *ITS2* markers were not informative when evaluating the levels of genetic variation. This is likely due to the high percentage of repeat sequences (63, 64), in the *Fasciola* genome, which leads to low-quality assemblies and difficulties in designing molecular markers that can provide better characterization of the parasite. While these markers are still being used, reports of low resolution are common in other countries (29, 45, 59, 65). To overcome this limitation, it is necessary to obtain a reference genome for *F. hepatica* that can be used to design more informative markers to reveal the parasite's evolutionary history. Mitochondrial genes have been shown to be informative for phylogenetic studies of *F. hepatica* due to their high mutation rate (66, 67). However, in our study, the use of the *COI* marker did not allow us to reconstruct phylogenetic relationships within Colombian samples (Figure 3), a situation similar to that found by Chaouadi et al. (68) in samples obtained in Algeria. To achieve higher resolution, it may be useful to integrate other mitochondrial markers such as nad1, as suggested by Bargues et al. (5). Although the β-tubulin 3 marker did not allow us to reconstruct phylogenetic relationships among Colombian samples (Figure 4), this marker presents opportunities for

new investigations related to pharmacological resistance processes. Previous studies have shown that β-tubulin is associated with resistance to triclabendazole in *F. hepatica* (69). Therefore, the diversity observed in this marker for the Colombian samples could be explored to analyze and understand the mechanisms of resistance to triclabendazole, which is an important factor to consider in the control of this parasite (70).

Previous studies have shown that there is genetic variability in *F. hepatica* specimens in Latin America, which are similar to those found in Europe (32, 44, 46, 71, 72). This is likely due to successive introductions of cattle from abroad during two historical periods. The first period was the colonial era, where European and Central American animals were brought and subsequently introduced towards South America through the Pacific coast or the terrestrial route from what is now Colombia and Venezuela to the rest of the South American countries. The latter route has been considered the most significant route in terms of the introduction probability of *F. hepatica* haplotypes into the continent. The second period was the post-colonial era, characterized by an increase in Imports of cattle from Europe, North America, and Asia to improve existing breeds in South American countries (45, 65). These introduction processes could have resulted in a wide haplotype diversity since metacercariae can infect different cattle species (73, 74). Additionally, *F. hepatica* infection does not generate premunition, leading to reinfections and the accumulation of the parasite inside the same host (75). This indicates that animal movements across borders could be the indirect source of introducing more than one haplotype capable of infecting multiple susceptible species. Therefore, using molecular tools as a diagnostic strategy in epidemiological surveillance protocols in border corridors is essential for *F. hepatica* identification.

During the colonial period in Colombia, cattle were distributed in both the plains and highlands of the country, similar to other Latin American countries. However, unlike other countries, there was a reduction in the number of cattle raisers during the independence period, and the remaining populations clustered around human dwellings. In the late 19th century, there was a significant increase in the number of cattle in Colombia, but with little participation from imported individuals. This suggests that the restoration of the cattle population in Colombia started from previously established individuals (76, 77). Despite the increased importation of stallions in the 20th century (78), the process of restocking and distribution of cattle in Colombia may have resulted in a founder effect that could explain the low genetic diversity of parasites, including *F. hepatica*, in the studied zones (15, 65). However, an archaeological study found evidence of *F. hepatica* in South America at least 2,300 years ago (79), which opens up a new hypothesis to be explored through molecular analysis of archaeological samples from other continents to clarify the time and route of entry of the parasite into South America. Nevertheless, further studies are still needed to explain the low genetic diversity of *F. hepatica* in Colombia.

The current study has provided new insights into the phylogenetic relationships and structure of *F. hepatica* in Colombia, revealing a low diversity of haplotypes for two markers. Despite the parasite's reported presence in multiple regions of the country, the expected excess of haplotypes that typically accompanies geographic expansion is not observed (80), as Table 3 illustrates. These findings differ from those in other countries where *Fasciola* population expansion is evident, such as Ecuador, Argentina, and Uruguay. This discrepancy may be due to differences in the molecular markers used in characterization, as well as to factors such as the arrival and movement of cattle in each country, sociocultural aspects, cattle handling practices, and the presence and distribution of intermediate host species (46, 67, 81). Further studies are required to gather more information and confirm the hypothesis that the population structure of *F. hepatica* is influenced by the mobility of the parasite's definitive host. In Colombia, the high mobility of cattle and other definitive hosts may result in a low population structure of *F. hepatica*, leading to a greater spread of the parasite. Insights gained from these studies will improve our understanding of the host-vector-pathogen triad and facilitate the management of fascioliasis by providing insights into the dynamics of the pathogen's population structure.

This study is the first to characterize the genetic structure of *Fasciola* in Colombia. We analyzed multiple departments and found that *F. hepatica* is exclusively circulating in the country, without strong indications of genetic structure. However, to broaden our comparisons, more sampling efforts are required to include other regions, using our results as a reference. Furthermore, additional studies are necessary to obtain a reference genome and identify suitable molecular markers that can enhance our understanding of the evolutionary history of *F. hepatica* and complement our current findings. Research on Limneidae snails, which are essential in the parasite's life cycle, is also necessary to better understand their distribution, implications, and potential role in the circulation of new haplotypes.

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 below: https://www.ncbi.nlm.nih.gov/genbank/, OQ518355-OQ518368, OQ513221-OQ513234, OQ513976-OQ513989, OQ532997-OQ533010, OQ513939-OQ513952.

Author contributions

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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.

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Supplementary material

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References

1. Linnaeus C. Systema naturæ: per regna tria naturaæ, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Tomo 1. Edición 10. Decima Reformata. *Holmiæ (Estocolmo): Impensis Direct Laurentii Salvii*. (1758):1–824. doi: 10.5962/bhl.title.542

2. Lalor R, Cwiklinski K, Calvani NED, Dorey A, Hamon S, Corrales JL, et al. Pathogenicity and virulence of the liver flukes *Fasciola hepatica* and *Fasciola gigantica* that cause the zoonosis fasciolosis. *Virulence*. (2021) 12:2839–67. doi: 10.1080/21505594.2021.1996520

3. Abdel-Fatah OR, Arafa WM, Wahba AA, el-Dakhly KM. Economic losses, morpho-molecular identification, and identity of *Fasciola* species recovered from Egypt. *J Parasit Dis*. (2022) 2016:623. doi: 10.1007/s12639-022-01526-x

4. Arias-Pacheco C, Lucas JR, Rodríguez A, Córdoba D, Lux-Hoppe EG. Economic impact of the liver condemnation of cattle infected with *Fasciola hepatica* in the Peruvian Andes. *Trop Anim Health Prod*. (2020) 52:1927–32. doi: 10.1007/s11250-020-02211-y

5. Bargues MD, Halajian A, Artigas P, Luus-Powell WJ, Valero MA, Mas-Coma S. Paleobiogeographical origins of *Fasciola hepatica* and *F. gigantica* in light of new DNA sequence characteristics of *F. nyanzae* from hippopotamus. *Front Vet Sci*. (2022) 9:990872. doi: 10.3389/fvets.2022.990872

6. Hayward AD, Skuce PJ, McNeilly TN. The influence of liver fluke infection on production in sheep and cattle: a meta-analysis. *Int J Parasitol*. (2021) 51:913–24. doi: 10.1016/j.ijpara.2021.02.006

7. Mehmood K, Zhang H, Sabir AJ, Abbas RZ, Ijaz M, Durrani AZ, et al. A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants. *Microb Path*. (2017) 109:253–62. doi: 10.1016/j.micpath.2017.06.006

8. Caravedo MA, White AC Jr, Morales ML, Lopez M, Tanabe MB, Baca-Turpo B, et al. Comparison of liver condemnation and bile microscopy as tools to estimate *Fasciola hepatica* prevalence and burden in the Anta Province of Cusco in Peru. *Vector Borne Zoonotic Dis*. (2021) 21:707–12. doi: 10.1089/vbz.2020.2753

9. World Health Organization. *Ending the Neglect to Attain the Sustainable Development Goals. A Road Map for Neglected Tropical Diseases 2021–2030*. Geneva: Department of Control of Neglected Tropical Diseases, World Health Organization, WHO Headquarters (2020).

10. Kasahara S, Ohari Y, Jin S, Calvopina M, Takagi H, Sugiyama H, et al. Molecular characterization revealed *Fasciola* specimens in Ecuador are all *Fasciola hepatica*, none at all of *Fasciola gigantica* or parthenogenic *Fasciola* species. *Parasitol Int*. (2021) 80:102215. doi: 10.1016/j.parint.2020.102215

11. Rizwan M, Khan MR, Afzal MS, Manahil H, Yasmeen S, Jabbar M, et al. Prevalence of fascioliasis in livestock and humans in Pakistan: a systematic review and Meta-analysis. *Trop Med Infect Dis*. (2022) 7:126. doi: 10.3390/tropicalmed7070126

12. Hodgkinson J, Cwiklinski K, Beesley NJ, Paterson S, Williams DJL. Identification of putative markers of triclabendazole resistance by a genome-wide analysis of genetically recombinant *Fasciola hepatica*. *Parasitology*. (2013) 140:1523–33. doi: 10.1017/S0031182013000528

13. Alba A, Vázquez AA, Sánchez J, Lounnas M, Pointier JP, Hurtrez-Boussès S, et al. Patterns of distribution, population genetics and ecological requirements of field-occurring resistant and susceptible *Pseudosuccinea columella* snails to *Fasciola hepatica* in Cuba. *Sci Rep*. (2019) 9:14359. doi: 10.1038/s41598-019-50894-7

14. Vilas R, Vázquez-Prieto S, Paniagua E. Infection, genetics and evolution contrasting patterns of population genetic structure of *Fasciola hepatica* from cattle and sheep: implications for the evolution of anthelmintic resistance. *Inf Gen Evol*. (2012) 12:45–52. doi: 10.1016/j.meegid.2011.10.010

15. Choi YJ, Fontenla S, Fischer PU, le TH, Costábile A, Blair D, et al. Adaptive radiation of the flukes of the family Fasciolidae inferred from genome-wide comparisons of key species. *Mol Biol Evol*. (2020) 37:84–99. doi: 10.1093/molbev/msz204

16. Periago MV, Valero MA, Panova M, Mas-Coma S. Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS). *Parasitol Res*. (2006) 99:368–78. doi: 10.1007/s00436-006-0174-3

17. Calvani NED, Šlapeta J. *Fasciola* species introgression: just a fluke or something more? *Trends Parasitol*. (2021) 37:25–34. doi: 10.1016/j.pt.2020.09.008

18. Ortega CM, Vásquez LR, Vargas R, Vergara D, Victoria I, Cañola L. Descripción epidemiológica de *Fasciola hepatica* en bovinos en el beneficiadero de Popayán, Cauca, Colombia. *Rev Med Hondur*. (2017) 85:1–114.

19. Becerra Rozo WM. Consideraciones sobre estrategias sostenibles para el control de *Fasciola hepatica* en Latinoamérica. *Rev Col Cienc Pec*. (2001) 14:28–35.

20. Bedoya J, Hurtado Y, Pérez J, Solano S, Úsuga MV, Vanegas M, et al. Primer registro de focos de fasciolosis y paramfistomosis en bovinos doble propósito, Gómez Plata, Antioquia. *Colombia Hechos Microbiol*. (2012) 3:31–9. doi: 10.17533/udea.hm.15059

21. Chaparro JJ, Ramírez NF, Villar D, Fernandez JA, Londoño J, Arbeláez C, et al. Survey of gastrointestinal parasites, liver flukes and lungworm in feces from dairy cattle in the high tropics of Antioquia. *Colombia Parasit Epidemio Cont*. (2016) 1:124–30. doi: 10.1016/j.parepi.2016.05.001

22. Correa S, Martínez YL, López JL, Velásquez LE. Evaluación de la técnica modificada de Dennis para el diagnóstico de fasciolosis bovina. *Biomedica*. (2016) 36:64–8. doi: 10.7705/biomedica.v36i2.2875

23. Mesa-Mesa KA, Fonseca-Sarmiento JC, Jaimes-Dueñez J. Frecuencia de fascioliasis y su impacto económico en una planta de beneficio de Santander, Colombia. *Braz J Anim Env Res*. (2022) 5:1819–28. doi: 10.34188/bjaerv5n2-029

24. Ortiz-Pineda MC, Archila-Barrera OA, Bulla-Castañeda DM, Díaz-Anaya AM, Giraldo Forero JC, Garcia-Corredor DJ, et al. Diagnóstico post mortem de *Fasciola hepatica* en bovinos faenados en la planta de beneficio de Sogamoso (Boyacá, Colombia). *Rev Investig Vet Peru*. (2021) 32:e21341 https://dx.doi.org/10.15381/rivep.v32i5.21341

25. Pinilla JC, Florez Muñoz AA, Uribe DN. Prevalence and risk factors associated with liver fluke *Fasciola hepatica* in cattle and sheep in three municipalities in the Colombian Northeastern Mountains. *Vet Parasitol Reg Stud Report*. (2020) 19:100364. doi: 10.1016/j.vprsr.2019.100364

26. Kooshan M, Hashemi T, Naghibi A. Use of somatic and excretory-secretory antigens of *Fasciola hepatica* in diagnosis of sheep by ELISA. *Am Eur J Agri Env Sci*. (2010) 7:170–5.

27. Valero MA, Panova M, Mas-Coma S. Phenotypic analysis of adults and eggs of *Fasciola hepatica* by computer image analysis system. *J Helminthol*. (2005) 79:217–25. doi: 10.1079/JOH2005301

28. Periago MV, Valero MA, El Sayed M, Ashrafi K, El Wakeel A, Mohamed MY, et al. First phenotypic description of *Fasciola hepatica*/*Fasciola gigantica* intermediate forms from the human endemic area of the Nile Delta. *Egypt Infect Genet Evol*. (2008) 8:51–8. doi: 10.1016/j.meegid.2007.10.001

29. Dos Reis SP, Pessoa LM, Strauss RE. Application of size-free canonical discriminant analisis to studies of geographic differentation. *Braz J Genet*. (1990) 13:509–20.

30. Dujardin J.P.. BAC Software. Institute de Reserches Pour le Développement. IRD, France; (2002). Available at: http://www.fsf.org/copyleft/gpl.html (Accessed September, 2022).

31. Aghayan S, Gevorgian H, Ebi D, Atoyan H, Addy F, Mackenstedt U, et al. *Fasciola* spp. in Armenia: genetic diversity in a global context. *Vet Parasitol*. (2019) 268:21–31. doi: 10.1016/j.vetpar.2019.02.009

32. Teofanova D, Kantzoura V, Walker S, Radoslavov G, Hristov P, Theodoropoulos G, et al. Genetic diversity of liver flukes (*Fasciola hepatica*) from Eastern Europe. *Inf Gen Evol*. (2011) 11:109–15. doi: 10.1016/j.meegid.2010.10.002

33. Shafiei R, Sarkari B, Sadjjadi SM, Mowlavi GR, Moshfe A. Molecular and morphological characterization of *Fasciola* spp. isolated from different host species in a

newly emerging focus of human Fascioliasis in Iran. *Vet Med Int*. (2014) 2014:405740. doi: 10.1155/2014/405740

34. Schwantes J, Quevedo P, D'Ávila M, Molento M, Graichen D. *Fasciola hepatica* in Brazil: genetic diversity provides insights into its origin and geographic dispersion. *J Helmint*. (2019) 94:E83. doi: 10.1017/S0022149X19000774

35. Maddison WP, Maddison DR. Mesquite: a modular system for evolutionary analysis. *Version*. (2019) 3:2018.

36. Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, et al. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol Biol Evol*. (2017) 34:3299–302. doi: 10.1093/molbev/msx248

37. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, et al. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Mol Biol Evol*. (2020) 37:1530–4. doi: 10.1093/molbev/msaa015

38. Kalyaanamoorthy S, Minh BQ, Wong TK, von Haeseler A, Jermiin LS. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods*. (2017) 14:587–9. doi: 10.1038/nmeth.4285

39. Schwarz G. Estimating the dimension of a model. *Ann Stat*. (1978) 6:4614. doi: 10.1214/aos/1176344136

40. Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS. UFBoot2: improving the ultrafast bootstrap approximation. *Mol Biol Evol*. (2018) 35:518–22. doi: 10.1093/molbev/msx281

41. Anisimova M, Gil M, Dufayard J-F, Dessimoz C, Gascuel O. Survey of branch support methods demonstrates accuracy, power, and robustness of fast likelihood-based approximation schemes. *Syst Biol*. (2011) 60:685–99. doi: 10.1093/sysbio/syr041

42. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol*. (2010) 59:307–21. doi: 10.1093/sysbio/syq010

43. Leigh JW, Bryant D. Popart: full-feature software for haplotype network construction. *Methods Ecol Evol*. (2015) 6:1110–6. doi: 10.1111/2041-210X.12410

44. Valero MA, Bargues MD, Calderón L, Artigas P, Mas-Coma S. First phenotypic and genotypic description of *Fasciola hepatica* infecting highland cattle in the state of Mexico. *Mexico Infect Genet Evol*. (2018) 64:231–40. doi: 10.1016/j.meegid.2018.06.032

45. Bargues MD, Valero MA, Trueba GA, Fornasini M, Villavicencio AF, Guamán R, et al. DNA multi-marker genotyping and CIAS morphometric phenotyping of *Fasciola gigantica*-sized flukes from Ecuador, with an analysis of the Radix absence in the NewWorld and the evolutionary Lymnaeid snail vector filter. *Animals*. (2021) 11:2495. doi: 10.3390/ani11092495

46. Bargues MD, Gayo V, Sanchis J, Artigas P, Khoubbane M, Birriel S, et al. DNA multigene characterization of *Fasciola hepatica* and *Lymnaea neotropica* and its fascioliasis transmission capacity in Uruguay, with historical correlation, human report review and infection risk analysis. *PLoS Negl Trop Dis*. (2017) 11:e0005352. doi: 10.1371/journal.pntd.0005352

47. Clement M, Snell Q, Walker P, Posada D, Crandall K. TCS: estimating gene genealogies. *IEEE*. (2002) 7. doi: 10.1109/IPDPS.2002.1016585

48. Amor N, Farjallah S, Merella P, Alagaili AN, Mohammed OB. Multilocus approach reveals discordant molecular markers and corridors for gene flow between North African populations of *Fasciola hepatica*. *Vet Parasitol*. (2020) 278:109035. doi: 10.1016/j.vetpar.2020.109035

49. Bargues MD, Mas-Coma S, Funatsu IR. *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology*. (2002) 123:115–27. doi: 10.1017/s0031182001008034

50. Lotfy WM, Brant SV, DeJong RJ, Le TH, Demiaszkiewicz A, Rajapakse RP, et al. Evolutionary origins, diversification, and biogeography of liver flukes (Digenea: Fasciolidae). *Am J Trop Med Hyg*. (2008) 79:248–55.

51. Reyna G, Sanabria R. Genetic diversity of Fasciola hepatica isolated from three definitive hosts in Cajamarca's valley, Perú In: *13th International Congress of Parasitology, México* (2014) Available at: https://dndi.org/events/2014/icopa-13/

52. Farjallah S, Sanna D, Amor N, Ben Mehel B, Piras MC, Merella P, et al. Genetic characterization of Fasciola hepatica from Tunisia and Algeria based on mitochondrial and nuclear DNA sequences. *Parasitol Res*. (2009) 105:1617–21. doi: 10.1007/s00436-009-1601-z

53. Khalafala RE. Prevalence and Phylogenetic analysis of *Fasciola* species in Upper Egypt Based on Ribosomal ITS-2 gene Sequencing. *EVMSPJ*. (2020) 16:142–58.

54. Shahbakhsh M, Reza N, Maryam G. Molecular characterization of Fasciola samples using sequences of Second Internal Transcribed Spacer-rDNA in different geographical localities of Sistan and Balouchestan province. *Iran. Int J Ent Path*. (2015) 4:10. doi: 10.17795/ijep33362

55. Khazan H, Halakou A, Bendehpour M, Taghipour N, Kazemi B. Identification of *Fasciola* species by PCR-RFLP assay in northern. *Iran. J Gorgan Univ Med Sci*. (2019) 19:99–104.

56. Ai L, Weng YB, Elsheikha HM, Zhao GH, Alasaad S, Chen JX, et al. Genetic diversity and relatedness of *Fasciola* spp. isolates from different hosts and geographic regions revealed by analysis of mitochondrial DNA sequences. *Vet Parasitol*. (2011) 181:329–34. doi: 10.1016/j.vetpar.2011.03.057

57. Lyngdoh D, Sharma S, Roy B, Tandon V. Animal Fascioliasis: perspectives from high altitudinal regions. *Vet Parasitol*. (2016) 232:21–31. doi: 10.1016/j.vetpar.2016.11.007

58. Valero MA, Perez-Crespo I, Khoubbane M, Artigas P, Panova M, Ortiz P, et al. *Fasciola hepatica* phenotypic characterization in Andean human endemic areas: valley versus altiplanic patterns analysed in liver flukes from sheep from Cajamarca and Mantaro. *Peru Infect Genet Evol*. (2012) 12:403–10. doi: 10.1016/j.meegid.2012.01.009

59. Bargues MD, Artigas P, Angles R, Osca D, Duran P, Buchon P, et al. Genetic uniformity, geographical spread and anthropogenic habitat modifications of lymnaeid vectors found in a one health initiative in the highest human fascioliasis hyperendemic of the Bolivian Altiplano. *Parasit Vectors*. (2020) 13:171. doi: 10.1186/s13071-020-04045-x

60. Beesley NJ, Caminade C, Charlier J, Flynn RJ, Hodgkinson JE, Martinez-Moreno A, et al. Fasciola and fasciolosis in ruminants in Europe: identifying research needs. *Transbound Emerg Dis*. (2018) 65:199–216. doi: 10.1111/tbed.12682

61. Cruz-Mendoza I, Quiroz-Romero H, Correa D, Gómez-Espinoza G. Transmission dynamics of *Fasciola hepatica* in the plateau region of Mexico. effect of weather and treatment of mammals under current farm management. *Vet Parasitol*. (2011) 175:73–9. doi: 10.1016/j.vetpar.2010.09.034

62. Stuen S, Ersdal C. Fasciolosis—an increasing challenge in the sheep industry. *Animals*. (2022) 12:1491. doi: 10.3390/ani12121491

63. Luo X, Cui K, Wang Z, Li Z, Wu Z, Huang W, et al. High-quality reference genome of *Fasciola gigantica*: insights into the genomic signatures of transposon-mediated evolution and specific parasitic adaption in tropical regions. *PLoS Negl Trop Dis*. (2021) 15:e0009750. doi: 10.1371/journal.pntd.0009750

64. Pandey T, Ghosh A, Todur VN, Rajendran V, Kalita P, Kalita J, et al. Draft genome of the liver fluke *Fasciola gigantica*. *Acs Omega*. (2020) 5:11084–91. doi: 10.1021/acsomega.0c00980

65. Mas-Coma S, Valero MA, Bargues MD. Human and animal Fascioliasis: origins and worldwide evolving scenario. *Clin Microbiol Rev*. (2022) 35:e0008819. doi: 10.1128/cmr.00088-19

66. Alsulami MN, Wakid MH, Al-matary M, Abdel-Gaber R, Wafa Abdullah I, Al-Megrin AO, et al. Identification and genetic characterization of *Fasciola hepatica* isolated from cattle in Jeddah, Saudi Arabia based on sequence analysis of mitochondrial (COI) gene. *Infect Drug Resist*. (2022) 15:4877–86. doi: 10.2147/IDR.S375671

67. Thang TN, Vázquez-Prieto S, Vilas R, Paniagua E, Ubeira FM, Ichikawa-Seki M. Genetic diversity of *Fasciola hepatica* in Spain and Peru. *Parasitol Int*. (2020) 76:102100. doi: 10.1016/j.parint.2020.102100

68. Chaouadi M, Scarpa F, Azzena I, Cossu P, Harhoura K, Aissi M, et al. New insights into the genetic variability of *Fasciola hepatica* (Trematoda) in Algeria and relationships with other geographic regions revealed by mitochondrial DNA. *Helminthologia*. (2022) 59:152–64. doi: 10.2478/helm-2022-0021

69. Beesley NJ, Cwiklinski K, Allen K, Hoyle RC, Spithill TW, la Course EJ, et al. A major locus confers triclabendazole resistance in Fasciola hepatica and shows dominant inheritance. *PLoS Pathog*. (2023) 19:e1011081. doi: 10.1371/journal.ppat.1011081

70. Kelley JM, Elliott TP, Beddoe T, Anderson G, Skuce P, Spithill TW. Current threat of Triclabendazole resistance in *Fasciola hepatica*. *Trends Parasitol*. (2016) 32:458–69. doi: 10.1016/j.pt.2016.03.002.

71. Carnevale S, Malandrini JB, Pantano ML, Soria CC, Rodrigues-Silva R, Machado-Silva JR, et al. First genetic characterization of *Fasciola hepatica* in Argentina by nuclear and mitochondrial gene markers. *Vet Parasitol*. (2017) 245:34–8. doi: 10.1016/j.vetpar.2017.08.006

72. Ichikawa-Seki M, Ortiz P, Cabrera M, Hobán C, Itagaki T. Molecular characterization and phylogenetic analysis of *Fasciola hepatica* from Peru. *Parasitol Int*. (2017) 65:171–4. doi: 10.1016/j.parint.2015.11.010

73. Andreyanov ON, Postevoy AN, Sidor EA. The effect of ambient temperature on biological properties and energy metabolism of *Fasciola hepatica* metacercariae. *Vet Parasitol*. (2021) 299:109576. doi: 10.1016/j.vetpar.2021.109576

74. González-Miguel J, Becerro-Recio D, Siles-Lucas M. Insights into *Fasciola hepatica* juveniles: crossing the Fasciolosis Rubicon. *Trends Parasitol*. (2021) 37:35–47. doi: 10.1016/j.pt.2020.09.007

75. Angles R, Buchon P, Valero MA, Bargues MD, Mas-Coma S. One health action against human Fascioliasis in the Bolivian Altiplano: food, water, housing, behavioural traditions, social aspects, and livestock management linked to disease transmission and infection sources. *Int J Environ Res Public Health*. (2022) 19:1120. doi: 10.3390/ijerph19031120

76. Sourdís Nájera A. Ganadería en Colombia Cinco Siglos Construyendo País. Bogotá, Federación Colombiana de Ganaderos. Fedegán; (2008). Available at: https://books.google.com.co/books/about/Ganader%C3%ADa_en_Colombia.html?id=0I1CQwAACAAJ

77. Ausdal V, Shawn. Potreros, ganancias y poder: una historia ambiental de la ganadería en Colombia 1850-1950. *Hist Crítica*. (2009) 39:126–49. doi: 10.7440/histcrit39E.2009.07

78. Pertuz Martínez AP, Elías Caro JE. Competitividad en el sector ganadero en Colombia: enfoque desde la historiografía económica, social y empresarial. *Pan Econ*. (2019) 27:453–80. doi: 10.32997/2463-0470-vol.27-num.2-2019-2637

79. Beltrame MO, Pruzzo C, Sanabria R, Pérez A, Mora MS. First report of prehispanic *Fasciola hepatica* from South America revealed by ancient DNA. *Parasitology*. (2020) 147:371–5. doi: 10.1017/Sphalo0031182019001719

80. Rosa IF, Oliveira DJ, Cruz VP, Ashikaga FY, Costa GO, Doretto LB, et al. Temporal genetic structure of a stock of *Prochilodus lineatus* (Characiformes: Prochilodontidae) in the Mogi-Guaçu River ecosystem, São Paulo, Brazil. *Neotrop Ichth*. (2022) 20:e210156. doi: 10.1590/1982-0224-2021-0156

81. Beesley NJ, Elizabeth A, Severo V-P, Román V, Esperanza P, Ubeira FM, et al. Evidence of population structuring following population genetic analyses of *Fasciola hepatica* from Argentina. *Int J Parasitol*. (2021) 51:471–80. doi: 10.1016/j.ijpara.2020.11.007

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Complete mitogenomes characterization and phylogenetic analyses of *Ceratophyllus anisus* and *Leptopsylla segnis*

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Fleas are one of the most common ectoparasites in warm-blooded mammals and an important vector of zoonotic diseases with serious medical implications. We sequenced the complete mitochondrial genomes of *Ceratophyllus anisus* and *Leptopsylla segnis* for the first time using high-throughput sequencing and constructed phylogenetic relationships. We obtained double-stranded circular molecules of lengths 15,875 and 15,785bp, respectively, consisting of 13 protein-coding genes, 22 transfer RNAs, 2 ribosomal RNAs, and two control regions. AT-skew was negative in both *C. anisus* (−0.022) and *L. segnis* (−0.231), while GC-skew was positive in both (0.024/0.248), which produced significant differences in codon usage and amino acid composition. Thirteen PCGs encoding 3,617 and 3,711 codons, respectively, isoleucine and phenylalanine were used most frequently. The tRNA genes all form a typical secondary structure. Construction of phylogenetic trees based on Bayesian inference (BI) and maximum likelihood (ML) methods for PCGs. The results of this study provide new information for the mitochondrial genome database of fleas and support further taxonomic studies and population genetics of fleas.

KEYWORDS

fleas, *Ceratophyllus anisus*, *Leptopsylla segnis*, mitogenome, phylogenetic

Introduction

Fleas are the most notorious blood-sucking ectoparasites that transmit a variety of zoonotic pathogens to infect domestic and wild animals (1). Fleas can host a wide range of species, including human, domestic pets, rodents, and ungulates, resulting in a shortened host lifespan, while ecologically related hosts might share flea species, which increases the public health impact of fleas and causes serious economic losses (2). Fleas play a significant role in maintaining the plague's natural foci (3). Several important pathogens have been identified in fleas, including plague, epidemic hemorrhagic fever, tularemia, leptospirosis, and murine typhus (4). Due to the significant medical, veterinary, and economic value of fleas, increasing attention has been placed on diseases transmitted by fleas, which currently cost global more than $15 billion annually (5). However, to date, phylogenetic relationships and clear classification among fleas have remained ambiguous, which has greatly hindered the control of fleas.

Ceratophyllus anisus belonging to the Siphonaptera order, Ceratophyllidae family, *Ceratophyllus* genus, is the main vector of plague. *C. anisus* can parasitize *Rattus norvegecus*,

Rattus tanezumi, *Apodemus agrarius*, and *Crocidura attenuata*, and occasionally human. *C. anisus* has been found in the former Soviet Union, Korea, and Japan, and is most widely distributed in Yunnan Province, China (6). *Leptopsylla segnis*, in the genus *Leptopsylla* of the family Leptopsyllidae, has been reported in Libya and Cyprus and is common in numerous provinces of China, where wild rodents are most often infested by it (7, 8). Due to the large number of pathogens carried by fleas and the low resolution of traditional taxonomic features for fleas, the choice of a rapid and accurate identification method is necessary for the control of fleas and flea-borne diseases (9).

With the development of molecular and phylogenetic studies, the mitochondrial genome has been used in ectoparasites taxonomy, systematics, and population genetics, which to some extent make up for the limitations of traditional morphology (10, 11). The mitochondrion, the organelles of eukaryotic cells that maintain the structure of life, possess their genetic material and can replicate autonomously outside the nucleus. Mitochondrial DNA (mtDNA) has the advantages of simple structure, little recombination, fast evolution rate, and maintains inheritance in the process of evolution, which makes it an effective tool for studying species identification, relatedness, and phylogeny (12, 13). However, the mitochondrial genome data of fleas is still extremely scarce, and the phylogenetic relationship has not been established, which is a major obstacle to the prevention and control of fleas and flea-borne diseases.

This study is the first to sequence and analyze the mitochondrial genomes of *C. anisus* and *L. segnis*, with the aim of contributing to their correct identification and classification, enriching the mitochondrial genome database of fleas, facilitating the prevention and control of diseases caused by them to minimize the risk to hosts and humans, and providing new and useful markers for further species identification and molecular epidemiological studies. Genetic markers for further species identification and molecular epidemiological studies. We also studied the phylogenetic relationships with the gene sequences of other flea species in NCBI, which provides an important basis for population genetic, phylogenetic and evolutionary analysis.

Materials and methods

Sample collection and DNA isolation

The *C. anisus* samples (two females and one male) used in this study were collected in June 2022 from wild *R. tanezumi* in Laojun Mountain, Lijiang City, Yunnan Province of China (26°53′N, 99°58′E). The adult fleas *L. segnis* specimens were collected in August 2022 in Jianchuan, Yunnan Province from *R. norvegicus* (26°57′N, 99°90′E) (one female and one male). Preliminary identification of the collected flea specimens based on morphological diagnostic features (14). One specimen each was selected for subsequent DNA extraction and mitochondrial genome sequencing, while the others were placed in the Museum of Parasitology, Dali University, under voucher numbers DLUP2206 and DLUP2208, respectively. Specimens for experiments were rinsed in 0.9% saline, fixed in 96% alcohol and stored at −80°C until used for DNA extraction (11). DNA extraction was performed on *C. anisus* and *L. segnis* samples using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) and following the manufacturer's instructions.

PCR amplification

The mitochondrial genomes of *C. anisus* and *L. segnis* were amplified using two sets of overlapping long-fragment RCR primers. Specific PCR primers were constructed from the *Xenopsylla cheopis* (MW310242) and *Pulex irritans* (NC063709) mitochondrial genomes, which correspond to the *COX1* and *12S rRNA* genes, using Primer Premier 5.0 software (CA1: 5′-TTC CCT ACC TGT GCT TGC AG-3′; 3′-AAG AAT TGG ATC TCC CCC GC-5′; CA2: 5′-GCT TGA AAC TTA AAG AAT TTG GCG G-3′, 3′-AAG AGC GAC GGG CAA TAT GT-5′; LS1: 5′-GCA GGA GGG GGT GAT CCT AT-3′; 3′-ATC GTC GAG GTA TTC CTG CT-5′; LS2: 5′-CTT GAA ACT TAA AGA ATT TGG CGG T-3′, 3′-TCC AGT ACA TCT ACT ATG TTA CGA C-5′). The long-range PCR was performed in a total volume of 50 μL, consisting of 10 μL 5× PrimerSTAR GXL Buffer (Takara, Japan), 4 μL of each primer, 4 μL of dNTPs, 1 μL of PrimerSTAR GXL DNA Polymerase (Takara, Japan), 4 μL of DNA template and 23 μL of ddH_2O under the following conditions: initial denaturation at 92°C for 2 min, followed by 30 cycles of denaturation at 92°C for 10 s, annealing at 68°C for 30 s and extension at 68°C for 10 min and the final extension step was subjected to 68°C for 10 min. The PCR products were tested using 1% agarose gel electrophoresis, which was purified and sequenced by Sangon Biotech Company (Shanghai, China).

Gene annotation and data analysis

Sequencing on the Illumina NovaSeq platform using AdapterRemoval software to eliminate low-quality data, assembly by software IDBA. The A5-miseq v20150522 program was used to assemble the complete mitochondrial genome and the MITOS WebServer[1] was used for genome annotation (15). MITOZ tool for mitochondrial genome prediction and online site tRNAscan-SE[2] for secondary structure prediction of transfer RNA (tRNA) (16, 17). Mitochondrial genome circle mapping with CGView Server.[3] The software DNAStar V7.1 was used for nucleotide composition analysis and the program CodonW was used to calculate the relative synonymous codon usages (RSCU). The formulas GC-skew = [G − C]/[G + C] and AT-skew = [A − T]/[A + T] were used to measure the relative base content skewness. The total mitogenome informations of *C. anisus* and *L. segnis* have been deposited in NCBI.

Phylogenetic analysis

Mitochondrial lineages from 15 fleas were determined by phylogeny based on the concatenated datasets of 13 PCGs (Table 1).

1 http://mitos.bioinf.uni-leipzig.de/index.py
2 http://lowelab.ucsc.edu/tRNAscan-SE/
3 https://paulstothard.github.io/cgview/

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Phylogenetic trees were constructed using the Bayesian inference (BI) and maximum likelihood (ML) methods in the Mrbayes v.3.2.7 (18) and MEGA 7.0 software, respectively. The BI approach selected GTR + G + I as the best model, running 10,000,000 generations in total, sampling every 1,000 generations. The ML analysis was based on 1,000 bootstrapped, which assessed branch reliability and nodal robustness. The resultant tree was visualized and edited with FigTree v1.4.2 (19).

TABLE 1 List of the 15 flea species and *Casmara patrona* analyzed in this paper with their GenBank numbers.

Species	Family	Length (bp)	Accession number
Ceratophyllus anisus	Ceratophyllidae	15,875	OQ366407.1
Leptopsylla segnis	Leptopsyllidae	15,785	OQ023576.1
Xenopsylla cheopis	Pulicidae	18,902	MW310242.1
Pulex irritans	Pulicidae	20,337	NC063709.1
Ctenocephalides canis	Pulicidae	15,609	ON109770.1
Ctenocephalides canis	Pulicidae	15,609	NC063710.1
Ctenocephalides felis	Pulicidae	20,873	NC049858.1
Ctenocephalides felis	Pulicidae	20,911	MW420044.1
Ceratophyllus wui	Ceratophyllidae	18,081	NC040301.1
Jellisonia amadoi	Ceratophyllidae	17,031	NC022710.1
Jellisonia amadoi	Ceratophyllidae	17,031	KF322091.1
Dorcadia ioffi	Vermipsyllidae	16,785	NC036066.1
Dorcadia ioffi	Vermipsyllidae	16,785	MF124314.1
Hystrichopsylla weida qinlingensis	Hystrichopsyllidae	17,173	NC042380.1
Hystrichopsylla weida qinlingensis	Hystrichopsyllidae	17,173	MH259703.1
Casmara patrona	Oecophoridae	15,393	NC053695.1

Results

Structure analysis of mitochondrial genome

The complete mitochondrial genomics of *C. anisus* and *L. segnis* were uploaded to Genbank in TBL format under the accession number OQ366407 and OQ023576. The *C. anisus* and *L. segnis* genomes are circular molecules of 15,875 bp and 15,785 bp in length, respectively, consisting of 13 protein-coding genes, 22 tRNAs, two rRNAs, and two D-loop (Figure 1). Fourteen tRNA genes and nine PCGs are located in the forward strand (+), and the remaining 14 genes are encoded in the reverse strand (−) (Table 2). The average AT content of *C. anisus* and *L. segnis* complete mitochondrial genome is 78.54% (78.89%) and GC content is 21.46% (21.11%), including *A* = 38.41% (40.37%), *T* = 40.14% (38.51%), *G* = 8.25% (13.17%) and *C* = 13.21% (7.94%; Table 3). The mitochondrial genome of *C. anisus* has 18 intergenic regions of 929 bp, accounting for 5.85% of the total length, and 13 overlapping regions totaling 28 bp. The genome of *L. segnis* has 19 spacer areas and 8 overlapping regions with a total of 894 bp and 21 bp (Table 2).

Protein-coding genes

The mitochondrial genomes of *C. anisus* and *L. segnis* consist of 13 protein-coding genes, with a total length of 11,014 bp and 11,134 bp, accounting for 69.4% and 70.5% of the total length, respectively. The PCGs of *C. anisus* use the standard ATN as the initiation codon, and stop codons are TAA except for *nad5* and *nad4* (TTA). Amino acid utilization and RSCU were calculated for the PCGs of the mitochondrial genomic of *C. anisus* and *L. segnis*, encoding 3,617 and 3,711 amino acids, and the most abundant amino acid was found to be Isoleucine and Phenylalanine, accounting for 9.95% and 9.75%, respectively (Figure 2). The

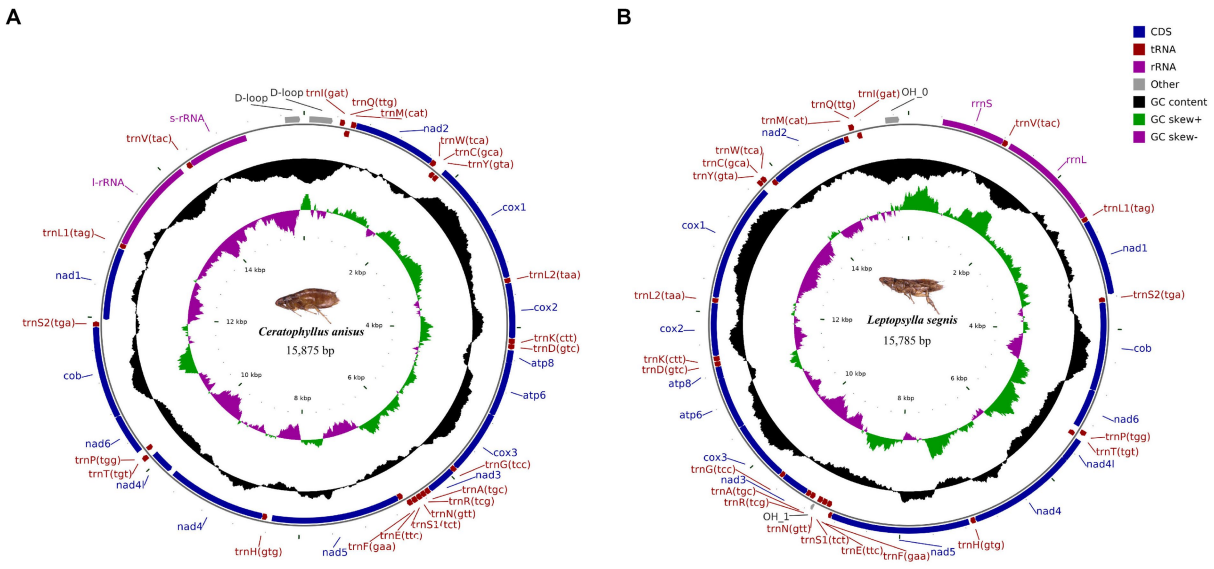

FIGURE 1
Circular map and organization of the mitochondrial genome of *Ceratophyllus anisus* **(A)** and *Leptopsylla segnis* **(B)**.

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TABLE 2 Organization of the *Ceratophyllus anisus* and *Leptopsylla segnis* mitochondrial genomes.

Gene	Strand	Position	Size(bp)	Initiation codon	Stop codon	Anticodon	Intergenic nucleotide
D-loop	N	1-347/117-287	347/171				91/321
trnI	N	439-501/609-672	63/64			GAT	7/5
trnQ	J	577-509/746-678	69/69			TTG	−1/41
trnM	N	577-643/788-854	67/67			CAT	
nad2	N	644-1657/855-1868	1014/1014	ATT/ATT	TAA/TAA		−2/−1
trnW	N	1656-1722/1868-1932	67/65			TCA	6/7
trnC	J	1789-1729/2001-1940	61/62			GCA	
trnY	J	1853-1790/2062-2002	64/61			GTA	−3/0
cox1	N	1851-3386/2063-3598	1535/1536	ATC/ATC	TAA/TAA		4/4
trnL2	N	3391-3454/3603-3666	64/64			TAA	1/1
cox2	N	3456-4136/3668-4348	681/681	ATG/ATG	TAA/TAA		2/2
trnK	N	4139-4208/4351-4420	70/70			CTT	−1/0
trnD	N	4208-4272/4421-4488	65/68			GTC	9/0
atp8	N	4282-4443/4489-4650	162/162	ATA/ATA	TAA/TAA		−7/−7
atp6	N	4437-5111/4644-5318	675/675	ATG/ATG	TAA/TAA		−1/−1
cox3	N	5111-5893/5318-6100	783/783	ATG/ATA	TAA/TAA		
trnG	N	5894-5956/6101-6159	63/59			TCC	−3/0
nad3	N	5957-6307/6160-6513	350/354	ATA/ATA	TAA/TAA		2/−2
trnA	N	6310-6373/6512-6575	64/64			TGC	−1/−1
trnR	N	6373-6435/6575-6637	63/63			TCG	−3/4
D-loop	N	0/6642-6679	/38				0/3
trnN	N	6433-6497/6683-6747	65/65			GTT	
trnS1	N	6498-6565/6748-6815	68/68			TCT	
trnE	N	6566-6630/6816-6878	65/63			TTC	−3/0
trnF	J	6694-6628/6944-6879	67/66			GAA	−2/2
nad5	J	8410-6693/8660-6947	1717/1714	ATT/ATT	TTA/TTA		61/1
trnH	J	8474-8412/8724-8662	63/63			GTG	−1/0
nad4	J	9810-8474/10063-8725	1337/1339	ATG/ATG	TTA/TTA		59/−7
nad4l	J	10097-9804/10350-10057	294/294	ATG/ATG	TAA/TAA		2/2
trnT	N	10100-10165/10353-10416	66/64			TGT	
trnP	J	10228-10166/10480-10417	63/64			TGG	11/2
nad6	N	10240-10746/10483-10992	507/510	ATT/ATT	TAA/TAA		0/−1
cob	N	10747-11886/10992-12131	1140/1140	ATG/ATG	TAA/TAA		2/2
trnS2	N	11889-11952/12134-12196	64/63			TGA	19/19
nad1	J	12910-11972/13151-12216	938/936	ATT/ATT	TAA/TAA		7/1
trnL1	J	12980-12918/13214-13153	63/62			TAG	0/1
rrnL	J	14198-12981/14489-13216	1218/1274				79/30
trnV	J	14346-14278/14585-14520	69/66			TAC	−1/−1
rrnS	J	15124-14346/15363-14585	779/780				519/420
D-loop	N	15643-15875/0	233/0				48/0

AT-skew and GC-skew of the PCGs of the *C. anisus* range from −0.202 (for *NAD1*) to −0.021 (for *ATP8*) and from −0.296 (for *NAD3*) to 0.320 (for *NAD5*), respectively. Among the 13 protein-coding genes, both *C. anisus* and *L. segnis* had nine genes in the forward chain (*NAD2*, *COX1*, *COX2*, *ATP8*, *ATP6*, *COX3*, *NAD3*, *NAD6*, *Cob*) and four genes in the reverse chain (*NAD1*, *NAD5*, *NAD4*, *NAD4L*), which are consistent with most metazoans such as fleas and ticks (5, 20).

TABLE 3 Composition and skewness of *Ceratophyllus anisus* and *Leptopsylla segnis* mitogenome.

Region	*A%*	*C%*	*G%*	*T%*	*A + T%*	*G + C%*	AT skew	GC skew
Whole genome	38.41/40.37	13.21/7.94	8.25/13.17	40.14/38.51	78.54/78.89	21.46/21.11	−0.022/0.024	−0.231/0.248
nad2	34.91/35.80	11.14/10.65	7.99/7.00	45.96/46.55	80.87/82.35	19.13/17.65	−0.137/−0.131	−0.165/−0.207
cox1	29.30/29.49	16.47/16.21	13.93/13.15	40.30/41.15	69.60/70.64	30.40/29.36	−0.158/−0.165	−0.084/−0.104
cox2	34.65/33.19	15.42/15.57	10.57/10.72	39.35/40.53	74.01/73.72	25.99/26.28	−0.099/−0.100	−0.187/−0.185
atp8	43.83/40.12	6.17/11.73	4.32/3.70	45.68/44.44	89.51/84.57	10.49/15.43	−0.021/−0.051	−0.176/−0.521
atp6	30.96/33.04	15.26/14.52	9.78/9.04	44.00/43.41	74.96/76.44	25.04/23.56	−0.174/−0.136	−0.219/−0.233
cox3	27.97/29.63	17.11/16.09	13.03/12.77	41.89/41.51	69.86/71.14	30.14/28.86	−0.199/−0.167	−0.135/−0.115
nad3	31.91/31.92	13.11/12.43	7.12/7.34	47.86/48.31	79.77/80.23	20.23/19.77	−0.200/−0.204	−0.199/−0.257
nad5	34.69/34.89	7.16/6.65	13.80/13.54	44.35/44.92	79.05/79.81	20.95/20.19	−0.122/−0.126	0.317/0.341
nad4	33.43/33.76	7.85/7.24	13.84/12.85	44.88/46.15	78.31/79.91	21.69/20.09	−0.146/−0.155	0.276/0.279
nad4l	36.05/34.35	2.72/3.40	13.61/13.61	47.62/48.64	83.67/82.99	16.33/17.01	−0.138/−0.172	0.667/0.600
nad6	35.90/35.10	10.26/11.57	5.72/4.90	48.13/48.43	84.02/83.53	15.98/16.47	−0.146/−0.160	−0.284/−0.405
cob	31.50/31.58	15.35/15.96	11.40/10.53	41.67/41.93	73.25/73.51	26.75/26.49	−0.139/−0.141	−0.148/−0.205
nad1	30.78/33.01	7.14/7.26	15.76/15.49	46.33/44.23	77.10/77.24	22.90/22.76	−0.202/−0.145	0.176/0.362
rrnl	40.48/41.52	6.16/5.73	12.89/12.32	40.48/40.42	80.95/81.95	19.05/18.05	0/0.013	0.353/0.365
rrns	41.98/40.90	6.55/6.41	12.45/12.31	39.02/40.38	81.00/81.28	19.00/18.72	0.037/0.006	0.311/0.315
trnI	41.27/40.62	7.94/7.81	12.70/12.50	38.10/39.06	79.37/79.69	20.63/20.31	0.040/0.020	0.230/0.231
trnQ	40.58/40.58	4.35/4.35	11.59/11.59	43.48/43.48	84.06/84.06	15.94/15.94	−0.034/−0.034	0.454/0.454
trnM	38.81/37.31	17.91/19.40	10.45/13.43	32.84/29.85	71.64/67.16	28.36/32.84	0.083/0.111	−0.263/−0.182
trnW	41.79/43.08	13.43/10.77	7.46/9.23	37.31/36.92	79.10/80.00	20.90/20.00	0.057/0.077	−0.286/−0.077
trnC	44.26/38.71	4.92/8.06	13.11/16.13	37.70/37.10	81.97/75.81	18.03/24.19	0.080/0.021	0.454/0.334
trnY	39.06/42.62	10.94/8.20	18.75/13.11	31.25/36.07	70.31/78.69	29.69/21.31	0.111/0.083	0.263/0.230
trnL2	34.38/32.81	12.50/12.50	14.06/14.06	39.06/40.62	73.44/73.44	26.56/26.56	−0.064/−0.106	0.059/0.059
trnK	32.86/32.86	15.71/15.71	17.14/15.71	34.29/35.71	67.14/68.57	32.86/31.43	−0.021/−0.042	0.044/0
trnD	47.69/50.00	7.69/5.88	9.23/7.35	35.38/36.76	83.08/86.76	16.92/13.24	0.148/0.153	0.091/0.111
trnG	44.44/42.37	6.35/8.47	7.94/11.86	41.17/37.29	85.71/79.66	14.29/20.34	0.038/0.064	0.111/0.167
trnA	37.50/40.32	6.25/9.68	9.38/9.68	46.88/40.32	84.38/80.65	15.62/19.35	−0.111/0	0.200/0
trnR	39.68/34.92	11.11/11.11	11.11/9.52	38.10/44.44	77.78/79.37	22.22/20.63	0.020/−0.120	0/−0.077
trnN	44.62/44.62	7.69/7.69	9.23/9.23	38.46/38.46	83.08/83.08	16.92/16.92	0.364/0.364	0.091/0.091
trnS1	42.65/39.71	10.29/10.29	10.29/10.29	36.76/39.71	79.41/79.41	20.59/20.59	0.281/0	0/0
trnE	41.54/39.68	7.69/9.52	4.62/6.35	46.15/44.44	87.69/84.13	12.31/15.87	−0.053/−0.057	−0.250/−0.120
trnF	35.82/40.91	10.45/6.06	14.93/13.64	38.81/39.39	74.63/80.30	25.37/19.70	−0.040/0.019	0.177/0.385
trnH	42.86/36.51	3.17/3.17	12.70/15.87	41.27/44.44	84.13/80.95	15.87/19.05	0.019/−0.219	0.600/0.667
trnT	42.42/39.06	4.55/6.25	9.09/9.38	43.94/45.31	86.36/84.38	13.64/15.62	−0.018/−0.074	0.333/0.200
trnP	38.10/42.19	4.76/3.12	15.87/14.06	41.27/40.62	79.37/82.81	20.63/17.19	−0.040/0.019	0.539/0.636
trnS2	42.19/42.86	6.25/7.94	12.50/12.70	39.06/36.51	81.25/79.37	18.75/20.63	0.039/0.080	0.333/0.230
trnL1	39.68/38.71	6.35/6.45	12.70/14.52	41.27/40.32	80.95/79.03	19.05/20.97	−0.020/−0.020	0.333/0.385
trnV	43.48/40.91	5.80/7.58	7.25/7.58	43.48/43.94	86.96/84.85	13.04/15.15	0/−0.039	0.111/0
OH	44.31/44.50	2.41/3.35	2.41/11.48	50.86/40.67	95.17/85.17	4.83/14.83	−0.069/0.045	0/0.548

Transfer RNAs and ribosomal RNAs

The mitochondrial genomes of *C. anisus* and *L. segnis* have 22 tRNA genes and two rRNA genes. The length of 22 tRNAs ranged from 61 bp for $tRNA^{Cys}$ (59 bp for $tRNA^{Gly}$) to 70 bp for $tRNA^{Lys}$ (70 bp for $tRNA^{Cys}$), with a total length of 1,433 bp (1,397 bp). The tRNA genes of both samples can form a complete typical canonical cloverleaf structure. There is an overlap between *ATP8* and *ATP6* with a length of 7 bp, which is typical of arthropods (21). The *16S rRNA* and *12S rRNA* genes of the *C. anisus* and *L. segnis* were separated by *Valine*,

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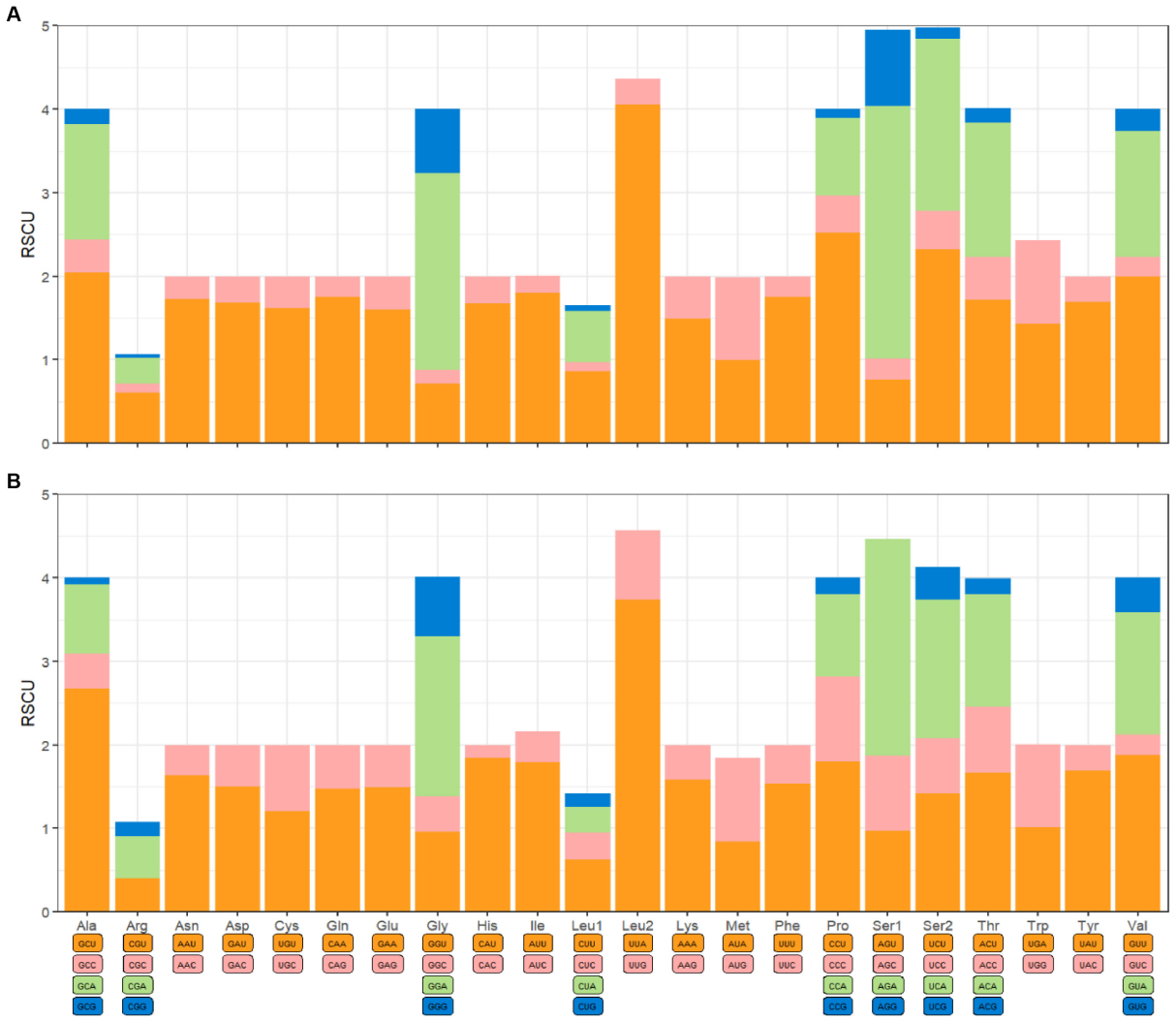

FIGURE 2
Relative synonymous codon usage (RSCU) of codons. **(A)** *Ceratophyllus anisus* and **(B)** *Leptopsylla segnis*. The horizontal coordinates represent all codons encoding each amino acid, and the vertical coordinates represent the sum of all RECU values.

and were 1,218 bp (1,274 bp) and 779 bp (780 bp) in length, respectively (Table 2), a structure consistent with that reported in the mitogenome of other flea species (22).

Phylogenetic analysis

To further analyze the phylogenetic relationships of fleas, we added the mitochondrial genomes of *C. anisus* and *L. segnis* to the analysis. Phylogenetic trees were constructed using the BI and ML methods for the concatenated nucleotide sequences of 13 PCGs of the mitochondrial genome of 15 fleas and *Casmara patrona* as an outgroup, and the topologies of the two methods were consistent. According to the topology analysis, *C. anisus* and *Ceratophyllus wui* are clustered in a branch with high statistical support and *L. segnis* is alone in a branch, forming a sister group with other families of fleas (Figure 3). The families Ceratophyllidae, Leptopsyllidae, Vermipsyllidae, Hystrichopsyllidae, and Pulicidae form monophyletic branches, which is consistent with the previous findings (23).

Discussion

As a temporary host and vector of some important human infectious diseases such as plague and endemic typhus, fleas are early warning indicators for judging the prevalence of plague and other human-animal infectious diseases (24). In recent years, plague has rebounded in some regions, with an increasing trend in incidence, which has always been a persistent and difficult problem worldwide. *C. anisus* and *L. segnis* are common species of fleas that play an important role in the transmission of zoonotic diseases.

The D-loop region has low evolutionary pressure, a large number of gene rearrangements, and rapid base substitutions, making it an effective molecular marker for population genetic studies. The frequency and location of the D-loop vary from species to species and tissue to tissue, and its length is influenced by the number of tandem repeat copies, which in turn affects the length of the entire mitogenome (25). One D-loop region was found for *X. cheopis*, *P. irritans*, and *C. wui*, and two D-loops existed for *Ctenocephalides felis*, *C. anisus*, and *L. segnis*. The two control regions were also found in some ticks

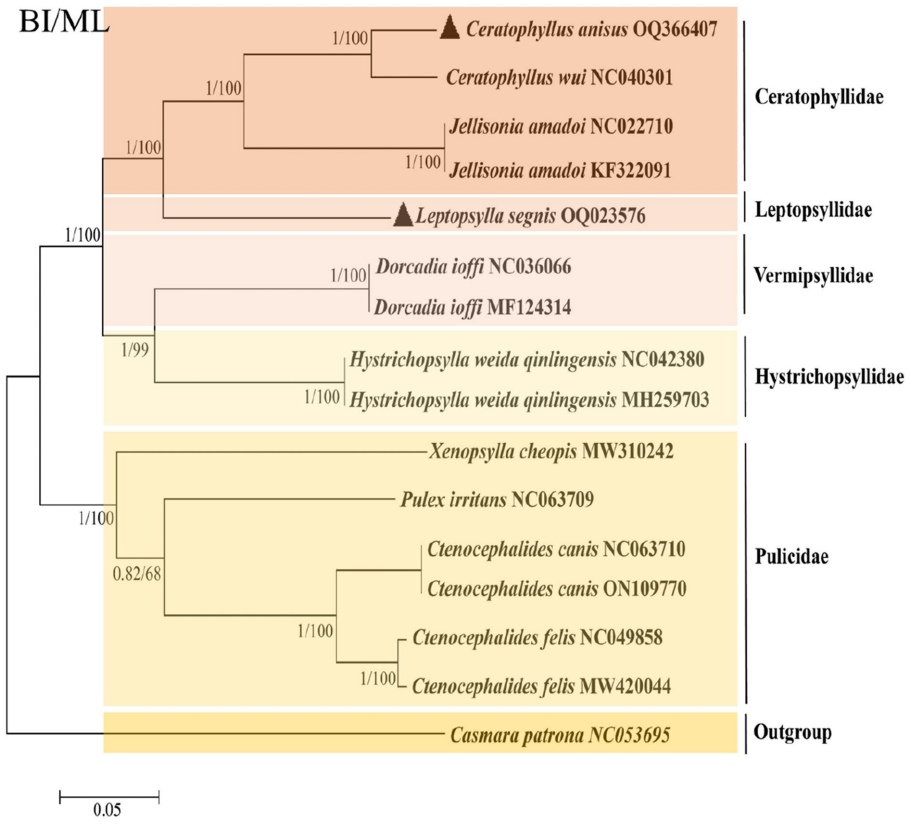

FIGURE 3
Phylogenetic analysis based on the nucleotide sequences of the 13 PCGs in the mitogenome. Each genus is represented by different colors. The solid black triangle represents the species in this study.

and sea cucumbers, and the mtDNA was replicated more efficiently, so it is speculated that the two D-loop regions acted synergistically during the evolutionary process (26). The mtDNAs of *C. anisus* and *L. segnis* have the same gene composition and arrangement as that of most flea species. Bases mismatch appears in most tRNA genes, and G-U wobble base pairs conform to the oscillating pairing principle, which is very important for maintaining the stability of the tRNA secondary structure (27).

The phylogenetic tree derived using all flea mitochondrial genomic data in the NCBI gene bank shows that the five families are divided into two distinct branches, with Ceratphyllidae, Leptosyllidae, Vermipsyllidae, and Hystrichopsyllidae clustered into one, and the Pulicidae family as the other branch. The branch where *L. segnis* is located and the *C. anisus* and *C. wui* branches form a sister group with high node support. The same species of fleas from different hosts and different geographical locations are clustered together in this phylogenetic tree with posterior probabilities and bootstrap values of 1 and 100, respectively, with a high degree of confidence.

The mtDNA is a valuable marker for population biology, species identification classification, and phylogenetic studies, especially for assessing genetic diversity and identifying cryptic species as well as population structure. There are still gaps in molecular data for *C. anisus* and *L. segnis*, which is a major obstacle to the development of flea species. In this study, we obtained the complete mitochondrial genome, which provides more accurate evidence for the phylogenetic relationships of flea species. To better understand the phylogenetic relationship among fleas, the mitochondrial genome study within the Siphonaptera order must be expanded. We expect that the complete mitogenomes of *C. anisus* and *L. segnis* will provide important genome information for molecular phylogenetic studies and contribute to clarifying the phylogeny and evolution of Siphonaptera.

Conclusion

In this study, the complete mitochondrial genomes of *C. anisus* and *L. segnis* are sequenced and annotated for the first time by long-range PCR combined with Illumina sequencing technology which will be helpful for future research on fleas. The results of this study contributed to the fleas, filling the flea mitochondrial genome database resources and laying the foundation for further understanding the phylogenetic relationships of the fleas. With the development of molecular biology, sequencing techniques using mitochondrial genomes as molecular markers have effectively bridged the morphological gap and have been widely used in species identification, kinship, and evolutionary studies.

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 Laboratory Animal Management Committee of Dali University and First Affiliated Hospital of Chengdu Medical College.

Author contributions

YL conceived the study and wrote the manuscript. BC, XL, and DJ carried out the experiments and analysed the data. TW and LG contributed to the collection of *C. anisus* and *L. segnis* and discussions. QZ and XY is responsible for the interpretation of experimental data, critical revision of important knowledge content, and final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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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. Maleki-Ravasan N, Solhjouy-Fard S, Beaucournu JC, Laudisoit A, Mostafavi E. The fleas (Siphonaptera) in Iran: diversity, host range, and medical importance. *PLoS Negl Trop Dis*. (2017) 11:e5206. doi: 10.1317/journal.pntd.0005260

2. Devevey G, Christe P. Flea infestation reduces the life span of the common vole. *Parasitology*. (2009) 136:1351–5. doi: 10.1017/S0031182009990746

3. Andrianaivoarimanana V, Kreppel K, Elissa N, Duplantier JM, Carniel E, Rajerison M, et al. Understanding the persistence of plague foci in Madagascar. *PLoS Negl Trop Dis*. (2013) 7:e2382. doi: 10.1371/journal.pntd.0002382

4. Brown LD. Immunity of fleas (order Siphonaptera). *Dev Comp Immunol*. (2019) 98:76–9. doi: 10.1016/j.dci.2019.03.019

5. Zhang Y, Nie Y, Deng YP, Liu GH, Fu YT. The complete mitochondrial genome sequences of the cat flea *Ctenocephalides felis felis* (Siphonaptera: Pulicidae) support the hypothesis that *C. felis* isolates from China and USA were the same *C. f. felis* subspecies. *Acta Trop*. (2021) 217:105880. doi: 10.1016/j.actatropica.2021.105880

6. Shinozaki Y, Shiibashi T, Yoshizawa K, Murata K, Kimura J, Maruyama S, et al. Ectoparasites of the Pallas squirrel, *Callosciurus erythraeus*, introduced to Japan. *Med Vet Entomol*. (2004) 18:61–3. doi: 10.1111/j.0269-283x.2004.0475.x

7. Wu AG, Zhong YH, Li YQ, Wu HS, Pu EN, Gao ZH, et al. Survey of Ectoparasitic fleas on *Rattus flavipectus* and *Rattus norvegicus* from 34 counties in Yunnan Province, China. *Sichuan J Zool*. (2015) 34:53–8.

8. Christou C, Psaroulaki A, Antoniou M, Toumazos P, Ioannou I, Mazeris A, et al. *Rickettsia typhi* and *Rickettsia felis* in *Xenopsylla cheopis* and *Leptopsylla segnis* parasitizing rats in Cyprus. *Am J Trop Med Hyg*. (2010) 83:1301–4. doi: 10.4269/ajtmh.2010.10-0118

9. Zurita A, Callejón R, García-Sánchez ÁM, Urdapilleta M, Lareschi M, Cutillas C. Origin evolution, phylogeny and taxonomy of *Pulex irritans*. *Med Vet Entomol*. (2019) 33:296–311. doi: 10.1111/mve.12365

10. Yssouf A, Socolovschi C, Leulmi H, Kernif T, Bitam I, Audoly G, et al. Identification of flea species using MALDI-TOF/MS. *Comp Immunol Microbiol Infect Dis*. (2014) 37:153–7. doi: 10.1016/j.cimid.2014.05.002

11. Lawrence AL, Brown GK, Peters B, Spielman DS, Morin-Adeline V, Šlapeta J. High phylogenetic diversity of the cat flea (*Ctenocephalides felis*) at two mitochondrial DNA markers. *Med Vet Entomol*. (2014) 28:330–6. doi: 10.1111/mve.12051

12. Chen SY, Qiu QG, Mo HL, Gong TF, Li F, He JL, et al. Molecular identification and phylogenetic analysis of *Ascarids* in wild animals. *Front Vet Sci*. (2022) 9:891672. doi: 10.3389/fvets.2022.891672

13. Wang HM, Li R, Deng YP, Liu GH, Fu YT. Comparative mitochondrial genomic analysis robustly supported that cat tapeworm *Hydatigera taeniaeformis* (Platyhelminthes: Cestoda) represents a species complex. *Front Vet Sci*. (2022) 9:931137. doi: 10.3389/fvets.2022.931137

14. He JH, Liang Y, Zhang HY. A study on the transmission of plague though seven kinds of fleas in rat type and wild rodent type plague foci in Yunnan. *Chin J Epidemiol*. (1997) 4:45–9.

15. Meng G, Li Y, Yang C, Liu S. MitoZ: a toolkit for animal mitochondrial genome assembly, annotation and visualization. *Nucleic Acids Res*. (2019) 47:e63. doi: 10.1093/nar/gkz173

16. Bernt M, Donath A, Jühling F, Externbrink F, Florentz C, Fritzsch G, et al. MITOS: improved de novo metazoan mitochondrial genome annotation. *Mol Phylogenet Evol*. (2013) 69:313–9. doi: 10.1016/j.ympev.2012.08.023

17. Lowe TM, Eddy SR. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res*. (1997) 25:955–64. doi: 10.1093/nar/25.5.955

18. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*. (2003) 19:1572–4. doi: 10.1093/bioinformatics/btg180

19. Chen Y, Chen S, Kang J, Fang H, Dao H, Guo W, et al. Evolving molecular epidemiological profile of human immunodeficiency virus 1 in the southwest border of China. *PLoS One*. (2014) 9:e107578. doi: 10.1371/journal.pone.0107578

20. Lu XY, Zhang QF, Jiang DD, Liu YF, Chen B, Yang SP, et al. Complete mitogenomes and phylogenetic relationships of *Haemaphysalis nepalensis* and *Haemaphysalis yeni*. *Front Vet Sci*. (2022) 9:1007631. doi: 10.3389/fvets.2022.1007631

21. Sun ET, Li CP, Nie LW, Jiang YX. The complete mitochondrial genome of the brown leg mite, *Aleuroglyphus ovatus* (Acari: Sarcoptiformes): evaluation of largest non-coding region and unique tRNAs. *Exp Appl Acarol*. (2014) 64:141–57. doi: 10.1007/s10493-014-9816-9

22. Hornok S, Beck R, Farkas R, Grima A, Otranto D, Kontschán J, et al. High mitochondrial sequence divergence in synanthropic flea species (Insecta: Siphonaptera) from Europe and the Mediterranean. *Parasit Vectors*. (2018) 11:221. doi: 10.1186/s13071-018-2798-4

23. Wei F, Jia X, Wang Y, Yang Y, Wang J, Gao C, et al. The complete mitochondrial genome of *Xenopsylla cheopis* (Siphonaptera: Pulicidae). *Mitochondrial DNA B*. (2022) 7:170–1. doi: 10.1080/23802359.2021.2017368

24. Sebbane F, Lemaître N. Antibiotic therapy of plague: a review. *Biomol Ther*. (2021) 11:724. doi: 10.3390/biom11050724

25. Nicholls TJ, Minczuk M. In D-loop: 40 years of mitochondrial 7S DNA. *Exp Gerontol*. (2014) 56:175–81. doi: 10.1016/j.exger.2014.03.027

26. Shao R, Aoki Y, Mitani H, Tabuchi N, Barker SC, Fukunaga M. The mitochondrial genomes of soft ticks have an arrangement of genes that has remained unchanged for over 400 million years. *Insect Mol Biol*. (2004) 13:219–24. doi: 10.1111/j.0962-1075.2004.00447.x

27. Watanabe Y, Kawai G, Yokogawa T, Hayashi N, Kumazawa Y, Ueda T, et al. Higher-order structure of bovine mitochondrial tRNA (SerUGA): chemical modification and computer modeling. *Nucleic Acids Res*. (1994) 22:5378–84. doi: 10.1093/nar/22.24.5378

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In vitro and *in vivo* evaluation of chlorhexidine salts as potential alternatives to potassium dichromate for *Eimeria maxima* M6 oocyst preservation

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Introduction: Coccidiosis caused by the *Eimeria* spp., an Apicomplexan protozoon, is a major intestinal disease that affects the poultry industry. Although most cases of coccidiosis are subclinical, *Eimeria* infections impair bird health and decrease overall performance, which can result in compromised welfare and major economic losses. Viable sporulated *Eimeria* oocysts are required for challenge studies and live coccidiosis vaccines. Potassium dichromate (PDC) is typically used as a preservative for these stocks during storage. Although effective and inexpensive, PDC is also toxic and carcinogenic. Chlorhexidine (CHX) salts may be a possible alternative, as this is a widely used disinfectant with less toxicity and no known carcinogenic associations

Methods: *In vitro* testing of CHX gluconate and CHX digluconate exhibited comparable oocyst integrity and viability maintenance with equivalent bacteriostatic and bactericidal activity to PDC. Subsequent use of CHX gluconate or digluconate-preserved Eimeria oocysts, cold-stored at 4°C for 5months, as the inoculum also resulted in similar oocyst shedding and recovery rates when compared to PDC-preserved oocysts.

Results and discussion: These data show that using 0.20% CHX gluconate could be a suitable replacement for PDC. Additionally, autofluorescence was used as a method to evaluate oocyst viability. Administration of artificially aged oocysts exhibiting >99% autofluorescence from each preserved treatment resulted in no oocyst output for CHX salt groups.

KEYWORDS

coccidiosis, *Eimeria maxima*, chlorhexidine, autofluorescence, potassium dichromate

1. Introduction

Eimeria is a genus of protozoan parasites that infect various animal species. The infection is prevalent in poultry, causing significant economic losses (1). The parasites cause intestinal coccidiosis, an extensive problem for the livestock industry that leads to severe economic losses. When shed from poultry in the feces, oocysts are unsporulated and, thereby, noninfective. The exogenous process in which oocysts mature in the environment is known as sporulation, an oxygen-intensive process. Only viable, sporulated oocysts will cause infection when ingested. Sporulation efficiency can be impacted by temperature, humidity, and oxygen availability (2). *Eimeria* oocyst degradation and sporulation assays are methods used to study the impact of compounds or conditions on integrity or exogenous development. However, they differ in their purpose and methodology. *Eimeria* oocyst degradation assays are used to determine the effectiveness of disinfectants or other treatments to damage the oocyst wall. In these assays, oocysts are treated with a chemical or physical agent, and then the percentage of degraded oocysts is determined by counting under a microscope. This assay aims to evaluate a treatment's efficacy in killing or degrading *Eimeria* oocysts. On the other hand, *Eimeria* oocyst sporulation assays are used to study the exogenous life cycle of the parasite. For this, fresh, unsporulated oocysts are subjected to specific temperatures, humidity, and oxygen levels to promote their sporulation while being exposed to different chemicals or alternative treatments *in vitro* (3). The percentage of sporulated oocysts is then determined by counting under a microscope. This assay aims to understand the factors that influence the sporulation of *Eimeria* oocysts.

Potassium dichromate (PDC) has been used extensively as a reagent to promote *in vitro* sporulation and to preserve oocysts for prolonged periods (4). PDC ($K_2Cr_2O_7$) is a strong oxidizing agent (5). PDC when used *in vitro* seems to promote the sporulation of Eimeria species oocysts, although the mechanism is not currently known (6). It is hypothesized that chromium ions may generate oxygen radicals in a manner similar to copper ions that promote sporulation (6, 7). The oxidizing agent also inhibits the growth of microorganisms, extending the viability of the oocysts (8). The purpose of PDC during *in vitro* sporulation is to provide a suitable environment and inhibit other oxygen-consuming microbes that may compete with the oocysts (3). PDC has several advantages as a reagent for storing *Eimeria* oocysts. It is easy to prepare, readily available, and inexpensive. The compound is stable and can be stored at room temperature for long periods without significant degradation (9). Furthermore, PDC has bactericidal and fungicidal properties to control microbial growth and preserve the oocysts' integrity (7). Nevertheless, PDC has some limitations in its application for storing *Eimeria* oocysts. PDC is highly toxic and is considered a hazardous material (10). The substance has been categorized as a known carcinogen associated with various health problems, including lung cancer, skin sensitization, and liver and kidney damage (11). Environmental hazards related to PDC have also been of concern (12, 13). In addition, the oxidization may also alter the antigenicity of the oocysts, affecting the results of experiments that require functionally intact oocysts (14).

Alternative commercially available disinfectants to store *Eimeria* oocysts have been proposed to overcome the limitations of PDC. For example, sodium hypochlorite (NaClO) has been used to surface sterilize oocysts prior to long-term preservation in PDC. The compound is less toxic than PDC and is effective in inactivating microorganisms. However, extended periods of exposure to sodium hypochlorite negatively impacted sporulation and altered the integrity of the oocysts (15, 16). Although ethanol concentrations below 20% and formalin concentrations at 5% or less did not affect *E. tenella* sporulation, *in vivo* studies were not conducted to confirm viability after initial exposure and storage in ethanol or formalin treatments (16). Higher concentrations of formalin or ethanol completely obliterated the oocysts or significantly damaged the oocyst wall and negatively impacted sporulation. As a result, further studies evaluating alternatives to PDC are needed.

Chlorhexidine (CHX) salts are widely used as antiseptics and disinfectants in the healthcare industry (17). CHX is a cationic antiseptic and disinfectant exhibiting bacteriostatic, bactericidal, and anti-fungal properties depending on concentration (18, 19). CHX is typically available in gluconate, digluconate, or diacetate salt forms (20). CHX salts are available in different concentrations, with higher concentrations more effective at killing microorganisms (20). CHX salts are less toxic than PDC and have no known carcinogenic properties (21). The salts are also less environmentally hazardous and can be disposed of safely (22).

Since one of the challenges with studying *Eimeria* is maintaining viable oocysts for experimentation, a method to determine *Eimeria* oocyst viability without the need for a host would be valuable. The viability of oocysts declines depending on storage conditions and the age of the stocks (23). However, viable and nonviable sporulated oocysts can appear very similar morphologically. Previously, a high-fidelity fluorescent microscopy technique was used for *Eimeria* oocysts as a predictor of nonviability (24). When excited, the cytoplasm and sporocysts of morphologically normal but noninfective (nonviable) oocysts exhibit strong autofluorescence, whereas infective (viable) oocysts do not. Thus, autofluorescence can be used to indicate nonviability for *E. maxima* oocysts (25).

The objectives of the present study were to (1) assess the impact of CHX salts (CHX gluconate and CHX digluconate) on *E. maxima* oocyst sporulation and degradation *in vitro,* (2) evaluate *E. maxima* oocyst infectivity *in vivo* after long term storage in CHX salts, and (3) utilize fluorescent microscopy to determine oocyst viability *in vitro* in relation to *in vivo* infectivity.

2. Materials and methods

2.1. Challenge strain and stock

A pure stock of sporulated *Eimeria maxima* M6 (EMM6) oocysts was provided by Dr. John. R. Barta, University of Guelph, Canada. To obtain a sufficient number of oocysts for the experiment described, an *in vivo* amplification was required. Approximately 500 sporulated EMM6 oocysts/mL/chicken were administered to chickens between 9–10 days of age and reared in battery cages for the duration of the study. The birds were reared from day-of-hatch and not previously exposed to *Eimeria* oocysts. A low-dose challenge was selected to optimize oocyst production and shedding (26). Fecal samples containing unsporulated oocysts were collected from d5-8 post-challenge. Fecal material was homogenized and suspended in a sterile saturated salt solution, blended for 30–60 s, then sieved to remove coarse debris. The filtrate was centrifugated at 1,250 × *g* for 10 min to

float oocysts away from fecal debris. Post-centrifugation, the supernatant containing oocysts was decanted, diluted in sterile distilled water (10x), and centrifugated again at 1,250 × *g* for 10 min to pellet the oocyst. The pellet of partially purified oocysts was resuspended in 2.0% or 2.5% potassium dichromate (w/v, aqueous) and transferred to Erlenmeyer flasks capped with sterile gauze at ~100,000 oocyst/mL to permit adequate aeration. The flask was placed on a rotary platform shaker operating at ~100 rpm at 26°C for 3–4 days or until microscopy confirmed sporulation. Following sporulation, the oocysts were resuspended in fresh 2.5% potassium dichromate and held at 4°C.

2.2. Chemicals

Chlorhexidine (CHX) salts evaluated in the present study included: CHX digluconate (Sigma, C9394), CHX diacetate salt hydrate (Sigma, C6143), and commercially available CHX gluconate (VETone brand). Aqueous CHX digluconate from a 20% stock solution was diluted in sterile H_2O to obtain appropriate concentrations for the assays described below. Aqueous CHX gluconate from a 2% solution was also diluted in sterile H_2O. A stock solution of CHX diacetate salt hydrate was made using ethanol and further diluted in sterile H_2O. As a control, potassium dichromate (PDC) (Sigma, 207,802) was used at a final concentration of 2.5% (w/v).

2.3. *In vitro* assay 1

The purpose of *in vitro* assay 1 was to evaluate the antimicrobial efficacy of CHX salts when compared to PDC. Bacteria from poultry feces was selected to be used. A negative 0.9% saline control, a positive 2.50% PDC control, 2.0% CHX gluconate, and 20% aqueous CHX digluconate were used. Additionally, 0.14% CHX diacetate was used (data not shown). CHX diacetate, when used, exhibited notable antibacterial effects but rapidly degraded oocysts during incubation and was thus excluded from further trials. The 2.0% CHX gluconate was diluted to concentrations of 0.03, 0.05, 0.10, 0.20, and 0.40%. The 20% aqueous CHX digluconate was diluted at 0.125, 0.25, 0.50, 1.0, and 2.0% concentrations. These concentrations were selected to investigate the lowest efficacious dose of each solution. The solutions were diluted using a sterile 0.9% saline solution. A 10% fecal slurry was made by combining 100 g of fresh poultry feces from unchallenged birds and 900 mL of sterile, deionized H_2O. The slurry was thoroughly mixed and allowed to separate at 1 g for 2 h. The resulting supernatant fluid was strained and kept for assay use. Before adding to the treatment solutions, the fecal slurry supernatant was serially diluted using 0.9% saline and drop-plated onto tryptic soy agar (TSA) plates (VWR, 880925) to quantify total aerobic bacteria present in the fecal slurry (7.13 Log_{10} CFUs/mL). For the assay, 4 mL of treatment solution and 1 mL of fecal slurry supernatant were combined into 15 mL conical tubes and incubated statically at 26°C (room temperature) for 116 h total. At 0 h, 21 h, 48 h, 75 h, and 116 h of incubation, each tube was line streaked onto a TSA plate using a sterilized inoculation loop. $N = 5$ replicate tubes and plates were used for each treatment dilution. Streaked plates were then incubated at 37°C aerobically for approximately 24 h before analysis.

TABLE 1 Incidence of total aerobic bacteria recovered from a poultry-derived fecal slurry treated with potassium dichromate (PDC), chlorhexidine (CHX) gluconate, or CHX digluconate at five time points of evaluation (*in vitro* assay 1).

Treatment	Total aerobic bacterial recovery				
	0h	21h	48h	75h	116h
Control saline solution	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
2.50% PDC	5/5 (100%)	4/5 (80%)	4/5 (80%)	2/5 (40%) *,**	0/5 (0%) *,**
CHX gluconate (%)					
0.03	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)
0.05	3/5 (60 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)
0.10	2/5 (40 %) *,**	3/5 (60 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)
0.20	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **
0.40	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **
CHX digluconate (%)					
0.125	0/5 (0%) *,**	4/5 (80%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
0.25	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	1/5 (20 %) **	0/5 (0 %) **
0.50	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **
1.0	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **
2.0	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **	0/5 (0 %) **

Data are expressed as positive bacterial growth/total number of samples evaluated (%). *Indicates significant differences within rows at $p < 0.05$. **Indicates significant differences within columns between all treatment groups at $p < 0.05$.

Plates with any bacterial growth present were considered positive. Data are shown in Table 1.

2.4. *In vitro* assay 2

The purpose of *in vitro* assay 2 was to further assess the antimicrobial activity of CHX gluconate and CHX digluconate at their lowest efficacious concentration, 0.20, and 0.25%. Apart from these altered concentrations, the methods and materials are similar to the previously described *in vitro* assay 1. A negative 0.9% saline control, a positive 2.50% PDC control, CHX gluconate at 0.20% concentration, and CHX digluconate at 0.25% concentration were used. Treatment solutions were diluted to the desired concentration using sterile saline. Once again, the supernatant fluid from a 10% fecal slurry made from 100 g of fresh poultry feces and 900 mL of sterile, deionized H_2O was used for the assay. The poultry feces and H_2O were mixed thoroughly and allowed to separate at 1 g for 2 h, and the strained supernatant,

TABLE 2 Total aerobic bacterial recovery from poultry-derived fecal slurries treated with potassium dichromate (PDC), chlorhexidine (CHX) gluconate, or CHX digluconate at two time points of evaluation (*in vitro* assay 2).

Treatment	Total aerobic bacterial recovery	
	0h	50h
Control saline solution	5/5 (100 %)	5/5 (100 %)
2.50% PDC	5/5 (100 %)	3/5 (60 %)
0.20% CHX gluconate	0/5 (0 %) *	0/5 (0 %) *
0.25% CHX digluconate	0/5 (0 %) *	0/5 (0 %) *

Data expressed as positive bacterial growth/total number of samples evaluated (%).
*Indicates significant differences within columns at $p < 0.05$.

TABLE 3 Impact of potassium dichromate (PDC), chlorhexidine (CHX) gluconate, or CHX digluconate on total oocysts at 1d, 4d, and 6d of aeration at 26°C (*in vitro* assay 3).

Treatment	Total oocysts (log_{10} /mL)			
	Initial day 0	1 day aeration	4 days aeration	6 days aeration
Control saline solution	5.87 ± 0.011	5.77 ± 0.016	5.79 ± 0.052	5.66 ± 0.004
2.50% PDC	5.89 ± 0.015	4.82 ± 0.011	5.80 ± 0.023	5.65 ± 0.004
0.20% CHX gluconate	5.88 ± 0.020	5.81 ± 0.023	5.78 ± 0.020	5.60 ± 0.019
0.20% CHX digluconate	5.91 ± 0.006	5.79 ± 0.012	5.79 ± 0.028	5.62 ± 0.019

Data express as mean ± SE.

containing 7.40 Log_{10} CFUs/mL, was kept for assay use. 4 mL of treatment solution and 1 mL of fecal slurry supernatant were combined in 15 mL conical tubes, thoroughly mixed, and statically incubated at 26°C for 50 h. Each tube was line streaked on TSA plates initially at 0 h and after 50 h incubation for bacteria recovery using a sterilized inoculation loop. For each treatment, there were $n = 5$ replicate tubes and plates. Streaked TSA plates were allowed to aerobically incubate at 37°C for approximately 24 h before analysis. Plates with bacterial growth present were considered positive. Data are shown in Table 2.

2.5. *In vitro* assay 3

The purpose of *in vitro* assay 3 was to determine whether CHX gluconate or digluconate would degrade oocysts during incubation at room temperature. A negative 0.9% saline control, a positive 2.50% PDC control, CHX gluconate at 0.20% concentration, and CHX digluconate at 0.20% concentration were selected to directly compare the two solutions. Treatments were diluted to the desired concentrations using sterile saline. As mentioned, CHX diacetate was also used but severely degraded the oocyst (data not shown). Each treatment was separated into 250 mL Erlenmeyer flasks and inoculated with approximately 75,000 oocysts/mL of sporulated EMM6 oocysts. The final volume was 20 mL. Oocyst dilution was completed using sterile saline. Flasks were then covered with sterile gauze pads and placed on a shaker plate to create gentle surface agitation in a 26°C incubator. The assay was allowed to incubate aerobically for 6d total, with 1 mL subsamples taken for enumeration at 0d, 24 h, 4d, and 6d. Oocysts were enumerated using the McMaster technique described by J.N. Hodgson (27) at a 1:100 dilution in a saturated salt solution, 600 µL/ chamber. McMaster slides containing samples were allowed to float undisturbed for 2–5 min before analysis. This method was repeated for each treatment at 0d, 24 h, 4d, and 6d. $N = 3$ replicate flasks per treatment. Data are shown in Table 3.

2.6. *In vitro* assay 4

The purpose of *in vitro* assay 4 was to further evaluate oocyst degradation during incubation in respective treatment solutions and calculate percent sporulation during this time. Fresh, unsporulated oocysts not exposed to PDC were collected for assay use. A negative 0.9% saline control, a positive 2.50% PDC control, CHX gluconate at 0.20% concentration, and CHX digluconate at 0.20% concentration were used. 250 mL Erlenmeyer flasks were used as vehicles. 15 mL of each treatment solution was inoculated with 5 mL of oocysts diluted in saline solution to achieve 10,000 oocysts/mL. Each flask was then covered with sterile gauze pads and placed on a shaker plate to achieve gentle surface agitation uniformly to oxygenate oocysts. The flasks were incubated aerobically at 26°C for 6d. Subsamples at a volume of 1 mL were collected at 0d, 4d, and 6d post-incubation to enumerate total oocysts, assess sporulation rate (%), and quantify viable aerobic bacteria for each treatment. Oocysts were enumerated using the previously described method at a 1:10 dilution. Percent sporulation was calculated by dividing sporulated oocysts by total oocysts (sporulated + unsporulated oocysts). Bacteria were quantified by serially diluting each solution using sterile saline and drop plating onto TSA plates. Inoculated plates were incubated aerobically at 37°C for 36 h before analysis. Data are shown in Table 4.

2.7. *In vivo* trial

For the *in vivo* trial, freshly propagated sporulated EMM6 oocysts not previously exposed to PDC were placed into cold storage at 4°C for 5 months in respective treatments. Oocysts were sporulated in their respective treatment solutions under the same conditions specified in the Challenge strain and stock section. These treatments included a negative 0.9% saline control, a positive 2.50% PDC control, 0.20% CHX gluconate, and 0.20% CHX digluconate. EMM6 oocysts that had undergone *in vitro* sporulation were washed and diluted in the treatment solutions to a concentration of 10,000 oocysts/mL, 20 mL total, and stored for 5 months at 4°C. Once a week, the storage containers were opened and gently agitated to aerate oocyst stocks. After 5 months of storage, a 7 mL subsample of each treatment was removed and placed into an incubator at 45°C to accelerate the aging process and achieve 100% oocyst autofluorescence uniformly in 11 days. It has been demonstrated that oocysts stored at 45°C for 6 days would result in 93.3% autofluorescence (25). Additional time was added to ensure that 100% autofluorescence would be uniformly achieved. Autofluorescence was determined using the Axio microscopy software and fluorescent imaging at 488 nm/509 nm excitation/emission. Oocysts that exhibited vibrant green when excited were considered autofluorescent (Figures 1, 2). An EMM6

TABLE 4 *Eimeria maxima* M6 oocyst degradation, sporulation (%), and total aerobic bacterial recovery after 4 and 6d of incubation at 26°C and treatment with potassium dichromate (PDC), chlorhexidine (CHX) gluconate, or CHX digluconate solutions (*in vitro* assay 4).

Treatment	Total oocysts (log_{10})	Sporulation (%)	Bacterial recovered (log_{10} CFU/mL)
Initial day 0			
Control saline solution	5.70	0.00	6.52 ± 0.14[a]
2.50% PDC	5.70	0.00	0.00 ± 0.00[c]
0.20% CHX gluconate	5.70	0.00	0.00 ± 0.00[c]
0.20% CHX digluconate	5.70	0.00	0.67 ± 0.67[c]
4 days aeration			
Control saline solution	5.66 ± 0.003[ab]	90.0 ± 0.47[a]	8.13 ± 0.18[a]
2.50% PDC	5.70 ± 0.036[ab]	82.7 ± 1.87[b]	0.00 ± 0.00[c]
0.20% CHX gluconate	5.76 ± 0.032[a]	89.4 ± 1.23[ab]	0.00 ± 0.00[c]
0.20% CHX digluconate	5.62 ± 0.018[b]	88.4 ± 1.95[ab]	2.00 ± 0.00[b]
6 days aeration			
Control saline solution	5.71 ± 0.024	89.9 ± 1.12[ab]	8.30 ± 0.17[a]
2.50% PDC	5.75 ± 0.015	85.2 ± 1.54[b]	0.00 ± 0.00[b]
0.20% CHX gluconate	5.74 ± 0.037	92.7 ± 1.27[a]	3.33 ± 1.76[b]
0.20% CHX digluconate	5.67 ± 0.043	80.8 ± 0.46[b]	2.00 ± 0.00[b]

Data express as mean ± SE. [a–c] Values within the same column by day and parameter that do not share a common letter differ significantly ($p < 0.05$).

challenge for each treatment, including the 100% autofluorescent variant of each and the non-aged variant (<~10% autofluorescence), was prepared at a dose of 250 sporulated oocysts/mL, administered orally three times over 3 days for a total of 750 oocysts/bird at d9, d10 and d11 of age. This dose was selected to maximize output and avoid the crowding effect of oocysts (26). The challenge was accomplished by diluting washed oocysts with sterile saline in a 50 mL conical tube. Oocysts underwent two sterile, deionized H_2O washes accomplished by centrifugation at 10 min at 845 g, discard of the supernatant fluid, and a final wash for the resulting oocyst pellet. Sterile saline was used to resuspend the oocyst pellet to the desired concentration. For the trial, 81 one-day-old male broiler chickens (Fayetteville, AR, United States) not previously exposed to *Eimeria* oocysts were randomly allocated to one of nine groups with three replicate cages per treatment ($n = 3$ chickens/ replicate). Chickens were placed in battery cages in a controlled, age-appropriate environment. Treatments consisted of Group (1) unchallenged, saline sham negative control, Group (2) challenged, 2.50% PDC-stored oocysts, Group (3) challenged, saline-stored oocysts, Group (4) challenged, 0.20% CHX gluconate-stored oocysts, Group (5) challenged, 0.20% CHX digluconate-stored oocysts, Group (6) challenged, 2.50% PDC-stored and 100% autofluorescent oocysts, Group (7) challenged, saline-stored and 100% autofluorescent oocysts, Group (8) challenged, 0.20% CHX gluconate-stored and 100% autofluorescent oocysts, and Group (9) challenged, 0.20% CHX digluconate-stored and 100% autofluorescent oocysts. Chicks received *ad libitum* access to water and feed for 23 days. All animal handling procedures complied with the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville, under protocol #22007. Broilers were orally challenged with respective treatment (250 oocysts/1 mL/chicken) at d9. From d5-9 after the challenge administered on d9 of age, all feces excreted were collected into 4 L beakers containing 2.50% PDC. Collections were performed twice daily at 9:00 AM and 6:00 PM. For total oocyst output, evaluation was determined on a volumetric basis. Oocysts were enumerated using the McMaster technique, as previously discussed. Data are shown in Figure 3.

2.8. Data and statistical analysis

All data were subjected to ANOVA as a completely randomized design using the GLM procedure of SAS (28). Treatment means were partitioned using Tukey's multiple range test at $p < 0.05$, indicating statistical significance.

Bacteria recovery from poultry fecal slurry positive samples was compared by a chi-square test of independence (29), testing all possible combinations to determine the significance ($p < 0.05$).

3. Results

The incidence of bacterial recovery from poultry-derived fecal slurries treated with PDC, CHX gluconate, or CHX digluconate at different concentrations and time points for *in vitro* assay 1 have been summarized in Table 1. The incidence of bacterial recovery from saline-treated samples was 100% at all time points evaluated. Samples treated with PDC had a significantly ($p < 0.05$) lower incidence of bacteria recovered at 75 and 116 h. The 0.10% CHX gluconate solution reduced bacterial recovery by 60% at 0 h. Nevertheless, CHX gluconate solutions at 0.20 and 0.40% inhibited bacterial growth at all points of evaluation (Table 1). The concentration of 0.125% CHX digluconate inhibited bacteria growth at 0 h. Interestingly, the rest of the higher concentrations of CHX digluconate inhibited bacteria growth at all evaluation points (Table 1). At 0 h, CHX gluconate at 0.10% or higher concentrations and all CHX digluconate concentrations significantly reduced bacterial recovery. Nevertheless, at 21, 48, 75, and 116 h of evaluation, 0.20 and 0.40% CHX gluconate and all concentrations of CHX digluconate (except the lowest, 0.125%) had similar results, with no bacteria being recovered. At 75 h recovery, 2.50% PDC reduced bacterial recovery to 40%, with 0% recovery at 116 h (Table 1).

Table 2 shows the incidence of bacterial recovery from poultry-derived fecal slurries treated with PDC, CHX gluconate, or CHX digluconate at two-time points for *in vitro* assay 2. Similar to *in vitro* assay 1, saline did not affect bacterial recovery at 0 h or 50 h, with bacterial recovery being 100% at both time points. There were no significant differences between PDC and the saline control. However, CHX gluconate at 0.20% and CHX digluconate at 0.25% inhibited bacterial recovery completely (Table 2).

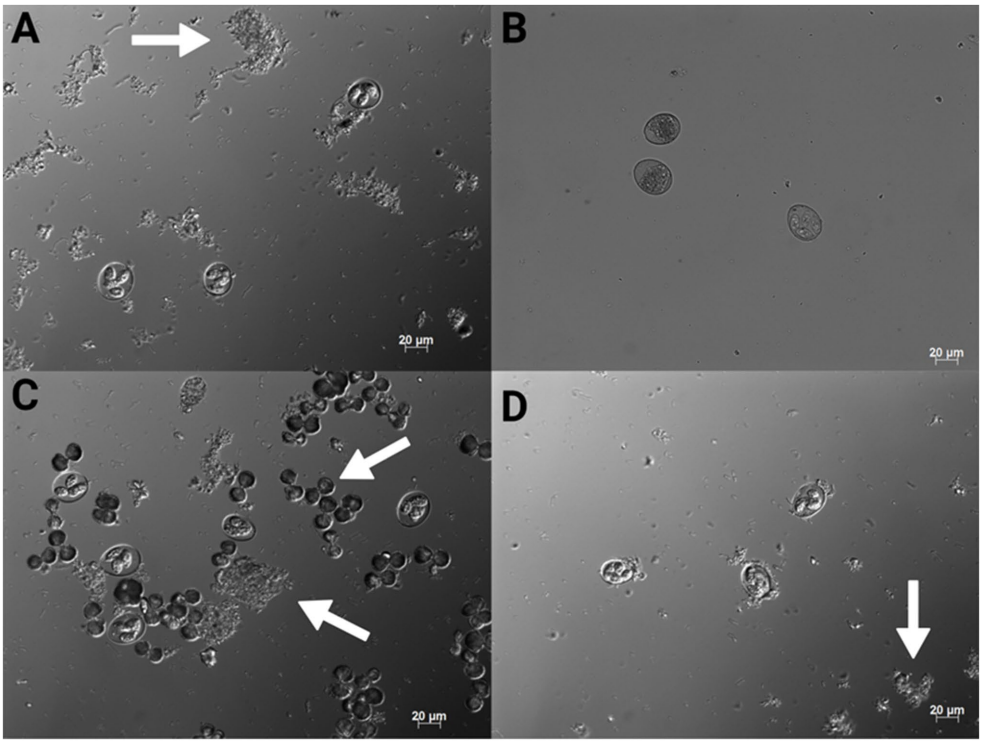

FIGURE 1
200x magnification conventional DIC photomicrographs of *Eimeria maxima* M6 stored in 0.9% saline **(A)**, 2.50% PDC **(B)**, CHX gluconate **(C)** and CHX digluconate **(D)**. The arrows indicate bacterial growth and also notable crystallization in **(C)** (Created with BioRender.com).

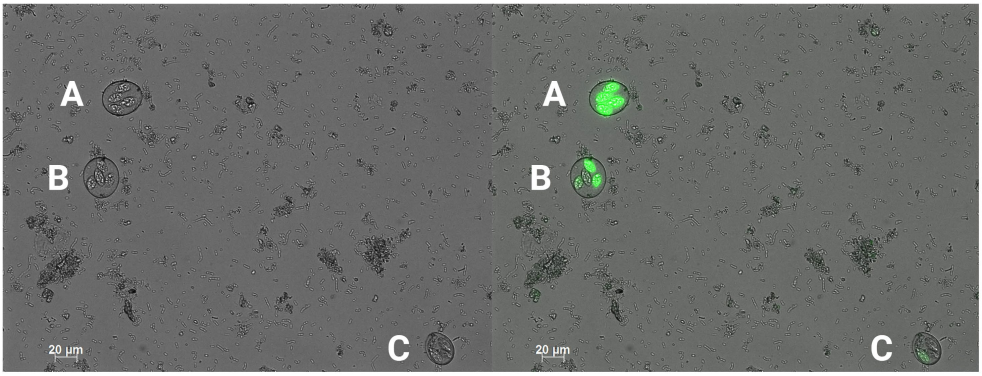

FIGURE 2
200x magnification conventional DIC photomicrograph (left) with a green fluorescent filter overlay (right). An example of a sporulated, fully autofluorescent oocyst (A), a sporulated, partially autofluorescent oocyst (B), and a sporulated, non-autofluorescent oocyst (C) (Created with BioRender.com).

Table 3 shows the effects of PDC, CHX gluconate, and CHX digluconate on EMM6 total oocyst concentration after 1d, 4d, or 6d of aeration *in vitro* assay 3. No significant differences in oocyst quantities were observed during the evaluation between groups (Table 3). However, oocysts evaluated in CHX digluconate exhibited poor oocyst wall integrity and were morphologically irregular compared to 2.50% PDC, saline, and CHX gluconate.

The effects of PDC, CHX gluconate, and CHX digluconate on EMM6 oocyst degradation, sporulation rate (%), and bacterial recovery after 4d and 6d of aeration for *in vitro* assay 4 have been presented in Table 4. At d4, a significant ($p < 0.05$) reduction in oocyst degradation was observed between CHX gluconate at 0.20% and CHX digluconate at 0.20% (Table 4). Sporulation (%) was significantly reduced with 2.50% PDC compared to the control saline solution. CHX digluconate at 0.25% significantly reduced bacterial recovery by 6.13 Log_{10} CFU/mL compared to the saline control. Furthermore, 2.50% PDC and 0.20% CHX gluconate completely inhibited bacterial growth (Table 4). At 6d, no significant differences were observed for oocyst degradation between all groups. Both 2.50% PDC and 0.20% CHX digluconate showed a significant reduction in sporulation (%)

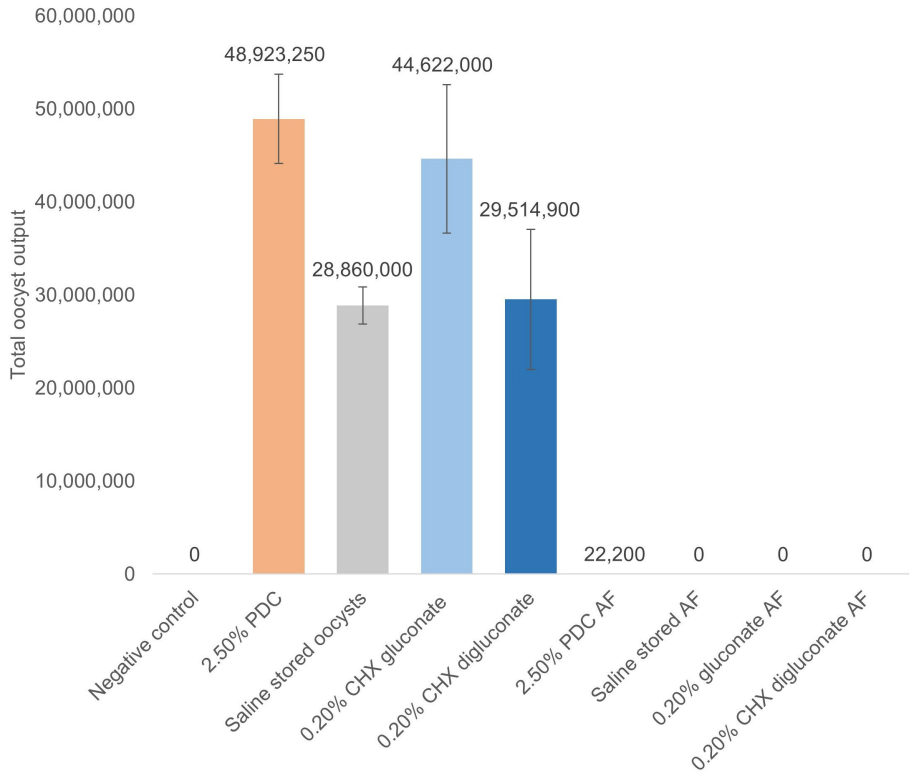

FIGURE 3
Total oocyst output from broilers challenged with non-autofluorescent or 100% autofluorescent (AF) EMM6 oocysts obtained from stocks stored for 5months at 4°C in potassium dichromate (PDC), chlorhexidine (CHX) gluconate, or CHX digluconate from d5-9 post-challenge. *N*=3 replicates/treatment group. Data express as mean±SE. $^{A-C}$ Differing letters indicate significant differences between treatment groups at $p< 0.05$. Means further separated using Student's *t* test. *In vivo trial*.

compared to CHX gluconate at 0.20%. Bacterial recovery was completely inhibited by 2.50% PDC, followed by CHX digluconate at 0.20% and CHX gluconate at 0.20% (Table 4).

The oocysts evaluated *in vitro* assay 4 were used for the inoculum in the *in vivo* study. Total oocyst output from broilers challenged with non-autofluorescent or 100% autofluorescent EMM6 oocysts obtained from stocks stored for 5 months at 4°C in 2.50% PDC, 0.20% CHX gluconate or 0.20% CHX digluconate has been presented in Figure 3. Total oocyst output was highest for chickens that received EMM6 oocysts stored for 5 months in 2.50% PDC, followed by CHX gluconate at 0.20%. There were no significant differences in total oocyst output post-challenge with the autofluorescent oocysts. Although not significant, there were oocysts recovered from two of the three replicates for the group that received the 100% autofluorescent oocysts stored in 2.50% PDC (Figure 3). This output could be due to sample contamination during handling and analysis, but additional studies to confirm these findings would be beneficial.

4. Discussion

Eimeria spp. oocysts have been traditionally stored in potassium dichromate (PDC), a compound that effectively eliminates microorganisms (4). However, toxic and carcinogenic properties of PDC have been described highlighting the need for a safer alternative for preserving *Eimeria* spp. oocysts (30). In the healthcare industry, chlorhexidine (CHX) salts are widely used as antiseptics and disinfectants. Disruption of membrane potential rather than ATPase inactivation is the primary method by which CHX is lethal to cells (31). CHX has been shown to have concentration-dependent antimicrobial effects on *E. coli* (32). The investigators demonstrated that the membrane integrity of *E. coli* was shown to decrease following treatment with 0.03 mmol/L of CHX. The internal cellular structure is damaged, possibly due to coagulation following biocidal CHX exposure suggesting that CHX could be used as a potential alternative to PDC.

In the present study, two independent *in vitro* fecal slurry assays demonstrated that 0.20% CHX gluconate or 0.25% CHX digluconate possessed greater antimicrobial activity than 2.50% PDC. Both 0.20% CHX gluconate and 0.25% CHX digluconate were more efficacious at each time point evaluated. By 116 h, there was no viable bacteria recovered from both CHX treatments and the 2.50% PDC control. At each time of analysis prior to 116 h, incidence of recovery was 0% for both 0.20% CHX gluconate and 0.25% CHX dicluconate apart from a 20% incidence at 75 h for 0.25% CHX digluconate. This demonstrates that CHX salts could not only successfully reduce the load at these concentrations, but also do so in a shorter period of time when compared to 2.50% PDC. On the other hand, while bacteria recovery after 4d of aeration at 26°C when EMM6 oocysts were treated with 2.50% PDC or 0.20% CHX gluconate were completely inhibited, 0.20% CHX digluconate was limited to a 6.13 log reduction of bacteria compared to the control saline solution. This could indicate a

difference in potency between CHX gluconate and CHX digluconate when used at the same concentration. Bacteria were not recovered at 6d of aeration from EMM6 oocysts stored in 2.50% PDC. Nevertheless, CHX gluconate or digluconate solutions showed a significant reduction in bacteria at 4.97 and 6.30 logs, respectively, compared to the control saline solution. None of the treatments impacted oocyst wall integrity after 1, 4, or 6d of incubation at 26°C or negatively impacted sporulation rates at 4 or 6 days of incubation compared to the PDC control in the present study. This indicates that CHX gluconate or digluconate could be used instead of PDC for *in vitro* sporulation of EMM6 oocysts, as they are both as effective, if not more effective, as PDC at reducing bacterial incidence and promoting sporulation *in vitro*.

PDC has been a reliable reagent for storing *Eimeria* oocysts for prolonged periods. Remarkably, 0.20% CHX gluconate was statistically comparable to 2.50% PDC in preserving oocyst infectivity during 5 months of *in vitro* storage at 4°C based on total oocyst output. Storage of EMM6 oocysts in 0.20% CHX digluconate markedly reduced total oocyst output compared to 2.50% PDC control and numerically lowered output compared to storage in 0.20% gluconate. This difference in oocyst output between CHX gluconate and CHX digluconate suggests that the CHX salt form can impact the viability of the oocysts during storage. Furthermore, these results show that CHX gluconate appears to be a better CHX salt for preserving EMM6 oocysts than CHX digluconate. The differences between the two salt forms and their effect on oocysts during long term cold storage should be evaluated. Although CHX diacetate results were not shown in the present manuscript, CHX diacetate degraded EMM6 oocysts and detrimentally affected sporulation of the remaining oocysts. Additionally, the pH did not appear to be a factor throughout the assay. This requires further investigation to better understand the mechanistic differences between the acetic acid and gluconic acid components of CHX salt forms. Perhaps the diacetate acts more readily on the oocyst wall components causing irreversible damage. CHX diacetate degraded EMM6 oocysts. The use of CHX diacetate for preserving oocysts should be avoided or investigated further.

As previously published (25), autofluorescence is a powerful tool in assessing the viability of *Eimeria* oocysts. Detectable autofluorescence in artificially aged oocysts further validates this method as an indicator for oocyst nonviability based on downstream total oocyst output. To effectively control and treat coccidiosis, it is essential to accurately determine the viability of *Eimeria* oocysts. A recent study in our laboratory showed that autofluorescence can distinguish viable from nonviable *Eimeria maxima* oocysts (25). Nonviable oocysts exhibit a strong autofluorescence signal, while viable oocysts exhibit a weak or absent signal. One of the key advantages of using autofluorescence to assess oocyst viability is that it is non-invasive and non-destructive. This means that oocysts can be assessed without being damaged, which is important when working with limited numbers of oocysts or when trying to maintain the integrity of oocyst populations for further study. This technique has the potential to greatly enhance our understanding of the biology and epidemiology of *Eimeria* and could help to improve the control and treatment of coccidiosis in a wide range of animal species (33, 34).

In general, the ideal preservation method should maintain oocysts' viability, infectivity, and antigenicity while minimizing toxicity and environmental impact. In summary, the results of this study suggest that 0.20% CHX gluconate is a promising and reliable alternative to PDC for storing *Eimeria* oocysts. CHX gluconate at 0.20% concentration was effective not only at killing bacteria, but also maintained oocyst wall integrity and infectivity while in storage at 4°C over 5 months. Furthermore, CHX salts are less toxic than PDC and have no known carcinogenic properties. The CHX salts are also less environmentally hazardous and can be disposed of safely. This manuscript successfully demonstrated that CHX salts, specifically CHX gluconate, could be a viable alternative to PDC for storing oocysts. However, further research is needed to determine whether CHX salts can alter the antigenicity of the oocysts. Overall, using CHX salts for storing *Eimeria* oocysts should be considered a safer and more environmentally friendly alternative to PDC.

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 the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville under protocol #22007.

Author contributions

JB, BH, GT-I, and BG conceptualized the study. LL, LB, KM, and MA-C handled the methodology. AF, MC, and CV oversaw the software. JB, GT-I, and BG validated the study. RS-C, IL, LG, and JL performed the formal analysis. BG and GT-I conducted the investigation. LL, BG, and GT-I prepared and wrote the original draft. XH-V, MJ-E, and GT-I contributed to review and editing of the manuscript. GT-I and BH were in charge of the project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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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. Cervantes HM, McDougald LR. Raising broiler chickens without ionophore anticoccidials. *J Appl Poult Res*. (2023) 32:100347. doi: 10.1016/j.japr.2023.100347

2. Conway DP, McKenzie ME. *Poultry coccidiosis: Diagnostic and testing procedures*. Ames, Iowa: Blackwell Publishing (2007). 164 p.

3. Felici M, Tugnoli B, Piva A, Grilli E. *In vitro* assessment of anticoccidials: methods and molecules. *Animals*. (2021) 11:1962. doi: 10.3390/ani11071962

4. Salama AA, Mostafa RE, Elgohary R. Effect of L-carnitine on potassium dichromate-induced nephrotoxicity in rats: modulation of PI3K/AKT signaling pathway. *Res Pharm Sci*. (2022) 17:153–63. doi: 10.4103/1735-5362.335174

5. Ornhan S, Turkmen R, Demirel HH, Akosman M, Fırat F. An investigation of the effect of chlorogenic acid on potassium dichromate-induced oxidative stress in rats. *Res Sq*. (2022) 39:989–97. doi: 10.21203/rs.3.rs-1231458/v1

6. Li MH, Ooi HK. Effect of chromium compounds on sporulation of *Eimeria piriformis* oocysts. *Exp Anim*. (2008) 57:79–83. doi: 10.1538/expanim.57.79

7. Rasoloson D, Shi L, Chong CR, Kafsack BF, Sullivan DJ. Copper pathways in plasmodium falciparum infected erythrocytes indicate an efflux role for the copper P-ATPase. *Biochem J*. (2004) 381:803–11. doi: 10.1042/BJ20040335

8. Weerasena OVDSJ, Amarasekara AS, Wijesundera RL. Fungicidal activity of synthetically modified cashew nut shell liquid. *J Nat Sci Counc Sri Lanka*. (1993) 21:253–8. doi: 10.4038/jnsfsr.v21i2.8109

9. Gong Z, Wei H, Chang F, Yin H, Cai J. Sporulation rate and viability of *Eimeria tenella* oocysts stored in potassium sorbate solution. *Parasitol Res*. (2021) 120:2297–301. doi: 10.1007/s00436-020-06792-3

10. Abbas RZ, Abbas A, Iqbal Z, Raza MA, Hussain K, Ahmed T, et al. *In vitro* anticoccidial activity of *Vitis vinifera* extract on oocysts of different *Eimeria* species of broiler chicken. *J Hell Vet Med Soc*. (2020) 71:2267–72. doi: 10.12681/jhvms.25071

11. Akinwumi KA, Oloyede ST, Eleyowo OO, Jubril AJ. Toxicological outcome of combined exposure to potassium dichromate and levonorgestrel in the kidney of female rats. *J Cancer Res Updates*. (2022) 11:33–8. doi: 10.30683/1929-2279.2022.11.05

12. Shaheen AM, Taha A, Salama A. Squalene is a potential hypothyroidism modulator: a study of its effect on the Keap1-Nrf2-ARE signaling pathway in potassium dichromate induced hypothyroidism model in rats. *Egypt J Chem*. (2022) 0:707–13. doi: 10.21608/ejchem.2022.125938.5591

13. Clemen-Pascual LM, Macahig RAS, Rojas NRL. Comparative toxicity, phytochemistry, and use of 53 Philippine medicinal plants. *Toxicol Rep*. (2022) 9:22–35. doi: 10.1016/j.toxrep.2021.12.002

14. 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

15. Olajide JS, Qu Z, Yang S, Oyelade OJ, Cai J. *Eimeria* proteins: order amidst disorder. *Parasit Vectors*. (2022) 15:38–16. doi: 10.1186/s13071-022-05159-0

16. Guimarães JS Jr, Bogado AL, da Cunha TC, Garcia JL. *In vitro* evaluation of the disinfection efficacy on *Eimeria tenella* unsporulated oocysts isolated from broilers. *Rev Bras Parasitol Vet*. (2007) 16:67–71.

17. Gadelhaq SM, Arafa WM, Abolhadid SM. *In vitro* activity of natural and chemical products on sporulation of *Eimeria* species oocysts of chickens. *Vet Parasitol*. (2018) 251:12–6. doi: 10.1016/j.vetpar.2017.12.020

18. Abniki R, Masoudi M, Tashakor A, Mansury D. Evaluating the effectiveness of chlorhexidine on gram-positive and gram-negative bacteria in Iran: a systematic review. *Res Sq*. (2023). doi: 10.21203/rs.3.rs-2410932/v1v1

19. Pawlowic MC, Vinayak S, Sateriale A, Brooks CF, Striepen B. Generating and maintaining transgenic *Cryptosporidium parvum* parasites. *Curr Protoc Microbiol*. (2017) 46:20B.2.1–20B.2.32. doi: 10.1002/cpmc.33

20. Lim KS, Kam PCA. Chlorhexidine-pharmacology and clinical applications. *Anaesth Intensive Care*. (2008) 36:502–12. doi: 10.1177/0310057X0803600404

21. Sharma C, Khajuria RR, Singh R. Salt water rinse and chlorhexidine against oral microbes. *J Adv Med Dent Sci Res*. (2022) 10:65–8. doi: 10.4103/JISPPD.JISPPD_299_16

22. Kamdem GSJN, Toukam M, Ntep DBN, Kwedi KGG, Brian NZ, Fokam ST, et al. Comparison of the effect of saline mouthwash versus chlorhexidine on oral flora. *Adv Oral Maxillofac Surg*. (2022) 6:100273. doi: 10.1016/j.adoms.2022.100273

23. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev*. (1999) 12:147–79. doi: 10.1128/CMR.12.1.147

24. Augustine PC. Effects of storage time and temperature on amylopectin levels and oocyst production of *Eimeria meleagrimitis* oocysts. *Parasitology*. (1980) 81:519–24. doi: 10.1017/s0031182000061904

25. Beer LC, Bielke LR, Barta JR, Faulkner OB, Latorre JD, Briggs WN, et al. Evaluation of auto fluorescent *Eimeria maxima* oocysts as a potential indicator of non-viability when enumerating oocysts. *Poult Sci*. (2018) 97:2684–9. doi: 10.3382/ps/pey124

26. Johnston WT, Shirley MW, Smith AL, Gravenor MB. Modelling host cell availability and the crowding effect in *Eimeria* infections. *Int J Parasitol*. (2021) 31:1070–81. doi: 10.1016/s0020-7519(01)00234-x

27. Hodgson JN. Coccidiosis: oocyst counting technique for coccidiostat evaluation. *Exp Parasitol*. (1970) 28:99–102. doi: 10.1016/0014-4894(70)90073-1

28. SAS Institute Inc. *SAS/share: 9.4 User's guide. 2nd* ed. Cary, NC: SAS Institute Inc. (2002). 112 p.

29. Zar JH. *Biostatistical analysis. 2nd* ed. Englewood Cliffs, NJ: Prentice Hall (1984). 718 p.

30. Chen F, Huang K, Qin S, Zhao Y, Pan C. Comparison of viability and infectivity of *Cryptosporidium parvum* oocysts stored in potassium dichromate solution and chlorinated tap water. *Vet Parasitol*. (2007) 150:13–7. doi: 10.1016/j.vetpar.2007.09.001

31. Kuyyakanond T, Quesnel LB. The mechanism of action of chlorhexidine. *FEMS Microbiol Lett*. (1992) 100:211–5. doi: 10.1111/j.1574-6968.1992.tb14042.x

32. Sheppard FC, Mason DJ, Bloomfield SF, Gant VA. Flow cytometric analysis of chlorhexidine action. *FEMS Microbiol Lett*. (1997) 154:283–8. doi: 10.1111/j.1574-6968.1997.tb12657.x

33. Miglianico M, Bolscher JM, Vos MW, Koolen KJ, de Bruijni M, Rajagopal DS, et al. Assessment of the drugability of initial malaria infection through miniaturized sporozoite assays and high-throughput screening. *Commun Biol*. (2023) 6:216. doi: 10.1038/s42003-023-04599-3

34. Rousseau A, Villena I, Dumètre A, Escotte-Binet S, Favennec L, Dubey JP. Evaluation of propidium monoazide–based qPCR to detect viable oocysts of *toxoplasma gondii*. *Parasitol Res*. (2019) 118:999–1010. doi: 10.1007/s00436-019-06220-1

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Identification of *Eimeria tenella* sporozoite immunodominant mimotopes by random phage-display peptide libraries–a proof of concept study

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Introduction: Coccidiosis, caused by parasites of numerous *Eimeria* species, has long been recognized as an economically significant disease in the chicken industry worldwide. The rise of anti-coccidian resistance has driven a search for other parasite management techniques. Recombinant antigen vaccination presents a highly feasible alternative. Properly identifying antigens that might trigger a potent immune response is one of the major obstacles to creating a viable genetically modified vaccine.

Methods: This study evaluated a reverse immunology approach for the identification of B-cell epitopes. Antisera from rabbits and hens inoculated with whole-sporozoites of *E. tenella* were used to identify Western blot antigens. The rabbit IgG fraction from the anti-sporozoite serum exhibited the highest reactogenicity; consequently, it was purified and utilized to screen two random Phage-display peptide libraries (12 mer and c7c mer). After three panning rounds, 20 clones from each library were randomly selected, their nucleotide sequences acquired, and their reactivity to anti-sporozoite *E. tenella* serum assessed. The selected peptide clones inferred amino acid sequences matched numerous *E. tenella* proteins.

Results and Conclusions: The extracellular domain of the epidermal growth factor-like (EGF-like) repeats, and the thrombospondin type-I (TSP-1) repeats of *E. tenella* micronemal protein 4 (EtMIC4) matched with the c7c mer selected clones CNTGSPYEC (2/20) and CMSTGLSSC (1/20) respectively. The clone CSISSLTHC that matched with a conserved hypothetical protein of *E. tenella* was widely selected (3/20). Selected clones from the 12-mer phage display library AGHTTQFNSKTT (7/20), GPNSAFWAGSER (2/20) and HFAYWWNGVRGP (8/20) showed similarities with a cullin homolog, elongation factor-2 and beta-dynein chain a putative *E. tenella* protein, respectively. Four immunodominant clones were previously selected and used to immunize rabbits. By ELISA and Western blot, all rabbit anti-clone serums detected *E. tenella* native antigens.

Discussion: Thus, selected phagotopes contained recombinant *E. tenella* antigen peptides. Using antibodies against *E. tenella* sporozoites, this study demonstrated the feasibility of screening Phage-display random peptide libraries for true immunotopes. In addition, this study looked at an approach for finding novel candidates that could be used as an *E. tenella* recombinant epitope-based vaccine.

KEYWORDS

Apicomplexa, 2nd-generation merozoites, reverse immunology, Et-MIC4, TRAP-family, Et EF-2, beta-dynein chain, ankyrin-repeat

Introduction

Avian coccidia belongs to the Eimeriidae family and the phylum Apicomplexa (1). *Eimeria tenella* is one of the most pathogenic species of avian coccidiosis, causing massive economic damage to the global poultry industry (2, 3). Several live vaccines consisting of either virulent or attenuated coccidian strains have been commercially developed in recent years (4). Live oocyst vaccines are a limited but useful option to prophylactic medicine; however, a recombinant vaccine with specific parasite antigens that develop strong protective coccidia-immunity would be preferable (5, 6). Several studies have identified potential protective antigens from *Eimeria* such as AMA1, EF-1α, EF-2, MIC-1, MIC-2, MIC-3, IMP-1, LDH1, SAG1, Gam22, Gam 56, Gam 82, Rhomboid-like Protein, Profilin and SO7, however, attempts to produce a successful commercial recombinant vaccine have been hindered until now (5–8).

Immune responses to *Eimeria* infections involve numerous aspects of innate and adaptive/acquired immunity (4). Although protective immunity to *Eimeria* includes both cellular and humoral immune pathways, it is commonly assumed that the primary role is based on a robust cell-mediated response, with antibodies presumably playing a minor role (4, 9). Nonetheless, it appears that antibodies play a significant role in protection under specific conditions (10–12). Class B epitopes have been found in all seven species of chicken coccidia, indicating that this antigen class may protect chickens from coccidiosis (8, 11, 13). As a result, an effective recombinant vaccine against coccidiosis should contain both lymphocyte type T and B antigens to elicit a successful cellular immune response (6–8). However, a better definition of protective B-cell epitopes from *Eimeria* species is still needed (14). It is critical to discover the involvement of specific proteins from different life-cycle stages of the *Eimeria*, possibly those engaged in the earliest steps of invasion and all those associated with pathogenesis and parasite survival (15, 16).

The main objective of epitope identification is to replace complex antigens used for immunization programs, antibody production, and even serodiagnosis (17–19). Additionally, with an appropriate methodology, it is easier to find a peptide that can induce either antibody production or T-cell induction (8, 11, 14, 20, 21).

Phage-display technology is a low-cost, high-performance screening approach for identifying peptides with high molecular affinity to specific antibodies (17, 22). Epitope discovery is critical in diagnostics, immunotherapy, drug discovery, and vaccine development (17, 22, 23). This methodology enables the selection of mimotopes, peptides that replicate a pathogen's native epitopes, even without prior knowledge of the natural ligand region (19). M13 Phage display vectors combine peptide gene sequences to coat protein genes, usually gIII or gVIII. Therefore, nucleotide sequencing of the phage DNA can be used to determine the amino acid sequence of the selected peptide (17). Rounds of screening (panning) can be used to select specific peptides with affinity to a ligand from Phage display libraries, exposing billions of peptides variants present in the libraries to an immobilized ligand and washing away unspecific phages, amplifying the bound phage by infecting *Escherichia coli* (17, 19).

The selection of peptides from random Phage display libraries by specific antibodies is an attractive strategy for the possible generation of pure epitope vaccines based on phagotopes (19, 21, 22). In recent years, screening Phage-display libraries with specific antibodies has become an attractive strategy for designing anticoccidial therapies to control this disease (9, 14, 24, 25).

Although monoclonal antibodies are excellent mimotope selectors (9, 14, 17, 26), polyclonal antibodies are generally favored because they are widely available and may find novel immunogenic epitopes (19, 22). Due to the pathogen's thousands of years of co-evolution to avoid the immune response, epitope selection with the host's polyclonal antiserum might be difficult (27, 28). An alternative approach to effective epitope identification is using antibodies generated in unnatural host species, where the hidden vital antigens for the natural host may be recognized, producing antibodies against them (18, 19, 21).

The phage display library technique is relatively new for peptide-based parasite vaccine development (18, 29), and it has never been used before in screening *E. tenella* sporozoite mimotopes for possible vaccinations against the disease. Therefore, in the present investigation, rabbit sera antibodies generated against sporozoites of *E. tenella* were used to screen two Phage display random peptides libraries in order to identify highly immunogenic epitopes involved in *E. tenella* infection. Using these heterologous antisera, we were able to identify highly immunoreactive sporozoite epitopes from *E. tenella*.

Materials and methods

Parasites

The wild-type strain of *E. tenella* used in this research was isolated from birds showing clinical signs of cecal coccidia on a broiler farm in Querétaro, Mexico's central state. The oocysts of *E. tenella* were purified using the method reported by Stephan et al. (30). Three-week-old Leghorn Specific Pathogen Free (SPF) chickens (SPAFAS Inc., Norwich, CT, United States) were used to propagate oocysts. Following established procedures, cecal oocysts were isolated, sporulated, and cleaned (31).

Sporozoites and second generation of merozoites antigens preparation

Sporocysts were released by vortexed sporulated oocysts (2.5×10^7/ml) at 2,000 X g using 1 mm diameter glass beads (Sigma-Aldrich, Inc., Burlington, MA, United States) for 1 min. A 50% Percoll gradient (density 1.13 g/mL, GE Healthcare, Piscataway, NJ, United States) was used to purify sporocysts (32). 1×10^7 purified sporocysts were

resuspended in an excystation medium and incubated at 42°C for 150 min. PBS (pH 7.4) with 0.75% (w/v) taurodeoxycholic acid (Sigma-Aldrich, Inc., Burlington, MA, United States) and 0.25% (w/v) trypsin from porcine pancreas Type II-S (Sigma-Aldrich, Inc., Burlington, MA, United States) comprised the excystation medium. Sporozoites (Sz) were washed and purified using a 60% Percoll gradient (density 1.13 g/mL, GE Healthcare, Piscataway, NJ, United States). Sporozoites were resuspended in sterile PBS for the immunization program, gradually frozen at −70°C at a 1°C/min rate, and then stored at −70°C until use.

Three 10-week-old hybrid pullets were gavage-inoculated with 5 × 10^5 sporulated oocysts of *E. tenella* and euthanized 112 h post-infection (PI) to collect second-generation merozoites (Mz). These birds' intestines were treated for merozoite isolation, as described previously by Liu et al. (33). The merozoites were purified using the method outlined by Geysen et al. (34). Each parasite (Sz and Mz) was suspended in sterile PBS with a protease inhibitor (cOmpleteTM Roche Applied Science, Mannheim, Germany). Five freeze/thaw cycles each disrupted both asexual zoite stages. The final suspension was centrifuged at 2,000 x g for 18 min at 4°C. The supernatants were collected, and the protein concentration was measured with the Bradford reagent (BioRad, Hercules, CA, United States) using a bovine serum albumin standard curve (Sigma-Aldrich, Inc., Burlington, MA, United States). Antigen suspensions were kept in 200 µL aliquots at −70°C until needed.

Rabbit and chicken antisera against whole sporozoites of *Eimeria tenella*

Two white New Zealand rabbits (2.5 kg) and two SPF White Leghorn chickens (1.1 kg) were immunized subcutaneously with 100 µg of purified whole sporozoites (5.3 × 10^6 sporozoites), diluted 1:1 with the IMS 1313 N VG PR nanoparticle adjuvant (Seppic Montanide™, France) in a total volume of 1 mL as previously was described by Juárez-Estrada et al. (35). Three further immunizations were given at two-week intervals. Both rabbits were euthanized by exsanguination 1 week after the last immunization. After clotting for 1 h at room temperature and overnight at 4°C, the blood was centrifuged at 2000 X g for 5 min, and serum samples were aliquoted and stored at −20°C until use. Both SPF Leghorn chickens were wing-bled, and serum was extracted by clotting it for 1 h at room temperature and overnight at 4°C, then centrifuged at 2000 X g for 5 min, aliquoted, and stored at −20°C until needed. According to the manufacturer's instructions, the rabbit serum's IgG fraction was purified by affinity chromatography using Sepharose 4 FF protein G (GE HLS Marlborough, Mass., United States).

Enzyme-linked immunosorbent assay (ELISA)

The reactogenicity of the bird's and rabbit's anti-sporozoite sera, the rabbit anticlone serum, and the purified rabbit IgG fraction to sporozoite and 2nd generation merozoite antigens were evaluated by ELISA, essentially, as Constantinoiu et al. (3) described it. 96-well microtiter plates (MaxiSorb, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 1 µg of sporozoite or merozoite antigen in 100 µL of carbonate buffer (0.1 M sodium bicarbonate and 0.1 M sodium carbonate buffer, pH 9.6). The control wells were incubated with only 100 µL of carbonate buffer. After four washes on a shaker with a saline solution (S) (120 mM NaCl, 25 mM Tris–HCl, pH 7.9) containing 1% Tween 20 (ST), non-specific binding sites were blocked by incubating for 1 h at 37°C in a static oven with 110 µL of 5% skim milk in ST (STM). Sera diluted in STM (1:10 and 1:100) were added to the test and control wells after four ST washes, and both were then incubated for 1 h at 37°C. Each plate included negative control sera from unimmunized SPF Leghorn chickens. Following incubation, the plates were washed four times with ST and incubated with their respective secondary antibody peroxidase conjugate diluted 1:2000 with STM (anti-rabbit, anti-chicken) (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, United States). After 1 h at 37°C, the plates were washed four times. The enzymatic reaction was developed by adding 100 µL of OPD chromogen (o-phenylendiamine dihydrochloride SIGMA, St. Louis MO, USA) at 5 µg/10 mL in citrate buffer (0.1 M citric acid, 0.1 M sodium citrate p.H. 4.5, and 20 µL of 30% hydrogen peroxide) for 10 min in a shaker under dark conditions. An ELISA microplate spectrophotometer (Epoch, BioTek, Winooski, VT, United States) was used to read the absorbance produced by substrate hydrolysis at 450 nm. All serum samples were examined in duplicate thrice times.

SDS-PAGE and Western blot

For SDS-PAGE analysis of both asexual zoite phases, 20 µg of each purified fraction was separated by 12% SDS-PAGE under standard reducing conditions. According to Constantinoiu et al. (36), the resolved proteins were stained with Coomassie brilliant blue (CBB) or electrotransferred on polyvinylidene difluoride membranes (PVDF) (Bio-Rad, Hercules, CA, USA). PVDF membranes were probed with anti-sporozoite of *E. tenella* sera from rabbits and Leghorn fowl. As a secondary antibody, horseradish peroxidase (HRP)-conjugated IgG goat anti-rabbit IgG, and (HRP)-conjugated IgG goat anti-chicken IgY (Jackson ImmunoResearch Laboratories, Philadelphia, PA, United States) were employed at a 1: 1500 (v/v) in 5% (w/v) STM. Tablets of DAB SigmaFast™ (3,3′-diaminobenzidine) (Sigma-Aldrich, St Louis MO, United States) at 10 mg/5 mL were used to visualize the PVDF membranes.

Screening of a phage-display library and characterization of selected clones

IgG fraction purified from rabbits previously immunized with sporozoites of *E. tenella* was used to screen commercially available dodecapeptide (Ph.D.-12TM) and cysteine-constrained heptapeptide (Ph.D.-C7CTM) M13 pIII libraries (New England Biolabs Inc., Ipswich, MA, United States). Three rounds of clone selection were performed for each screening using progressively decreasing quantities of antibodies (20 µg, 10 µg, and 5 µg, respectively). Briefly, two wells in a 96-well polystyrene microtiter plate (Maxisorb, Nunc, Roskilde, Denmark) were coated and incubated at 4°C overnight (O/N) with IgG in sterile phosphate chloride buffer (PCB) (0.1 M potassium chloride, 3 mM sodium chloride, 5 mM sodium phosphate dibasic, 1 mM potassium phosphate monobasic). Wells were washed five times with 0.1% (v/v) Tween 20 in 0.01 M phosphate-buffered

saline (TPBS), pH 7.2, then blocked with BSA (5 µg/mL in distilled H2O). Every plate was incubated for 1 h at room temperature with 1 × 10^{10} plaque-forming units (pfu) of each phage library. After washing with TPBS, the bound phages were eluted with 100 µL of 0.1 M glycine-HCl, pH 2.2 (hereafter referred to as "eluate") and immediately neutralized with 15 µL of 1 M Tris–HCl, pH 9.1. The eluate phages were amplified and titer through infection of the *E. coli* strain ER2738 (New England Biolabs Inc., Ipswich, MA, United States). The phages were titled as plaque-forming units by ml (pfu/ml). For the next round of phage-display screening, every phage-display library eluate was amplified and concentrated with polyethylene glycol-8000 PEG/2.5 M NaCl (20%/40%) following the manufacturer's instructions. The phage input and output titers ratio was used to calculate the specific phage enrichment level.

Phage clone selection and nucleotide sequence analysis

An aliquot of approximately 100 pfu from the third-round panning was combined with *E. coli* ER2738 and plated on LB agar with IPTG (isopropyl-β-D thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside). After 37°C O/N growth, twenty separate isolated phage plaques per library were randomly collected ($n = 40$). Each plaque was grown in 2XYT broth containing a dilution of 1/1000 of *E. coli* strain ER2738 grown O/N and incubated for 5 h at 37°C at 250 rpm. Following standard procedures, single-stranded phage DNA was isolated using an iodide buffer extraction, precipitated with 1 volume of ethanol, and resuspended in nuclease-free distilled water (Thermo Fisher Scientific Inc. Waltham, MA, United States). Purified phage DNA was sequenced with the Big Dye Terminator v1.1 cycle sequencing kit (Thermo Fisher Scientific, Waltham, MA, United States) using the pIII primer (5'-CCAGACGTTAGTAAATG-3′).

Bioinformatic analysis

Using the Clone Manager Professional Edition program, version 9.0 (Scientific and educational software, Cary, NC, United States), the amino acid sequences of the peptide were determined from the nucleotide sequences. The protein BLAST software[1] was used to screen the selected clones in the published sequences of the *Eimeria tenella* genome. The settings for the search were: Database: non-redundant protein sequence (nr); organism: *Eimeria tenella* (taxid: 5802); algorithm: blast (protein–protein BLAST).

Immune reactivity assessment of phage–displayed clones to rabbit and chicken anti-sporozoite serum by ELISA

ELISA reactogenicity to the selected phage clones was assessed using the pooled anti-sporozoite serum from both rabbits immunized with sporozoites of *E. tenella*. Briefly, phage–displayed clones were bound to microtiter plates (MaxiSorb, Nunc, Roskilde, Denmark) and developed with the anti-sporozoite sera diluted (1:10 and 1:100). Wild-type M13 phage and a clone of M13 phage (Ph.D. 12mer and c7c libraries) from screening with pseudorabies virus rabbit IgGs were used as negative controls. The total phage elution of the third round panning of both libraries was amplified and used as a positive control. After four washes with SS, the anti-sporozoite antibodies were detected using a secondary anti-rabbit IgG or anti-chicken IgY peroxidase conjugate diluted at 1:2,000 in blocking solution for 1 h at 37°C (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, United States). The plate was then washed four more times with SS before adding 100 µL of TMB chromogen at 1 mg/10 mL in citrate buffer (3,3′,5,5′-Tetramethylbenzidine, Sigma-Aldrich, Inc., Burlington, MA, United States), the addition of 2 N sulfuric acid stopped this reaction. An ELISA reader (Epoch, BioTek, Winooski, VT, United States) was used to determine the color intensity at 450 nm. Otherwise, the antisera reactivity of phage-immunized rabbits was determined in phage-ELISA format using 1 µg/well of *E. tenella* sporozoite and merozoite as antigens.

Rabbit antisera against each selected phage clone

Rabbit antisera for every one of the four most relevant phage clones were obtained. Candidate phage clones were selected based on the following criteria: (i) highest frequency selection during the screening process, and (ii) highest reactivity in the ELISA test (e.g., Ph.D. 12mer 15: HFAYWWNGVRGP; Ph.D. 12 mer 18: AGHTTQFNSKTT; Ph.D. c7c 1: CNTGSPYEC; Ph.D. c7c 7: CSISSLTHC). One rabbit by each clone was subcutaneously immunized with 1 mL containing 2 × 10^{12} pfu in sterile PBS, mixed 1:1 with the adjuvant ISA 50 V (Seppic Montanide ™, France). Six immunizations were applied at intervals of 2 weeks each. The immunological reactogenicity and specificity of every antiserum from phage-immunized rabbits were evaluated by ELISA and Western blot, as already described before.

Statistical analysis

Data were analyzed using the SAS/STAT 9.2 software (SAS Institute Inc., Cary, NC). Overall differences between groups were analyzed using ANOVA test. The mean value with respective standard deviation (SD) was calculated. A post-hoc analysis (Tukey's test at $p < 0.05$) was performed after the overall analysis showed significant differences between all groups.

Results

Characterization of rabbit and bird anti-sporozoite sera by ELISA

Time-course of the reactogenicity of the anti-sporozoite sera from rabbits and birds immunized with whole-sporozoites of *E. tenella* was evaluated. Figure 1 shows the kinetics of *E. tenella* anti-sporozoite sera

1 https://blast.ncbi.nlm.nih.gov/Blast.cgi

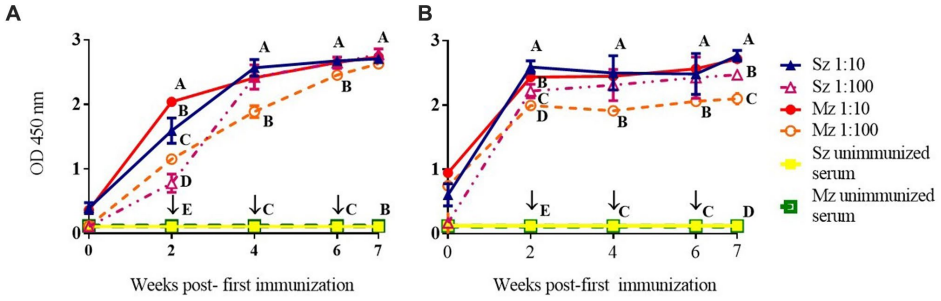

FIGURE 1
Immunization with whole-sporozoites of *E. tenella* to rabbits **(A)** and chickens **(B)**. The dynamics of antibody production in rabbits **(A)** and chickens **(B)** were assessed by ELISA against sporozoite (Sz) and second generation of merozoite (Mz) antigens, after immunization with sporozoites of *E. tenella*, at weeks 0, 2, 4, and 6 (↓). The rabbit and chicken antisera were diluted at 1:10 (▲) and 1:100 (△) againt Sz antigen, and the rabbit and chicken antisera were diluted at 1:10 (●) and 1:100 (○) against Mz antigen. Preimmune sera were diluted 1:10 and exposed to Sz (□) or Mz (■) antigens. Figures represent mean ODs (± SD). Different letters on the same data indicate statistical differences: $*p < 0.05$ (ANOVA followed by the Tukey post-hoc test).

production in rabbits (Figure 1A) and birds (Figure 1B), assessed by its reactivity to sporozoite and merozoite; we can observe that rabbit and bird antisera cross-react to both asexual zoite stages in a similar pattern. Similar levels of antibody production were observed in rabbits and chickens; however, bird titers reached a plateau after 2 weeks, whereas rabbit titers did not reach a plateau until 4 weeks. Even though only sporozoites were used for immunization schedule, the immune response to the second generation of merozoites exhibited similar reactivity to the sporozoite antigen and followed the same dynamic pattern, indicating the presence of the same or cross-reacted antigens between both asexual zoite stages. Antisera diluted 1:100 showed better reactivity against the sporozoite than the merozoite (Figure 1B). At 7 week post-first immunization titers in rabbits against both asexual zoite stages are more homogenous than titers observed in birds at this same time indicating more affinity from rabbit antibodies toward both antigens.

Reactivity in Western blot from two life-cycle stages of *Eimeria tenella* using anti-sporozoite sera

Reactogenicity of the anti-sporozoite sera from rabbits and birds immunized with whole-sporozoites of *E. tenella* against polypeptides of two asexual zoite stages of *E. tenella* was evaluated. Figure 2A shows the sporozoite and merozoite proteins separated on a 12% SDS-PAGE and stained with Coomassie brilliant blue. Although a complex protein profile of sporozoite and merozoite proteins was detected, it is possible to identify some common bands above 35 kDa. In the sporozoite, two specific distinct proteins below 25 kDa are evident, while in the merozoite, a band closer to 10 kDa is notoriously abundant. High molecular weight proteins seem shared among both asexual stages zoites but with some differences in concentration. The Western blot analysis with anti-sporozoite sera from rabbits (Figure 2B) and birds (Figure 2C) showed that both antisera recognized a similar polypeptide pattern in the sporozoite antigen, mainly polypeptides with molecular weight higher than 35 kDa, among which a 40 kDa protein is relevant. In the sporozoite, the most intense bands detected weighted 21 kDa, 23 kDa, and 24 kDa. In contrast, in the 2nd generation merozoite antigen, the antisera from rabbits or chickens indistinctly detected only a few high molecular weight antigens (approximately 70 kDa and 124 kDa). The most relevant here is that each rabbit antiserum had to be proportionally diluted four times more than the bird antiserums in order to obtain the clearest image of the PVDF membrane, which indicates a superlative reactogenicity of these antibodies toward both antigens subject to evaluation.

Phage-display library screening with rabbit purified IgGs

In order to select recombinant Phage-display clones with specific affinity for rabbit anti-sporozoite antibodies, the IgG fraction was purified from the rabbit's antisera by affinity chromatography with protein G sepharose. We diluted the purified IgG fraction from 30 µg/well to 0.23 µg/well to test by indirect ELISA their reactivity against the sporozoite or the 2nd generation of the Mz antigens. Figure 3 shows greater reactivity to the Sz antigen compared to the Mz antigen.

Using this same purified IgG fraction three screening rounds were performed for the 12-mer and cyclic 7-mer phage-display libraries. Table 1 shows the number of phage clones selected during each screening round. The selected phages of the 12-mer Ph.D. library increased from 2.0×10^5 pfu in the first round to 6.9×10^6 pfu in the third round. Similarly, phages obtained from the cyclic 7-mer Ph.D. library increased from 1.5×10^5 pfu in the first round to 2.2×10^7 pfu in the third round, indicating a selection of specific phage clones through the three screening rounds.

Characterization of the phage-display selected clones

After the third screening round of each Phage-display random peptide libraries, 20 single phage clones were randomly selected and amplified. DNA sequencing was used to examine all of the selected clones. Tables 2, 3 provide the deduced amino acid sequences of the selected clones. DNA sequencing of the 12-mer Ph. D. library-selected phage clones revealed two immunodominant clones with the AGHTTQFNSKTT (7 times) and HFAYWWNGVRGP (8 times)

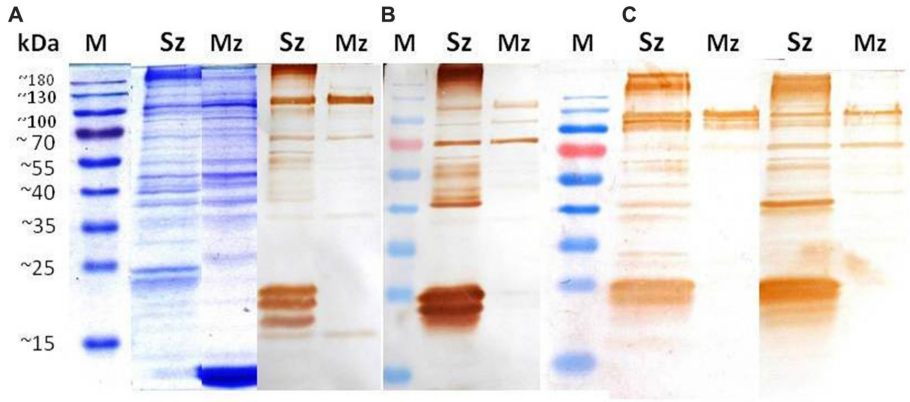

FIGURE 2
Identification of sporozoite and merozoite antigens by rabbit and chicken anti-sporozoite sera. Sporozoite (Sz) and merozoite (Mz) proteins were separated on a 12% SDS-PAGE and stained with Coomassie brilliant blue **(A)**. Western blot analysis with anti-sporozoite sera from two rabbits **(B)** and two birds **(C)**. The antisera were diluted 1/8000 in **(B)** and 1/2000 in **(C)**.

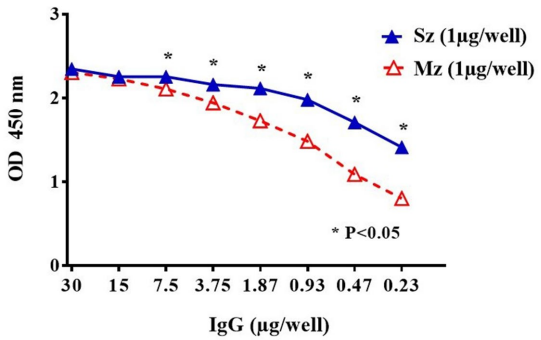

FIGURE 3
Indirect ELISA titration of purified rabbit anti-sporozoite IgGs to sporozoite (Sz ▲) and second generation of merozoite (Mz △). A two-fold IgG dilution was evaluated starting from 30 µg/well. Means in ODs (± SD) from duplicate wells are shown. Asterisks indicate statistical differences: *$p < 0.05$ (ANOVA, followed by Tukey post-hoc test).

peptide sequences. One clone with the amino acid sequence PNSAFWAGSER was obtained twice (Table 2). The clones selected from the cyclic 7-mer library were more diverse than those selected from the 12-mer library, four clones were selected twice, and one clone was selected three times (Table 3). All selected clones matched with one or two main proteins of *E. tenella* previously quoted in the NCBI gen bank. Some of these proteins are crucial in adhesion and invasion events of the sporozoite to the enterocyte, however, most of them have been described as hypothetical proteins.

Genome sequence analysis of the phage-display selected clones

The *E. tenella* genome was BLAST screened with the sequence of every selected peptides. The peptides obtained from the 12 mer library found clear identities (Table 2). The AGHTTQFNSKTT peptide matched with a Cullin homolog. It was found that the HFAYWWNGVRGP peptide has an identity with the Dynein beta chain. The phage clone sequence GPNSAFWAGSER showed three matches with the Elongation Factor 2 (EF-2). The peptides obtained from the cyclic 7-mer library also found matches in the *E. tenella* genome (Table 3). The CNTGSPYEC clone had ten matches with the extracellular domains of EtMIC4's epidermal growth factor-like repeats. Furthermore, the amino acid sequence of the phage clone CMSTGLSSC matched the thrombospondin type-I repeats of EtMIC4. In contrast, the CSISSLTHC clone showed several matches with a hypothetical protein conserved in *E. tenella*. Finally, as seen in Table 3, at least three 7-mer peptides share the CLK and KL motifs; interestingly, two of these phage clones showed the highest reactivity in the ELISA test (Figure 4C).

Reactivity of the phage-display selected clones to anti-sporozoite rabbit and bird sera

The anti-sporozoite sera from rabbits and Leghorn chicks were used to test the recognizing level of every Phage clone selected from each Phage display random peptide libraries. Figure 4 shows the antigen reactivity of the selected Phage clones to rabbit and bird anti-sporozoite sera evaluated by indirect ELISA. Most of the selected phage clones interacted specifically with anti-sporozoite sera and had greater OD values than negative control sera (Figure 4). Every Phage clone showed different level of reactogenicity toward anti-sporozoite sera. Although with some differences almost all Phage clones showed reactogenicity in the same order to both anti-sporozoite sera, it indicates that the sporozoite has immunodominant antigens recognized by both species.

Phage-display selected clones induce anti-sporozoite and anti-merozoite antibodies

If the peptide clones selected with the anti-sporozoite antibodies represent mimotopes present in *E. tenella* antigens, these phage clones will be able to induce antibodies against *E. tenella* antigens. In that

TABLE 1 Enrichment of phage clone output in three screening rounds.

	Ph.D. 12 mer (pfu/mL)			Ph.D. c7c (pfu/mL)		
Coating antibody	Eluted phage titer	Increase in phage titer (fold)	Amplified phage titer	Eluted phage titer	Increase in phage titer (fold)	Amplified phage titer
1st Pannig (10 µg/well)	2.0×10^5	1.0	3.0×10^{13}	1.5×10^5	1.0	4.8×10^{13}
2nd Pannig (5 µg/well)	2.8×10^6	14.0	3.0×10^{12}	3.3×10^5	2.2	5.0×10^{12}
3rd Panning (2.5 µg/well)	6.9×10^6	34.5	N.A.	2.2×10^7	146.7	N.A.

pfu plaque forming units; N.A. not amplified.

TABLE 2 Peptide deduced aminoacid sequences on phage clones selected from 12 mer after three screening rounds.

12-mer Phage selected clones	*AA sequence	Identified protein, sequence ID, residues length and number of matches	Position/protein coverage
1	LHRGNEAVYAWP	Hypothetical protein, conserved [*E. tenella*] Sequence ID: XP_013228393.1 Length: 1103 Matches: 1	Position 553/6/7 (86%)
2,7,8,12,14,18,20	AGHTTQFNSKTT	Cullin homog, putative [*E. tenella*] Sequence ID: XP_013232647.1 Length: 208 Matches: 1	Position 54/6/7 (86%)
3	NRPDSAQFWLHH	Hypothetical protein, conserved [*E. tenella*] Sequence ID: XP_013228556.1 Length: 9860 Matches: 3	Position 1,326/7/9 (78%) Position 812/6/14 (43%)
11,17	GPNSAFWAGSER	Hypothetical protein, conserved [*E. tenella*] Sequence ID: XP_013233745.1 Length: 1116 Matches: 3	Position 76/6/7 (86%) Position 984/5/8 (63%)
6	FPVNNMQLWQVT	Hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase, putative [*E. tenella*] Sequence ID: XP_013228556.1 Length: 9860 Matches: 3	Position 262/8/11 (73%)
4,5,9,10,13,15,16,19	HFAYWWNGVRGP	Dynein beta chain, flagellar outer arm, putative [*E. tenella*] Sequence ID: XP_013232219.1 Length: 959 Matches: 1	Position 523/5/5 (100%)

*Consensus amino acid residues are highlighted in bold letters. Position and protein coverage for AA sequence of every selected phage clone on NCBI standard protein BLAST protein►protein sequence of the *Eimeria tenella* taxid 5,802.

sense, four Phage clones were selected and used to immunize one rabbit each. Every anti-clone rabbit serum was used to assess the recognition of the sporozoite and merozoite native antigens. These four Phage clones were selected for rabbit immunization based on their (i) ELISA reactivity to anti-sporozoite antibodies, (ii) frequency of clone selection, and (iii) bioinformatic similarity with *E. tenella* proteins. In this way, phage clones 1 and 7 of the Ph.D. c7c library, and phage clones 15 and 18 of the Ph.D. 12 mer library were selected for rabbit immunization (Table 2). Figure 5 shows the time course reactogenicity of the antiserum from every rabbit immunized with the selected phage clones to both asexual zoite stages of *E. tenella*. Although with different time course reactivity against every life cycle of *E. tenella* antigen, overall the rabbit anti-clone serums showed high titer recognizing both antigens tested, it was observed a time course light tendency to recognice more to Mz than Sz antigen, however at last of the immunization shedule the reactogenicity of every anti-clone serum agains both antigens was indistinghishable.

Recognition of *Eimeria tenella* sporozoite and 2nd generation of merozoite antigens by anti-phage serums

The reactivity level and specific polypeptide recognition of every rabbit anti-phage serum to Sz and Mz antigens was investigated by Western blot. Figure 6 shows the reactivity of the anti-phage 1 (Figure 6C), anti-phage 7 (Figure 6D), anti-phage 15 (Figure 6E), and anti-phage 18 (Figure 6F) serums. Figure 6A shows both *E. tenella* asexual zoite stages protein profile and BSA resolved by 12% SDS-PAGE electrophoresis. It was a control group for every PVDF membrane studied. Figure 6B shows the reactivity of the antiserum pool from two rabbits immunized with wild-type M13 used as a negative control.

All anti-phage serums notably recognized both Sz and Mz antigens (Figure 6). The anti-M13 negative control sera recognized bands of approximately 38 kDa, 20 kDa, and 13 kDa in the Mz proteins and only a ≈25–27 kDa range band in the Sz extract. These bands were also detected in all blots tested with all anti-phage serums (Figure 6). The anti-phage 1 serum detected prominent bands of 78 kDa, 68 kDa, 48 kDa, and 40 kDa in the Sz proteins (Figure 6C, lane Sz). In addition, a pair of approximately 23 and ≈25–27 kDa antigens (as was already seen with M13 antiserum) were recognized. Two bands of 68 kDa and 60 kDa were recognized in the Mz proteins; both share the same molecular weight with antigens detected in the sporozoites. A band of 40 kDa was observed, which is also shared in the Sz antigens (Figure 6A).

The phage clone 7 antiserum (Figure 6D) detected at the Mz extract a pattern of bands closely similar to the anti-phage 1 serum. In contrast, very few bands were detected in the Sz proteins. A light band of 40 kDa (also present in the Mz) and a band of ≈25–27 kDa (present

TABLE 3 Peptide deduced aminoacid sequences on phage clones selected cyclic 7 mer library after three screening rounds.

Cyclic 7-mer Phage selected clones	*AA sequence	Identified protein, sequence ID, residues length and number of matches	Position/protein coverage
1,5	CNTGSPYEC	Microneme protein 4 [*E. tenella*] Sequence ID: CAC34726.2 Length: 2340 Matches: 10	Position 297, 991/5/8 (63%) Position 769, 810, 938, 124/6/9 (67%)
6	CMSTGLSSC	Thrombospondin type 1 domain-containing protein, putative [*E. tenella*] Sequence ID: XP_013235772.1 Length: 3774. Matches: 5	Position 3,575/7/9 (78%)
7,15,20	CSISSLTHC	Hypothetical protein, conserved [*E. tenella*] Sequence ID: XP_013233649.1 Length: 961. Matches: 3	Position 412/7/8 (88%)
8	CRSANIYTC	Hypothetical protein, conserved [*E. tenella*] Sequence ID: XP_013232311.1 Length: 1348. Matches: 1	Position 1,270/5/7 (71%)
9,16	CHPVSGQKC	Hypothetical protein ETH_00011430	Position 208;/6/7(86%)
10,18	**CLK**FWKPNC	Hypothetical protein, conserved [*E. tenella*] Sequence ID: XP_013228358.1 Length: 323 Matches: 1	Position 185/5/7(71%)
7′	**CLKL**GEKWC	Hypothetical protein ETH_00039830 [*E. tenella*] Sequence ID: XP_013231892.1 Length: 271 Matches: 1	Position 128/6/7 (86%)
10′	CA**KL**CLNNC	Asparaginyl-tRNA synthetase, putative [*E. tenella*] Sequence ID: XP_013227989.1 Length: 516 Matches: 1	Position 319/6/9 (67%)
11	CHQTKTKFC	Hypothetical protein ETH_00037435 [*E. tenella*] Sequence ID: XP_013229950.1 Length: 251 Matches: 1	Position 137/6/6 (100%)
13	CHNETQKMC	Dynein heavy chain 3, axonemal, related [*E. tenella*] Sequence ID: XP_013232337.1 Length: 1111 Matches: 1	Position 462/6/9 (67%)
17	CVGISALLC	Adenosine transporter, putative [*E. tenella*] Sequence ID: XP_013231508.1 Length: 443 Matches: 1	Position 197/7/9 (78%)
1′,3′	CPTNQHHLC	Regulator of chromosome condensation RCC1 (Precursor), related [*E. tenella*] Sequence ID: XP_013236104.1 Length: 812 Matches: 1	Position 26/6/8 (75%)
2′	CMNNFNITC	Hypothetical protein, conserved [*E. tenella*] Sequence ID: XP_013233414.1 Length: 1063 Matches: 1	Position 1/6/8 (75%)

*Consensus amino acid residues are highlighted in bold letters. Position and protein coverage for AA sequence of every selected phage clone on NCBI standard protein BLAST protein►protein sequence of the *Eimeria tenella* taxid 5,802.

in the anti-M13 sera). The 40 kDa antigen was detected in the Sz and Mz proteins by the anti-phage 1 and anti-phage 7 serums. Anti-phage 15 (Figure 6E) and anti-phage 18 (Figure 6F) serums detected a similar antigenic pattern in the Mz proteins and a pair of bands of approximately 68 kDa and 70 kDa. These bands were also detected in the Sz antigen. A specific band of 40 kDa was detected in the Sz and Mz proteins with the anti-phage 18. This antiserum recognized a prominent band of 45 kDa. This same antiserum also recognized bands of 34, 33, 27, 24, and 17 kDa in the Sz extract (Figure 6F, lane Sz). Although with different rank of reactivity and molecular mass, the four anti-phage clone serums recognized more bands in the Sz and Mz antigens than recognized by the sera from rabbits immunized with a wild-type M13 strain that was used as a negative control.

Discussion

Several studies have shown that chickens become clinically resistant to artificial trickle infections with *E. tenella* oocysts (37–39). When antisera of these chickens have been used to probe immunoblots on sporozoites and merozoites of *E. tenella*, a similar pattern of antigens has been identified on both asexual zoite stages (35, 36, 38). We discovered that rabbits and hens inoculated subcutaneously with a vaccine of complete *E. tenella* sporozoites induce a humoral response that detects the same particular antigens in both asexual life-cycle phases of *E. tenella*. Our Western blot observation is consistent with previous studies, showing that antibodies generated against one asexual zoite stage cross-react with antigens from another asexual zoite stage (10, 26).

Although rabbits and chickens responded equally to both *Eimeria* antigens, rabbit antisera showed a much greater antibody response to both antigens than antisera derived from SPF White Leghorn birds. Antiserum from a rabbit had to be diluted 1:8,000, whereas antiserum from a Leghorn chicken only had to be diluted 1/2,000. This has been done to improve the signal-to-noise ratio of the images of Western blot membranes. This is consistent with previous observations that sporozoites injected subcutaneously are more immunogenic than oocysts inoculated naturally (orally) to infected chickens (40). Furthermore, it has been shown that heterologous hosts, such in this case the rabbits, recognize more critical epitopes of the sporozoites than those recognized by natural hosts (40–42). In addition, it should be noted that the evolutionary distance between birds and rabbits permits the production of unique, specific antibodies against, for example, antigens of common pathogens in birds.

Pathogen-specific monoclonal or polyclonal antibodies can be employed in phage display combinatorial library screenings to find

recombinant specific peptides of immunodominant epitopes with high affinity to these antibodies. Due to the scarcity of information on the nature of *E. tenella* protective antigens, we used rabbit anti-sporozoite antibodies as a probe in our study to discover key immunogenic epitopes that could be protective against *E. tenella* infection (6, 8, 11). Indirect ELISA testing revealed that the majority of the 20 selected clones from each library strongly reacted to anti-sporozoite rabbit sera, showing that the selected recombinant peptide binds to a particular antibody against the *E. tenella* sporozoite. The DNA sequencing of the selected phage clones revealed that some phage clones were identical and had been selected many times. In the panning of random Phage-display libraries, the relative abundance of monospecific antibodies in a polyclonal mixture of an immune serum is expected to direct phage clone selection (17). The frequency of the selected recombinant peptides here suggests that these phagotopes are immunogenic and diverse. The peptides selected from the cyclic 7-mer library (suitable for selecting conformational epitope) were more varied than those obtained from the lineal 12-mer library.

Typically, many *Eimeria* antigens identified as vaccine candidates often play a role in host/parasitic interaction, most likely because these antigens are naturally exposed during parasite invasion/replication mechanisms and are thus accessible targets for the host immune response (7, 16, 33, 43–46). Proteins released from micronemes, organelles situated at the apical tip of apicomplexan parasites, whose contents are crucial for parasite gliding, motility, and adhesion to host cells, as well as entrance into infected cells, are among the most intensively studied (6, 16, 44, 47–49). In order to identify and determine the function of the target *Eimeria* antigens, Sasai et al. (50) employed monoclonal antibodies (mab's) against recombinant EtMIC2 protein. These mabs first detected the micronemes of the sporozoite and merozoite of *Eimeria* sp. In another study, Witcombe et al. (51) demonstrated that the persistence of maternal protection in offspring chicks was connected with the reactivity of protective maternal (polyclonal) IgY antibodies with a high-weight molecular TRAP (thrombospondin-related anonymous protein)-family microneme protein termed EmTFP250.

The epidermal growth factor-like modules (EGF-like) and thrombospondin type 1 domain (TSP-1) motifs are found in high repetition in the EmTFP250, a feature also observed in other Apicomplexa parasites such as Plasmodium and Toxoplasma (49). EmTFP250 is related to *E. tenella* microneme protein 4 (EtMIC4) (52), and it is believed that, as a protein complex together with EtMIC5, it plays a crucial function in host cell adhesion and invasion (53). Moreover, EtMIC4, unlike the other *E. tenella* microneme proteins studied to date, appears to be present both on the sporozoite surface and within the micronemes in a constitutive manner (47, 54, 55). EtMIC4 contains adhesive domains conserved in higher eukaryotes, which include the EGF-like and the TSP-1 motifs (52, 54, 55). Interestingly, the genomic screening of *E. tenella* NCBI-Blast with the Ph.D. c7c mer selected peptide sequences found multiple matches with the EGF-like and TSP-1 modules of EtMIC4. The results obtained in the present study suggest that the screening strategy of the Ph.D. c7c mer library increased the possibility of identifying conformational peptides with cysteine-restricted motifs, such as EGF-like and TSP-1 of EtMIC4 (54, 55). These findings demonstrated that these peptides were immunodominants when the *E. tenella* intact sporozoites were inoculated as an immunogen in an animal different from a natural host (40, 55).

Comparative analysis of the phagotope CSISSLTHC disclosed matches with a conserved hypothetical protein of *E. tenella*. Blake et al. (15) previously reported that at least 70% of *E. tenella* genes are currently classified as uncertain functions or are well characterized as conserved hypothetical proteins. Until recently, only a few genes of the conserved hypothetical proteins in *Eimeria* sp. have been examined and tested for function and immunogenicity (15). A single-family of two 7mer peptides share the consensus motif C-LKLxxxN-C; both were selected in the same phage-display random panning (Table 3), and the phage clone CAKLCLNNC showed the highest reactivity in the indirect ELISA, likely indicate that they are mimics of an immunodominant epitope of the *E. tenella* sporozoite.

The use of phage displays as a means of mapping epitopes in Apicomplexa could be an innovative strategy. However, the intricacy in the protein composition of every stage zoite of *E. tenella* makes mapping of epitopes represented by the mimotopes somewhat complicated (9, 44, 47). Further studies using random Phage display libraries involving heterologous antibodies against different life-cycle stages of *E. tenella* as ligands are required.

Liu et al. (33) analyzed the proteome of second-generation merozoites using antisera from chickens previously infected with *E. tenella*. However, these antibodies only detected a small number of microneme and merozoite surface antigens in addition to other housekeeping proteins like enolasa, beta-tubulin, and heat shock protein 70. Recent research by Liu et al. (45) used antisera from *E. tenella*, *E. acervulina*, and *E. maxima* to perform a Western blot of two-dimensional gels containing sporozoite proteins from every *Eimeria* species. Fifty-four immunodominant proteins were identified in *E. tenella*, and 18 ortholog proteins were identified among the three *Eimeria* species. Five of the 18 ortholog proteins shared sequence similarity of more than 93% and were identified as common immunodominant antigens; these proteins included elongation factor 2 (EF-2), ubiquitin-conjugating enzyme domain-containing protein (UCE), 14–3-3 protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and transhydrogenase.

Some of the *E. tenella* native protein sequences available in GenBank are similar to the deduced oligopeptide sequences of the phage clones selected from the Ph.D. 12 mer library; however, only two of them have been previously identified as immunodominant epitopes of the *E. tenella* sporozoites (6, 45). For example, the phagotope AGHTTQFNSKTT matched with cullin homolog putative protein of *E. tenella*, a protein that recruits particular targets to ubiquitin ligase in a multisubunit protein complex beneficial for ubiquitination (45, 56). This phagotope may be connected to UCE, a protein previously discovered as a common immunodominant antigen for *Eimeria* sp. by Liu et al. (45). Otherwise, the most reactive phagotope in the ELISA assay (Figure 4A) matched with dynein beta chain, flagellar outer arm, a putative protein of *E. tenella*, which creates force toward the minus ends of the microtubules in the inner membrane (44), the latter protein is essential for *Eimeria* invasion of enterocytes, while the former protein has already been identified as an immunodominant peptide by Liu et al. (44).

The phagotope GPNSAFWAGSER, which ranked second in indirect ELISA reactivity, matched with a putative protein that contain ankyrin-like repeats of *E. tenella*, in some Apicomplexa members this protein contributes to regulate the conoid stability, motility and cell invasion (57). There is no prior evidence that these proteins are involved in coccidiosis protection (6, 7). The phage clone

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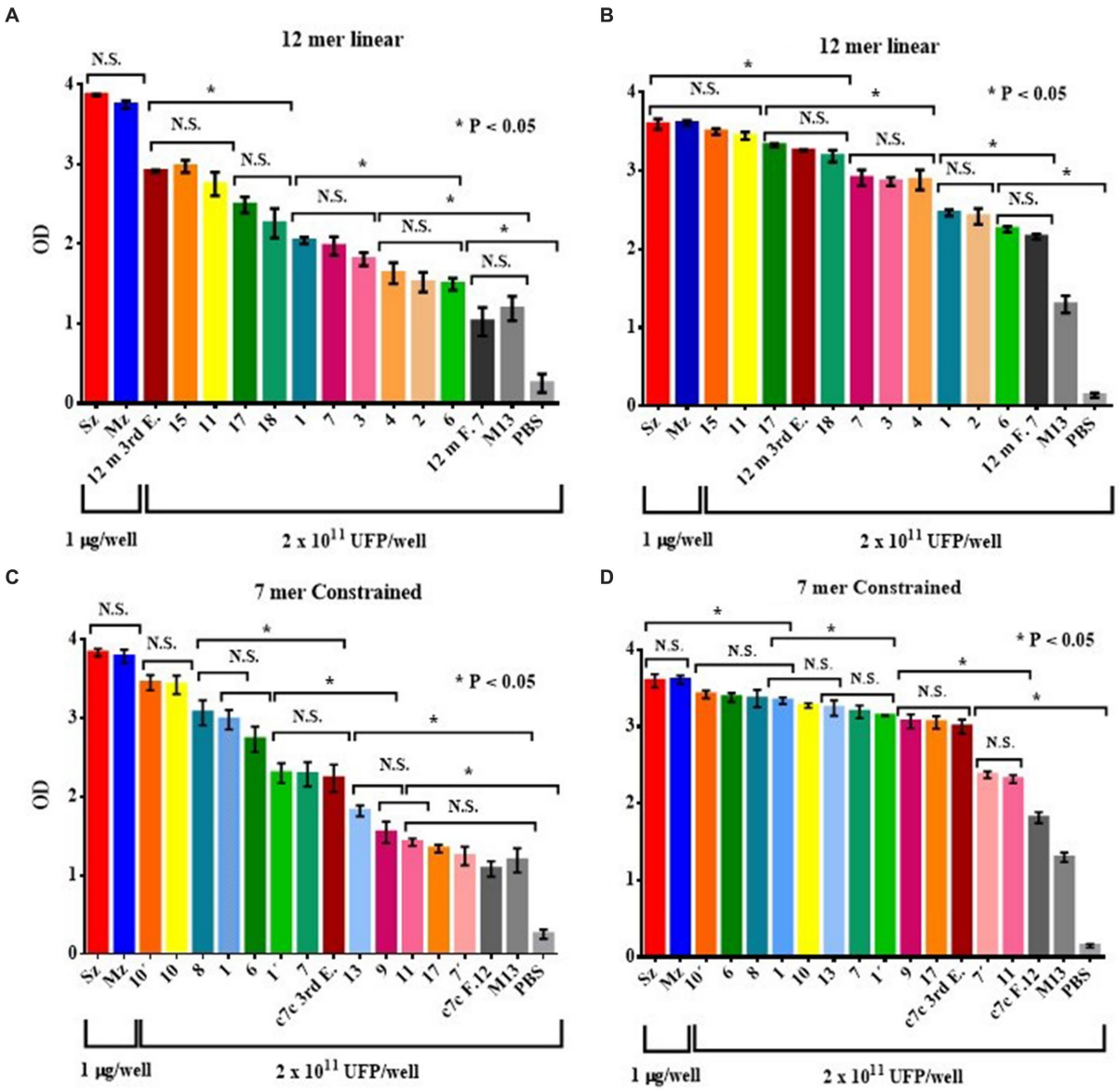

FIGURE 4
ELISA of the reactogenicity of rabbit **(A,C)** and chicken **(B,D)** anti-sporozoite sera to selected phage clones from the 12-mer **(A,B)** and c7c mer **(C,D)** libraries. The selected phage clones were tested by ELISA with rabbit and chicken anti-sporozoite sera. The wild-type phage M13mp19, an irrelevant phage clone for each library (selected with rabbit antisera anti-Pseudorabies virus, 12 m F.7 and c7c F.12), and PBS were used as negative controls. Reactivity of rabbit **(A)** and chicken antisera **(B)** against phage clones selected from the PhD 12-mer; rabbit antisera against selected clones from the c7c library **(C)**; and reactivity of the chicken antisera to selected clones from the c7c library **(D)** are shown. Bars represent mean ODs (± SD). NS, not significant. Asterisks indicate statistical differences: $*p < 0.05$ by ANOVA, applying the Tukey post-hoc test.

GPNSAFWAGSER was also related to the putative *E. tenella* elongation factor-2 (EtEF-2) protein, which, according to Liu et al. (45), is a common immunodominant antigen in *E. tenella*, *E. acervulina*, and *E. maxima*.

While this particular phage clone had not previously been characterized and tested as a vaccine candidate, Panebra and Lillehoj (58) recently tested a recombinant vaccine platform with the same type of protein (EaET-1) in a priming/challenge trial, with promising results for cross-protective immunity against *Eimeria* sp. Lately, Liu et al. (46) described the elongation factor 1-β, putative, partial (EF-1 β), another protein from this group, as an immunodominant antigen common to sporozoites of *E. acervulina*, *E. tenella*, and *E. necatrix*. These findings support the vaccination potential of different elongation factor antigens for coccidiosis and open up new avenues for developing multivalent vaccines against *Eimeria* sp., which is crucial for the poultry industry.

In the search for immunodominant antigens, the selected peptide clones are expected to represent epitope mimics or mimotopes present in *E. tenella* antigens (reviewed in Adda et al. [17]). One way to corroborate this is to obtain antibodies against these peptides and then evaluate their immune reactogenicity to native *E. tenella* antigens. Our ELISA and Western blot data demonstrated that each rabbit anti-phage serum successfully detected specific antigens in the *E. tenella* sporozoite and 2nd generation of merozoite antigens. The latter

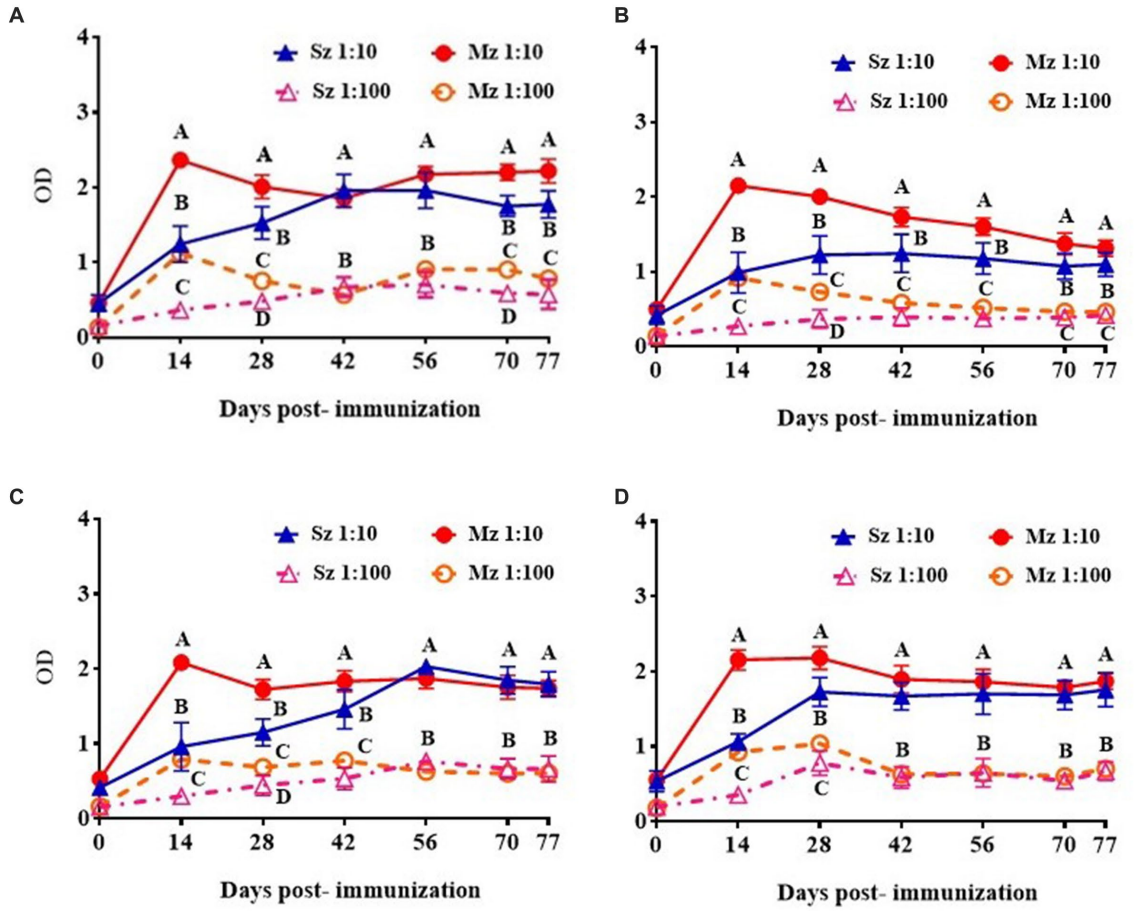

FIGURE 5
ELISA reactivity of rabbit antiserum of selected clones to sporozoite and second generation of merozoite antigens. Rabbit anti-phage clones serum was diluted 1/10 and 1/100 and tested against sporozoite and merozoite antigens. The antiserum response from different phage clones is shown: **(A)** Phage clone 1, C-NTGSPYE-C; **(B)** Phage clone 7, C-SISSLTH-C; **(C)** Phage clone 15, HFAYWWNGVRGP and **(D)** Phage clone 18, AGHTTQFNSKTT. Figures represent mean ODs values with respective ± SD. Different letters on the same date indicate statistical differences: $*p < 0.05$ (ANOVA followed by Tukey post-hoc test).

supports the idea that the selected peptides correspond to true mimotopes, evidencing the immunogenic and protective potential of these phagotopes.

Interestingly, these mimotopes are presumably present in more than one life-cycle stage of *E. tenella*, indicating that either every mimotope is expressed by both parasite stages or the antiserum recognizes more than one mimotope paralogue. Antiserums from rabbits immunized with phagotope CNTGSPYEC or AGHTTQFNSKTT recognized more antigens in the sporozoite than in merozoite. All of these recognized antigens concatenate well with antigens initially identified by rabbit antibodies used to screen both Ph.D. libraries (Figure 2B).

In the present study, the rabbit antiserums against both phagotopes recognized more than one antigen band, indicating that some proteins in the crude preparation of sporozoite and merozoite of *E. tenella* are under proteolytic activity like it would be doing *in vivo* infection (47). Typically, micronemal and rhoptry proteins are often proteolytically cleaved during biogenesis and post-exocytosis to allow sporozoite cell identification and attachment to host cell membranes (14, 44, 47). Fragments generated from each *E. tenella* denaturized protein (Sz and Mz) could be keeping the same epitopes in scattered small pieces of different molecular weights. Each of these fragments could be maintaining these epitopes similarly as they were in the original quaternary structure of *the E. tenella* native protein, which would explain this multivalent recognition. Another possible explanation is that the anti-clone serum cross-reacts with the same specific antigen located in both life-cycle phases of *E. tenella*, and this antigenic determinant is in several polypeptides with different molecular mass. Further identification of biological targets in the intact *E. tenella* sporozoites and 2nd generation of merozoites with every anti-clone serum might be illuminating.

Our observations confirm that mimotopes can produce native antigen-specific antibodies in active vaccination programs against distinct pathogen targets (19, 21, 22, 29). Curiously, different anti-phage serums reacted to the same prominent band in the sporozoite, which had a molecular weight of approximately ≈25–27 kDa (Figure 6). This antigenic band was also found in our immunostained membranes of Western blot probed with rabbit and chicken anti-sporozoite sera; however, this polypeptide was not observed in blottings of Sz and Mz antigens tested with antisera of chickens naturally (orally) immunized with *E. tenella* by Constantinoiu et al. (36), despite this band (≈25–27) was evident in their CBB stained gels.

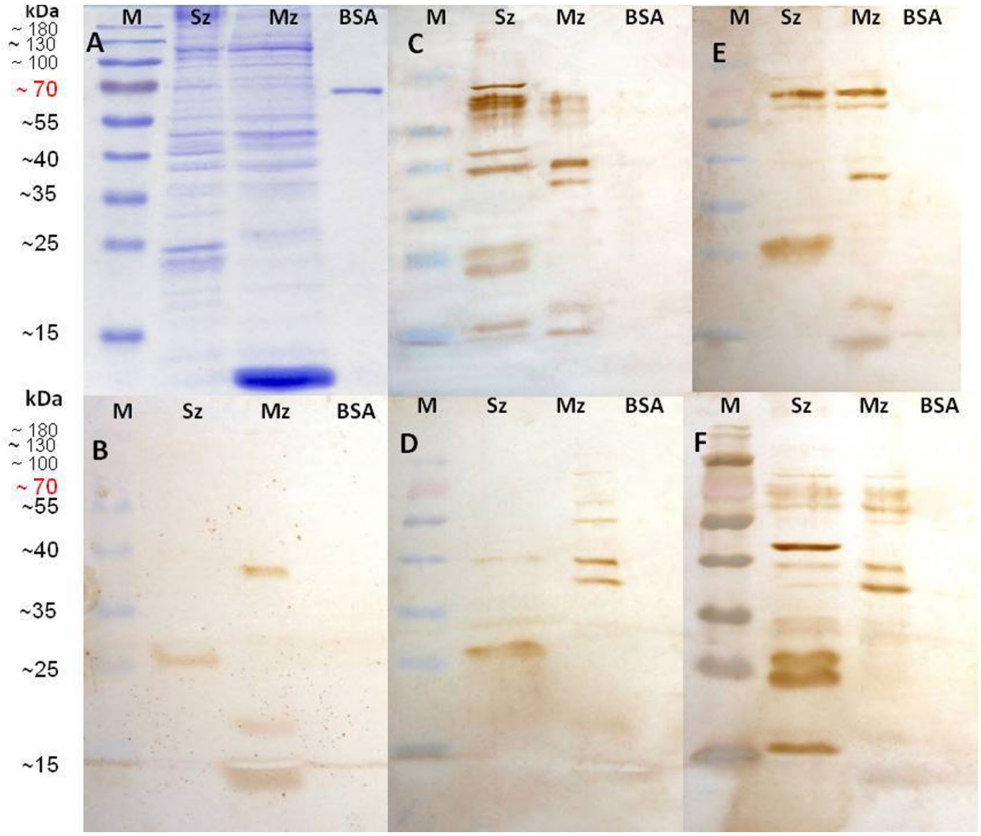

FIGURE 6
Identification of sporozoite and second generation of merozoite antigens by every rabbit anti-phage clone serum. Sporozoite (Sz), Merozoite (Mz), and Bovine Serum Albumin (BSA) proteins were separated on a 12% SDS-PAGE and stained with Coomassie brilliant blue **(A)**. Western blots are shown with wild-type M13 phage rabbit antisera used as negative control **(B)**; rabbit anti-phage clone 1 (C-NTGSPYE-C) serum **(C)**; rabbit anti-phage clone 7 (C-SISSLTH-C) serum **(D)**; rabbit anti-phage clone 15 (HFAYWWNGVRGP) serum **(E)**, and rabbit anti-phage clone 18 (AGHTTQFNSKTT) serum **(F)**.

In the early 1990s, surface antigens like TA4, a 25-kDa polypeptide encompassing 17 and 8 kDa, were identified (6, 7). TA4 was later identified as the sporozoite-specific glycosyl-phosphatidylinositol (GPI) anchored surface antigen (SAG)1, which can bind cultured epithelial cells and may play a role in parasite attachment to the enterocyte prior to invasion (59). When used as a recombinant protein immunogen, DNA vaccine, or a *Salmonella typhimurium*-vectored vaccine, SAG1 has been shown to induce partial protective immunity (6, 14, 60). Previously, using two-dimensional electrophoresis and mass spectrometry, de Venevelles et al. (43) identified a sporozoite antigen TA4 precursor with a predicted molecular weight of 25.04 kDa but an experimental molecular weight of 26.86 kDa, which is more similar to the molecular mass of the band that we identified here. The latter helps us better understand the changes reported by several researchers throughout time regarding the specific molecular weight of this highly immunogenic peptide observed in our blots. In the meantime, further studies are being conducted to evaluate the immunogenicity of all these characterized phagotopes in priming/challenge trials.

Conclusion

Both phage display libraries used here successfully discovered novel candidates of B- cells antigens from *E. tenella*. This attractive reverse immunology approach leads to identifying high immunogenic *E. tenella* sporozoite mimotopes and their coding DNA sequence. The isolated clones revealed relatedness to dynein beta chain, flagellar outer arm putative, cullin homolog putative, elongation factor 2, microneme 4 protein of *E. tenella*, and a conserved hypothetical protein of *E. tenella*. The phagotopes were recognized by antisera from rabbits immunized with complete sporozoites of *E. tenella*, indicating their potential use as immunotopes.

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 FMVZ-UNAM's Institutional Review Board for Husbandry and Care in Animals (CICUA) examined and authorized the animal study through the Ph.D. internal board.

Author contributions

MJ-E and RA-M: conceptualization, methodology, software, and writing original draft preparation. MJ-E, GT-I, DG, and AG-V: visualization and investigation. MJ-E, LL, and GT-I: data curation and statistical analysis. MJ-E, GT-I, and RA-M: funding acquisition, reviewing, and editing. GT-I and RA-M: supervision. All authors contributed to the article and approved the submitted version.

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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.

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References

1. Ogedengbe ME, El-Sherry S, Ogedengbe JD, Chapman HD, Barta JR. Phylogenies based on combined mitochondrial and nuclear sequences conflict with morphologically defined genera in the eimeriid coccidia (Apicomplexa). *Int J Parasitol*. (2018) 48:59–69. doi: 10.1016/j.ijpara.2017.07.008

2. Blake DP, Knox J, Dehaeck B, Huntington B, Rathinam T, Ravipati V, et al. Re-calculating the cost of coccidiosis in chickens. *Vet Res*. (2020) 51:115. doi: 10.1186/s13567-020-00837-2

3. Constantinoiu CC, Molloy JB, Jorgensen WK, Coleman GT. Development and validation of an ELISA for detecting antibodies to *Eimeria tenella* in chickens. *Vet Parasitol*. (2007) 150:306–13. doi: 10.1016/j.vetpar.2007.09.019

4. Dalloul RA, Lillehoj HS. Poultry coccidiosis: recent advancements in control measures and vaccine development. *Expert Rev Vaccines*. (2006) 5:143–63. doi: 10.1586/14760584.5.1.143

5. Blake DP. *Eimeria* genomics: where are we now and where are we going? *Vet Parasitol*. (2015) 212:68–74. doi: 10.1016/j.vetpar.2015.05.007

6. Blake DP, Pastor-Fernández I, Nolan MJ, Tomley FM. Recombinant anticoccidial vaccines–a cup half full? *Infect Gen Evol*. (2017) 55:358–65. doi: 10.1016/j.meegid.2017.10.009

7. Venkatas J, Adeleke MA. A review of *Eimeria* antigen identification for the development of novel anticoccidial vaccines. *Parasitol Res*. (2019) 118:1701–10. doi: 10.1007/s00436-019-06338-2

8. Yang X, Song X, Liu J, Chen Q, An T, Liu Q. Protection of hatchlings against coccidiosis by maternal antibodies to four recombinant proteins of *Eimeria tenella*, *Eimeria acervulina* and *Eimeria maxima*. *Vet Parasitol*. (2022) 312:109813. doi: 10.1016/j.vetpar.2022.109813

9. Krücken J, Hosse RJ, Mouafo AN, Entzeroth R, Bierbaun S, Marinovski P, et al. Excystation of *Eimeria tenella* sporozoites impaired by antibody recognizing gametocyte oocyst antigens GAM22 and GAM 56. *Eukaryot Cell*. (2008) 7:202–11. doi: 10.1128/EC.00292-07

10. Danforth HD, McAndrew SJ. Hybridoma antibody characterization of stage-specific and stage-cross reactive antigens of *Eimeria tenella*. *J Parasitol*. (1987) 73:985–92. doi: 10.2307/3282522

11. Wiedmer S, Alnassan AA, Volke B, Thabet A, Daugschies A, Lendner M, et al. Passive immunization with *Eimeria tenella* gametocyte antigen 56 (EtGam56) specific antibodies and active immunization trial with the epitope containing peptide. *Vet Parasitol*. (2017) 247:100–7. doi: 10.1016/j.vetpar.2017.09.019

12. Juárez-Estrada MA, Tellez-Isaias G, Sánchez-Godoy FD, Alonso-Morales RA. Immunotherapy with egg yolk *Eimeria* sp.-specific immunoglobulins in SPF Leghorn chicks elicits successful protection against *Eimeria tenella* infection. *Front Vet Sci*. (2021) 8:758379. doi: 10.3389/fvets.2021.758379

13. Wallach M. Role of antibody in immunity and control of chicken coccidiosis. *Trends Parasitol*. (2010) 26:382–7. doi: 10.1016/j.pt.2010.04.004

14. Jahn D, Matros A, Bakulina AY, Tiedemann J, Shubert U, Giersberg M, et al. Model structure of the immunodominant surface antigen of *Eimeria tenella* identified as a target for sporozoite neutralizing monoclonal antibody. *Parasitol Res*. (2009) 105:655–68. doi: 10.1016/j.peptides.2009.09.019

15. Zhai Q, Huang B, Dong H, Zhao Q, Zhu S, Liang S, et al. Molecular characterization and immune protection of a new conserved hypothetical protein of *Eimeria tenella*. *PLoS One*. (2016) 11:e0157678. doi: 10.1371/journal.pone.0157678

16. Burrell A, Tomley FM, Vaughan S, Marugan-Hernandez V. Life cycle stages, specific organelles and invasion mechanisms of *Eimeria* species. *Parasitology*. (2020) 147:263–78. doi: 10.1017/S0031182019001562

17. Adda CG, Andeers RF, Tilley I, Foley M. Random sequence libraries displayed on phage: identification of biologically important molecules. *Comb Chem High Throughput Screen*. (2002) 5:1–14. doi: 10.2174/1386207023330561

18. Ellis SE, Newlands GFJ, Nisbet AJ, Matthews JB. Phage-display library biopanning as a novel approach to identifying nematode vaccine antigens. *Parasite Immunol*. (2012) 34:285–95. doi: 10.1111/j.1365-3024.2011.01317.x

19. Gazarian K, Gazarian T, Sánchez BJ, Alonso MR. Immunogenic peptides from phage display libraries with potential of protecting mice against the pseudorabies virus. *Vet Microbiol*. (2011) 154:29–36. doi: 10.1016/j.vetmic.2011.06.020

20. Hernández-Chiñas U, Gazarian T, Gazarian K, Mendoza-Hernández G, Xicohtencatl-Cortes J, Eslava C. Peptide sequences identified by phage display are immunodominant functional motifs of pet and pic serine proteases secreted by Escherichia coli and *Shigella flexneri*. *Peptides*. (2009) 30:2127–35. doi: 10.1016/j.peptides.2009.09.019

21. Prudencio CR, Marra AOM, Cardozo R, Goulart LR. Recombinant peptides as new immunogens for the control of the bovine tick, *Rhipicephalus* (Boophilus) *microplus*. *Vet Parasitol*. (2010) 172:122–31. doi: 10.1016/j.vetpar.2010.04.012

22. Denisova G, Denisov D, Evelegh C, Weissgram M, Beck J, Foley SR, et al. Characterizing complex polysera produced by antigen-specific immunization through the use of affinity-selected mimotopes. *PLoS One*. (2009) 4:e5309. doi: 10.1371/journal.pone.0005309

23. Pande J, Szewczyk MM, Ashok K, Grover A. Phage display: concept, innovations, applications and future. *Biotech Adv*. (2010) 28:849–58. doi: 10.1016/j.biotechadv.2010.07.004

24. Abi-Ghanem D, Waghela SD, Caldwell DJ, Danforth HD, Berghman LR. Phage display selection and characterization of single-chain recombinant antibodies against *Eimeria tenella* sporozoites. *Vet Immunol Immunopathol*. (2008) 121:58–67. doi: 10.1016/j.vetimm.2007.08.005

25. Zhao Y, Amer S, Wang J, Wang C, Gao Y, Kang G, et al. Construction, screening and identification of a phage display antibody library against the *Eimeria acervulina* merozoite. *Biochem Biophys Res Commun*. (2010) 393:703–7. doi: 10.1016/j.bbrc.2010.02.063

26. Ko C, Smith CK 2nd, McDonel M. Identification and characterization of a target antigen of a monoclonal antibody directed against *Eimeria tenella* merozoites. *J Mol Bioch Parasitol*. (1990) 41:53–64. doi: 10.1016/0166-6851(90)90096-5

27. Deitsch KW, Lukehart SA, Stringer JR. Common strategies for antigenic variation by bacterial, fungal and protozoan pathogens. *Nat Rev Microbiol*. (2009) 7:493–503. doi: 10.1038/nrmicro2145

28. DosReis GA. Evasion of immune responses by *Trypanosoma cruzi*, the etiological agent of Chagas disease. *Braz J Med Biolog Res*. (2011) 44:84–90. doi: 10.1590/s0100-879x2011007500005

29. Costa LE, Goulart LR, Pereira NC, Lima MI, Duarte MC, Martins VT, et al. Mimotope-based vaccines of *Leishmania infantum* antigens and their protective efficacy against visceral leishmaniasis. *PLoS One*. (2014) 9:e110014. doi: 10.1371/journal.pone.0110014

30. Stephan B, Rommel M, Daugshies A, Haberkorn A. Studies of resistance to anticoccidials in *Eimeria* field isolates and pure *Eimeria* strains. *Vet Parasitol*. (1997) 69:19–29. doi: 10.1016/s0304-4017(96)01096-5

31. Tomley F. Techniques for isolation and characterization of apical organelles from *Eimeria tenella* sporozoites. *Methods*. (1997) 13:171–6. doi: 10.1006/meth.1997.0509

32. Dulski P, Turner M. The purification of sporocysts and sporozoites from *Eimeria tenella* oocysts using percoll density gradients. *Avian Dis*. (1988) 32:235–9. doi: 10.2307/1590810

33. Liu L, Xu L, Yan F, Yan R, Song X, Li X. Immunoproteomic analysis of the second generation merozoite proteins of *Eimeria tenella*. *Vet Parasitol*. (2009) 164:173–82. doi: 10.1016/j.vetpar.2009.05.016

34. Geysen J, Ausma J, Van den Bossche H. Simultaneous purification of merozoites and schizonts of *Eimeria tenella* (Apicomplexa) by percoll flotation and assessment of cell viability with a double fluorescent dye assay. *J Parasitol*. (1991) 77:989–93. doi: 10.2307/3282754

35. Juárez-Estrada MA, Gayosso-Vázquez A, Tellez-Isaias G, Alonso-Morales RA. Protective immunity induced by an *Eimeria tenella* whole sporozoite vaccine elicits specific B-cell antigens. *Animals*. (2021) 11:1344. doi: 10.3390/ani11051344

36. Constantinoiu CC, Molloy JB, Jorgensen WK, Coleman GT. Antibody response against endogenous stages of an attenuated strain of *Eimeria tenella*. *Vet Parasitol*. (2008) 154:193–204. doi: 10.1016/j.vetpar.2008.03.029

37. Long PL, Johnson J, McKenzie ME, Perry E, Crane MS, Murray PK. Immunization of young broiler chickens with low level infections of *Eimeria tenella*, *E. acervulina*, or *E. maxima*. *Avian Pathol*. (1986) 15:271–8. doi: 10.1080/03079458608436287

38. Constantinoiu CC, Molloy JB, Jorgensen WK, Coleman GT. Characterization of the antibody response in birds following infection with wild-type and attenuated strains of *Eimeria tenella* and *Eimeria necatrix*. *Vet Parasitol*. (2011) 175:47–51. doi: 10.1016/j.vetpar.2010.09.025

39. Soutter F, Werling D, Tomley FM, Blake DP. Poultry coccidiosis: design and interpretation of vaccine studies. *Front Vet Sci*. (2020) 7:101. doi: 10.3389/fvets.2020.00101

40. Talebi A. Inhibition of *Eimeria acervulina* sporozoite invasion by rabbit and chicken antisera using ISI assay. *Arch Razi Inst*. (2001) 52:49–59.

41. Talebi A, Mulcahy G. High-resolution mapping of B-cell epitopes within an antigenic sequence from *Eimeria tenella*. *Infect Immun*. (1994) 62:4202–7. doi: 10.1128/iai.62.10.4202-4207.1994

42. Talebi A, Mulcahy G. Cross-reactivity among antisera raised against five avian *Eimeria* species in the natural host and in rabbits. *Avian Pathol*. (1995) 24:533–44. doi: 10.1080/03079459508419092

43. de Venevelles P, Chich JF, Faigle W, Loew D, Labbé M, Girard-Misguich F, et al. Towards a reference map of *Eimeria tenella* sporozoite proteins by two-dimensional electrophoresis and mass spectrometry. *Int J Parasitol*. (2004) 34:1321–31. doi: 10.1016/j.ijpara.2004.08.002

44. Besteiro S, Dubremetz JF, Lebrun M. The moving junction of apicomplexan parasites: a key structure for invasion. *Cell Microbiol*. (2011) 13:797–805. doi: 10.1111/j.1462-5822.2011.01597.x

45. Liu L, Huang X, Liu J, Li W, Ji Y, Tian D, et al. Identification of common immunodominant antigens of *Eimeria tenella*, *Eimeria acervulina* and *Eimeria maxima* by immunoproteomic analysis. *Oncotarget*. (2017) 8:34935–45. doi: 10.18632/oncotarget.16824

46. Liu J, Tuo W, Wu X, Xiong J, Yu E, Yin C, et al. Immunoproteomic and mass spectrometric analysis of *Eimeria acervulina* antigens recognized by antisera from chickens infected with *E. acervulina*, *E. tenella* or *E. necatrix*. *Parasit Vectors*. (2020) 13:93. doi: 10.1186/s13071-020-3965-y

47. Dowse T, Soldati D. Host cell invasion by the apicomplexans: the significance of microneme protein proteolysis. *Curr Op in Microb*. (2004) 7:388–96. doi: 10.1016/j.mib.2004.06.013

48. Lamarque M, Besteiro S, Papoin J, Roques M, Vulliez-Le Normand B, Morlon-Guyot J, et al. The RON2-AMA1 interaction is a critical step in moving junction-dependent invasion by apicomplexan parasites. *PLoS Pathog*. (2011) 7:e1001276. doi: 10.1371/journal.ppat.1001276

49. Morahan BJ, Wamg L, Coppel RL. No TRAP, no invasion. *Trends Parasitol*. (2009) 25:77–84. doi: 10.1016/j.pt.2008.11.004

50. Sasai K, Fetterer RH, Lillehoj H, Matsuura S, Constantinoiu CC, Matsubayashi M, et al. Characterization of monoclonal antibodies that recognize the *Eimeria tenella* microneme protein MIC2. *J Parasitol*. (2008) 94:1432–4. doi: 10.1645/GE-1558.1

51. Witcombe DM, Ferguson DJP, Belli SI, Wallach MG, Smith NC. *Eimeria maxima* TRAP family protein EmTFP250: subcellular localisation and induction of immune responses by immunization with a recombinant C-terminal derivative. *Int J Parasitol*. (2004) 34:861–72. doi: 10.1016/j.ijpara.2004.03.006

52. Periz J, Ryan R, Blake DP, Tomley FM. *Eimeria tenella* microneme protein EtMIC4: capture of the full-length transcribed sequence and comparison with other microneme proteins. *Parasitol Res*. (2009) 104:717–21. doi: 10.1007/s00436-008-1301-0

53. Periz J, Gill AC, Hunt L, Brown P, Tomley FM. The microneme proteins EtMIC4 and EtMIC5 of *Eimeria tenella* form a novel, ultra-high molecular mass protein complex that binds target host cells. *J Biol Chem*. (2007) 282:16891–8. doi: 10.1074/jbc.M702407200

54. Tomley F, Billington KJ, Bumstead JN, Clark JD, Monaghan P. EtMIC4: a microneme protein from *Eimeria tenella* that contains tandem arrays of epidermal growth factor-like repeats and thrombospondin type-I repeats. *Int J Parasitol*. (2001) 31:1303–10. doi: 10.1016/s0020-7519(01)00255-7

55. Réfega S, Misguich FG, Bourdieu C, Péry P, Labbé M. Gene discovery in *Eimeria tenella* by immunoscreening cDNA expression libraries of sporozoites and schizonts with chicken intestinal antibodies. *Vet Parasitol*. (2003) 113:19–33. doi: 10.1016/s0304-4017(03)00033-5

56. Wan KL, Chong SP, Ng ST, Shirley MN, Tomley FM, Jangi MS. A survey of genes in *Eimeria tenella* merozoites by EST sequencing. *Int J Pasitol*. (1999) 29:1885–92. doi: 10.1016/s0020-7519(99)00160-5

57. Long S, Anthony B, Drewry LL, Sibley LD. A conserved ankyrin repeat-containing protein regulates conoid stability, motility and cell invasion in *Toxoplasma gondii*. *Nat Commun*. (2017) 8:2236. doi: 10.1038/s41467-017-02341-2

58. Panebra A, Lillehoj HS. *Eimeria tenella* elongation factor-1α (EF-1α) coadministered with chicken IL-7 (chIL-7) DNA vaccine emulsified in Montanide gel 01 adjuvant enhanced the immune response to *E. acervulina* infection in broiler chickens. *Avian Dis*. (2019) 63:342:–350. doi: 10.1637/11976-092418-Reg.1

59. Reid AJ, Blake DP, Ansari HR, Billington K, Browne HP, Bryant J, et al. Genomic analysis of the causative agents of coccidiosis in domestic chickens. *Genome Res*. (2014) 24:1676–85. doi: 10.1101/gr.168955.113

60. Zimmermann J, Saalbach I, Jahn D, Giersberg M, Haehnel S, Wedel J, et al. Antibody expressing pea seeds as fodder for prevention of gastrointestinal parasitic infections in chickens. *BMC Biotechnol*. (2009) 9:79. doi: 10.1186/1472-6750-9-79

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Assessing the effects of a mixed *Eimeria* spp. challenge on performance, intestinal integrity, and the gut microbiome of broiler chickens

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A mixed *Eimeria* spp. challenge model was designed to assess the effects of challenge on broiler chicken performance, intestinal integrity, and the gut microbiome for future use to evaluate alternative strategies for controlling coccidiosis in broiler chickens. The experimental design involved broiler chickens divided into two groups: a control group (uninfected) and a positive control group, infected with *Eimeria acervulina* (EA), *Eimeria maxima* (EM), and *Eimeria tenella* (ET). At day-of-hatch, 240 off-sex male broiler chicks were randomized and allocated to one of two treatment groups. The treatment groups included: (1) Non-challenged (NC, n=5 replicate pens); and (2) challenged control (PC, n=7 replicate pens) with 20 chickens/pen. Pen weights were recorded at d0, d16, d31, d42, and d52 to determine average body weight (BW) and (BWG). Feed intake was measured at d16, d31, d42, and d52 to calculate feed conversion ratio (FCR). Four diet phases included a starter d0–16, grower d16–31, finisher d31–42, and withdrawal d42–52 diet. At d18, chickens were orally challenged with 200 EA, 3,000 EM, and 500 ET sporulated oocysts/chicken. At d24 (6-day post-challenge) and d37 (19-day post-challenge), intestinal lesion scores were recorded. Additionally, at d24, FITC-d was used as a biomarker to evaluate intestinal permeability and ileal tissue sections were collected for histopathology and gene expression of tight junction proteins. Ileal and cecal contents were also collected to assess the impact of challenge on the microbiome. BWG and FCR from d16–31 was significantly (p<0.05) reduced in PC compared to NC. At d24, intestinal lesion scores were markedly higher in the PC compared to the NC. Intestinal permeability was significantly increased in the PC group based on serum FITC-d levels. Cadherin 1 (CDH1), calprotectin (CALPR), and connexin 45 (Cx45) expression was also upregulated in the ileum of the PC group at d24 (6-day post-challenge) while villin 1 (VIL1) was downregulated in the ileum of the PC group. Additionally, *Clostridium perfringens* (ASV1) was enriched in the cecal content of the PC group. This model could be used to assess the effect of alternative coccidiosis control methods during the post-challenge with EA, EM, and ET.

KEYWORDS
coccidiosis, chickens, intestinal permeability, performance, challenge model

1. Introduction

Coccidiosis, a parasitic disease caused by protozoan parasites of the genus *Eimeria*, is a significant health concern in the poultry industry. This disease affects chickens worldwide, leading to substantial economic losses and posing challenges to poultry producers (1). The economic impact of coccidiosis is multifaceted, encompassing direct costs associated with mortality, decreased productivity, and increased medication expenses, as well as indirect costs related to reduced feed conversion efficiency and impaired flock performance (2, 3). Moreover, the shift away from antibiotics for chicken coccidia control has been driven by several factors, including concerns over antibiotic resistance, regulatory changes, and the need for sustainable farming practices (4). To address this concern, there has been a global push to reduce the use of antibiotics in livestock production, including the poultry industry (5). Already, regulatory bodies in many countries have implemented restrictions on antibiotics in animal feed, including those commonly used for coccidia control. These regulations have encouraged the development and adoption of alternative strategies for coccidia control (6). The purpose of the present study is to evaluate the effect of a mixed *Eimeria* spp. challenge model on performance, intestinal integrity, and the gut microbiome of broiler chickens for future application assessing potential intervention strategies to control coccidiosis. This study focused on different aspects such as exploring additional parameters and providing unique insights into the mechanisms of coccidiosis. Our intention was to develop a comprehensive model that can be used to evaluate the effectiveness of different non-drug-based alternatives for controlling coccidiosis in broiler chickens.

2. Materials and methods

2.1. Experimental design

At day-of-hatch, 240 off-sex male broiler chicks were randomized and allocated to one of two treatment groups. The treatment groups included: (1) Non-challenged (NC, $n = 5$ replicate pens); and (2) challenged control (PC, $n = 7$ replicate pens) with 20 chickens/pen. Pen weights were recorded at d0, d16, d31, d42, and d52 to determine average body weight (BW) and body weight gain (BWG). Feed intake was measured at d16, d31, d42, and d52 to calculate the feed conversion ratio. Four diet phases included a starter d0–16, grower d16–31, finisher d31–42, and withdrawal d42–52 diet. The experimental diets were formulated to approximate the nutritional requirements of broiler chickens as recommended by the NRC (7) and adjusted to the breeder's recommendations (8). At d18, chickens were orally challenged with 200 *Eimeria acervulina* (EA), 3,000 *Eimeria maxima* (EM), and 500 *Eimeria tenella* (ET) sporulated oocysts/chicken. At d24 (6-day post-challenge), intestinal lesion scores based on the method described by Johnson and Reid (9) were recorded for four chickens/pen ($n = 20$ for NC; $n = 28$ for PC). The entirety of the gastrointestinal tract was scored with scores being specifically assigned to the duodenal, jejunal/ileal, or cecal sections of the intestine. Additionally, on the same day, four random chickens per pen were selected and orally gavaged with 8.32 mg/kg of body weight of fluorescein isothiocyanate-dextran (FITC-d, MW 3–5 KDa; Sigma-Aldrich Co). One hour after FITC-d administration, chickens were humanely euthanized by CO_2 inhalation. Blood samples were collected from the femoral vein and centrifuged ($1{,}000 \times g$ for 15 min) to separate the serum. Serum levels of FITC-d were used as a biomarker to evaluate intestinal permeability as described by Baxter et al. (10), and ileal tissue sections were collected to evaluate gene expression of tight junction proteins ($n = 8$/group). Ileal and cecal contents were also collected at d24 for 16S rRNA sequencing and microbiome analysis ($n = 8$/group). At d37 (19-day post-challenge), lesion scores were also recorded for four chickens/pen ($n = 20$ for NC; $n = 28$ for PC) but no additional samples were collected at this time. Animal handling and experimental procedures were approved by the University of Arkansas Division of Agriculture Institutional Animal Care and Use Committee (#21134).

2.2. *Eimeria* spp. strains

Eimeria maxima M6 and *E. tenella* oocysts were donated by Dr. John. R. Barta, University of Guelph, Canada, and wild-type *E. acervulina* oocysts were used for the challenge. Single oocyst-derived stocks of *E. maxima* M6, a strain recovered from a broiler flock in Florida, United States, in the mid-1990s (11) and was propagated *in vivo* in *Eimeria*-free chickens and sporulated *in vitro* to obtain a challenge stock. The *E. acervulina* strain was obtained from broiler chickens in Arkansas, United States and species confirmation was based on oocyst morphology and intestinal pathology. A preliminary dose titration study was conducted to determine the challenge dose for the trial (Table 1). The multi-species challenge dose was selected based on % reduction in body weight gain during the challenge period and lesion scores 6-day post-challenge. A 25% reduction of body weight gain during the challenge period (compared to the non-challenged control) and a lesion score of ~2 is target for subclinical coccidiosis challenge models (12). Thus, based on preliminary dose titration results, chickens in the PC group were orally challenged with 200 EA, 3,000 EM, and 500 ET sporulated oocysts/chicken at d18.

2.3. RNA extraction, reverse transcription, and qPCR

Total RNA was extracted from 50 mg of ileal tissue ($n = 8$/treatment) collected at d24 or 6-day post-challenge. The tissue was homogenized in 1 mL Trizol following the manufacturer's protocol (Invitrogen, Waltham, MA, United States). RNA was then resuspended in 40 μL nuclease-free water and then treated with DNase 1 (New

TABLE 1 Preliminary dose titration study conducted to determine the challenge dose for the trial.

Treatment	% BWG reduction post-challenge compared to NC	Duodenal Lesion score	*p* value	Jejunal/ileal Lesion score	*p* value	Cecal Lesion score	*p* value
Non-challenged Control (NC)	0.00	0	-	0	1.00	0	-
Dose 1: Challenged with 60 EA, 4,000 EM, and 250 ET	37.77	0.17 ± 0.09	0.9301	2.83 ± 0.17	<0.0001	1.03 ± 0.21	0.0010
Dose 2: Challenged with 90 EA, 6,000 EM, and 500 ET	34.83	0.50 ± 0.13	0.2244	2.70 ± 0.17	<0.0001	0.90 ± 0.19	0.0049
Dose 3: Challenged with 120 EA, 8,000 EM, and 500 ET	48.06	0.87 ± 0.27	0.0085	3.43 ± 0.13	<0.0001	1.90 ± 0.23	<0.0001
Dose 4: Challenged with 150 EA, 10,000 EM, and 750 ET	50.72	1.23 ± 0.31	<0.0001	3.40 ± 0.13	<0.0001	1.70 ± 0.24	<0.0001

Lesion scores: Means reported as mean ± standard error. ANOVA used to determine significant differences at $p < 0.05$ between the non-challenged control group and each challenged group by intestinal segment scored. Means further separated using Student's *t*-test.

England Biolabs, Ipswich, MA, United States). RNA was repurified using the Trizol RNA isolation protocol and resuspended in 35 µL nuclease-free water. RNA concentration and purity were determined using a Nanodrop 1000 (Nanodrop Technology, Willmington, DE, United States). To obtain template cDNA for qPCR, 1 µg of RNA was added to PrimeScript RT Master Mix (Takara Bio USA Inc., San Jose, CA, United States) per the manufacturer's instructions. cDNA was diluted 1:10 with nuclease-free water. Power SYBR Green Master Mix (Life Technologies, Carlsbad, CA, United States) was used for real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system). The oligonucleotide primers for adhesion, tight junction, and gap junction genes have been listed in Table 2 and were previously described by Tabler et al. (13). The qPCR conditions were as follows: 50°C for 2 m, 95°C for 10 m, 40 cycles at 95°C for 15 s, and 58–61°C for 1 m (varied by primer). Data were analyzed by the delta–delta Ct method (14) using 18S as reference.

2.4. Microbiome

At d24 or 6-day post-challenge, ileal and cecal contents were collected ($n = 8$/treatment). Samples were stored at RT in RNA/DNA shield. Total genomic DNA of ileal and cecal content samples were extracted using the DNeasy Power Lyzer PowerSoil Kit (Qiagen, Germantown, MD, United States) according to the manufacturer's protocol. The concentration of DNA was measured using a NanoDrop One (Thermo Fisher Scientific, Madison, WI, United States). The extracted DNA was then diluted to 10 ng/µL. The V4 region of the 16S rRNA was amplified using primer sequences (forward: 5′-GTGCCAGCMGCCGCGGTAA-3′ and reverse: 5′-GGACTAC HVGGGTWTCTAAT-3′) attached with gene-specific Illumina adapters for each sample. The PCR products were determined on a 1% agarose gel and then normalized using a commercial normalization plate [SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA, United States)]. All purified PCR amplicons were pooled together to generate a sequencing library. After the concentration and quality of the library were confirmed by KAPA Illumina Library Quantification Kits (Roche, Indianapolis, IN, United States) and an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, United States), the library was sequenced on a MiSeq sequencer (MiSeq Reagent Kit v2, 500 cycles; Illumina, San Diego, CA, United States). To prevent contaminations from reagents, a mock community, ZymoBIOMICS™ Microbial Community Standard (Zymo, Irvine, CA, United States) and negative of DNA extraction and PCR amplification were included in sequencing as well. The sequencing files obtained from the Illumina sequencer were pre-processed, quality filtered ($Q > 30$), and analyzed using the QIIME2 (2021.4 release) software (15). The Deblur algorithm was used for sequence trimming, denoising, chimera removal, and feature binning at the amplicon sequence variants (ASV) level (16). A naive Bayes classifier was employed for the assignment of all sequences into bacterial taxonomy using the Greengenes (v13_8 clustered at 99% identity) reference database. The raw data are available in the NCBI SRA database with the BioProject ID PRJNA.

2.5. Statistics

All data, excluding microbiome data, were analyzed by a one-way ANOVA using the GLM procedure of SAS (17). Means were further separated using Student's T test with significance at $p < 0.05$. Alpha diversity, including the Shannon Index and the number of Observed ASVs, was compared using a two-tailed Wilcoxon signed-rank test between two groups. Beta diversity based on Bray-Curtis and Jaccard distances was tested using an analysis of similarity (ANOSIM). The outputs of diversity were visualized using the "ggplot2" package in R (v4.1.2). The linear discriminant analysis (LDA) effect size (LEfSe), an analytical tool for discovering and interpreting biomarkers of high-dimensional data, was used to identify the signature bacteria associated with the growth stages and intestinal segments. LDA score>2 was used as a criterion for judging the significant effect size (18). The signature bacteria were visualized in a heat map using the "heatmap" function in R.

3. Results

3.1. Performance

The results of the evaluation of body weight (BWG), feed intake (FI), and feed conversion ratio (FCR) in broiler chickens in a mixed *Eimeria* spp. challenge model is summarized in Table 3. Significant ($p < 0.05$) differences in average BWG and FCR between NC and PC

TABLE 2 Primers used for real-time quantitative PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
18S	TCCCCTCCCGTTACTTGGAT	GCGCTCGTCGGCATGTA	60
CDH1	GGGAGCGCGTTGCCTACTA	GAGGGCTGCCCAGATCTGA	57
CALPR	GCTGGAGAAAGCCATTGATGTC	CCCCTCCCGTCTCGAGTAC	61
Cx45	TCCACCTTCGTTGGCAAAA	TCAGAACGATCCGAAAGACGAT	58
VIL1	TGCCGGTGCCCACTAAAA	TCGACAGCAGCACGTAGCA	63
ZO-1	GGGAACAACACACGGTGACTCT	AGGATTATCCCTTCCTCCAGATATTG	80
ZO-2	GCAATTGTATCAGTGGGCACAA	CTTAAAACCAGCTTCACGCAACT	69
ZO-3	CAAAGCAAGCCGGACATTTAC	GTCAAAATGCGTCCGGATGTA	63
OCLN	CGCAGATGTCCAGCGGTTA	GTAGGCCTGGCTGCACATG	59
LCN2	TGCAGCTTGCAGGGAGATG	GCTTCTTGTCCTTGAACCAGTTG	69
GJA1	TGGCAGCACCATCTCCAA	GGTGCTCATCGGCGAAGT	56
PATJ	GGATCCAGCAACGTGTCCTATT	GCATCCAGTGGAGTGTCTTTCC	114
JAMA	TCACCTCGGAGACAAAGGAAGT	ACGCAGAGCACGGGATGT	60

CDH1, cadherin 1; CALPR, calprotectin; Cx45, connexin 45; VIL1, villin 1; ZO-1-3, zonula occludens; OCLN, occludin; LCN2, lipocalin 2; GJA1, gap junction protein alpha 1; PATJ, PALS1-associated tight junction protein; and JAMA, junctional adhesion molecule A.

TABLE 3 Evaluation of body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) in broiler chickens in a mixed *Eimeria* spp. challenge model.

Item	Non-challenged	Challenged
BW, g/broiler		
d0	46.71 ± 0.19	47.35 ± 0.25
d16	532.91 ± 26.28	560.24 ± 26.90
d31	1850.22 ± 55.59	1708.35 ± 65.00
d42	3234.69 ± 43.09	3125.15 ± 81.60
d52	4279.19 ± 59.83	4233.74 ± 99.27
BWG, g/broiler		
d0–d16	486.20 ± 26.26	512.89 ± 26.95
d16–d31	1317.31 ± 32.35[a]	1148.12 ± 43.68[b]
d31–d42	1384.46 ± 22.23	1416.80 ± 30.23
d42–d52	1044.50 ± 77.35	1108.59 ± 63.38
d0–d52	4232.48 ± 59.81	4186.39 ± 99.16
FI, g/broiler		
d0–d16	809.96 ± 24.37	835.89 ± 28.47
d16–d31	2117.81 ± 43.80	2030.36 ± 59.33
d31–d42	2556.50 ± 34.02	2650.84 ± 66.22
d42–d52	2142.48 ± 28.68	2153.72 ± 52.93
d0–d52	7626.75 ± 102.32	7670.79 ± 176.30
FCR		
d0–d16	1.67 ± 0.04	1.63 ± 0.04
d16–d31	1.43 ± 0.01[b]	1.61 ± 0.04[a]
d31–d42	1.53 ± 0.03	1.48 ± 0.02
d42–d52	2.10 ± 0.17	1.98 ± 0.11
d0–d52	1.61 ± 0.02	1.64 ± 0.02

Data expressed as mean ± SE.
[a,b]Non-matching superscripts within rows indicates significant difference at $p < 0.05$.

groups were observed from d16-31, with the NC having markedly higher BWG and lower FCR compared to the PC. There were no significant differences in feed intake observed between the NC and PC groups during any period evaluated (Table 3). There were no significant differences in mortality between the groups (data not shown).

3.2. Lesion scores

Table 4 shows the coccidiosis lesion scores at d24 (6-day post-challenge) and d37 (19-day post-challenge). On d24, chickens in the PC group had significantly ($p < 0.05$) higher lesion scores for EA, EM, and ET compared to the NC. Nevertheless, by d37, only significant lesion scores were observed for EM in the PC group compared to non-challenged chickens (Table 4).

Figure shows macroscopic and microscopic lesions observed in chickens challenged with the mixed culture of *Eimeria* spp. *Eimeria acervulina* primarily affects the duodenum and upper small intestine. Macroscopic lesions include congestion, oedema, and thickening of the intestinal mucosa (Figure 1A). Microscopic examination reveals partial destruction of the duodenal and upper jejunal villi. The damaged villi may exhibit blunting or fusion. The mucosal epithelium may show sloughing or detachment, leading to erosion of the intestinal surface (Figure 1B).

Eimeria maxima, the most noticeable macroscopic lesions, occur in the small intestine. The affected intestinal segments, particularly the jejunum, appear thickened, congested, and edematous. They may exhibit hemorrhages and appear dark red or black due to the presence of blood. The mucosa may also have a velvety or grainy appearance (Figure 1C). Microscopic examination shows severe damage to the intestinal mucosa. The villi, finger-like projections on the mucosal surface, become shortened and broadened. The epithelial cells lining the villi may be detached, leading to denuded areas. Infiltration of inflammatory cells, such as lymphocytes and heterophils, may also be observed (Figure 1D).

TABLE 4 Evaluation of intestinal lesions scores associated with *Eimeria acervulina*, *Eimeria maxima*, or *Eimeria tenella* at d24 (6-day post-challenge) and d37 (19-day post-challenge) in broiler chickens.

Age (days)	Section of intestine	Lesion scores	
		Non-challenged	Challenged[1]
24	Duodenum	0.00 ± 0.00^{b}	0.79 ± 0.11^{a}
	Jejunum/ileum	0.00 ± 0.00^{b}	3.29 ± 0.15^{a}
	Cecum	0.00 ± 0.00^{b}	1.57 ± 0.23^{a}
37	Duodenum	0.00 ± 0.00	0.00 ± 0.00
	Jejunum/ileum	0.05 ± 0.05^{b}	0.43 ± 0.10^{a}
	Cecum	0.00 ± 0.00	0.04 ± 0.04

Data expressed as mean ± SE.
[a,b]Non-matching superscripts within rows indicates significant difference at $p < 0.05$.
[1]At d18, PC chickens were orally challenged with 200 EA, 3,000 EM, and 500 ET sporulated oocysts/chicken.

Eimeria tenella affects the ceca. Macroscopic lesions in infected chickens involve hemorrhages, congestion, and thickening of the cecal mucosa. The ceca may become distended and contain blood, fibrin, and a characteristic reddish mucoid exudate (Figure 1E). Microscopic Lesions cause severe damage to the cecal mucosa. The cecal epithelium is destroyed, resulting in the formation of ulcers. The base of these ulcers often contains a reddish fibrin necrotic material. Inflammatory infiltrations, including heterophils, lymphocytes, and plasma cells, are commonly observed in the affected areas (Figure 1F).

3.3. Intestinal permeability and gene expression (tight and gap junctions)

The serum FITC-d and gene expression of tight and gap junctions are summarized in Table 5. Significantly increased serum FITC-d levels were observed for the PC group 6-day post-challenge, which was attributed to the severity of intestinal lesions in the PC group as compared to the NC group. Similarly, significant differences were observed in relative mRNA expressions of cadherin 1 (CDH1), calprotectin (CALPR), connexin 45 (Cx45), and villin 1 (VIL1) between the NC and PC. CDH1, CALPR, and Cx45 were upregulated in the PC group compared to the NC, whereas VIL1 was downregulated in the PC group compared to the NC. However, no significant differences were observed for the other tight or gap junction genes evaluated between both experimental groups (Table 5).

3.4. Microbiome

The challenge with mixed *Eimeria* spp. did not affect the alpha diversity of the microbial community in the ileal lumen of the PC group compared to the NC group (Figure 2). However, the PC group had a lower alpha diversity [Shannon Index and the number of Observed Amplicon Sequence Variants (ASVs)] in the cecal lumen compared to the NC group. There were no differences in the community structure within the ileal contents of the NC or PC observed on the principal coordinate analysis (PCoA) based on Bray-Curtis and Jaccard distances [Analysis of similarities (ANOSIM): $R = 0.10$, $p = 0.108$; $R = 0.17$, $p = 0.073$; Figure 2]. In contrast, the cecal microbiome of the PC group was distinct compared to NC (ANOSIM: $R = 0.33$, $p = 0.003$; $R = 0.22$, $p = 0.001$). Moreover, higher alpha diversity in the cecal luminal contents was observed in both NC and PC groups, and distinct clusters between cecum and ileum-based Bray-Curtis and Jaccard distances were observed in NC and PC groups (ANOSIM: $R = 0.99$, 0.43, 0.79, 0.33, $p < 0.05$).

At the phylum level, Firmicutes was the dominant bacteria across all samples (Figure 3). Bacteroidetes was also a major phylum in the cecal community. In the ileum, PC had a higher abundance of Proteobacteria (2.43%) compared to NC (1.51%) and a lower abundance of Actinobacteria (0.16%) compared to NC (1.83%). A similar pattern was observed in the cecal community, with Proteobacteria, Actinobacteria, and Bacteroidetes abundance in the NC (0.67, 4.07 and 5.37%) vs. the PC (6.46, 2.16, and 10.41%). At the genus level, the dominant genera across all samples were *Lactobacillus* (28.00%), *Clostridium* (22.02%), *Faecalibacterium* (5.48%), and *Ruminococcus* (5.32%; Figure 4). Although inter-bird variation within treatment groups was observed, treatment effects on the microbiome composition were observed. The average abundance of *Lactobacillus* in the PC (33.58%) was lower than that in the NC (58.99%), while *Clostridium* was higher in the PC (47.12%) compared to the NC (26.72%). For the cecal microbiome, higher abundances of *Faecalibacterium* (12.79%), *Ruminococcus* (13.94%), and *Oscillospira* (5.02%) were observed in the NC, while *Lactobacillus* (11.86%), *Clostridium* (13.78%), *Bacteroides* (9.74%), and *Streptococcus* (3.96%) were higher in the PC.

Linear discriminant analysis Effect Size (LEfSe) was employed to identify bacterial biomarkers for each group at the ASV level (Figure 5). In the ileum, the signature ASVs for the NC were *Lactobacillus helveticus* (ASV3), *Peptostreptococceceae* unclassified (ASV29), *Streptophyta* (ASV49), and *Corynebacterium stationis* (ASV74), while PC had greater abundances of *Clostridium sordellii* (ASV10), *Clostridium butyricum* (ASV11), *Ruminococceceae* unclassified (ASV51), and *Bacteroides* unclassified (ASV18; Figure 5A). In the cecum, the NC was enriched with *Dorea* (ASV13), *Coprobacillus* (ASV33, ASV58), *Clostridiales* unclassified (ASV61, 73), *Bacteroides ovatus* (ASV65), *Oscillospira* (ASV64), *Coprococcus* (ASV53), and *Lachnospiraceae* unclassified (ASV90, ASV59), while the PC had higher abundances of *Clostridium perfringens* (ASV1, ASV12) and *Enterobacteriaceae* unclassified (ASV8; Figure 5B).

4. Discussion

Eimeria maxima, *Eimeria acervulina*, and *Eimeria tenella* are three common species that affect chickens (19). Each species produces distinct macroscopic and microscopic lesions in the intestines of infected birds. It is important to note that the severity of lesions may vary depending on the stage of infection, host immunity, and the presence of concurrent infections. FITC-d is a serum biomarker that has been used to assess intestinal permeability in chickens (20). During an *Eimeria* challenge, the parasites invade the intestinal lining, causing damage to the epithelial cells and disrupting the gut homeostasis leading to increased intestinal permeability and allowing molecules, such as FITC-d to leak from the gut lumen into the bloodstream. As a result, serum FITC-d becomes elevated (21). This leakage can occur due to the destruction

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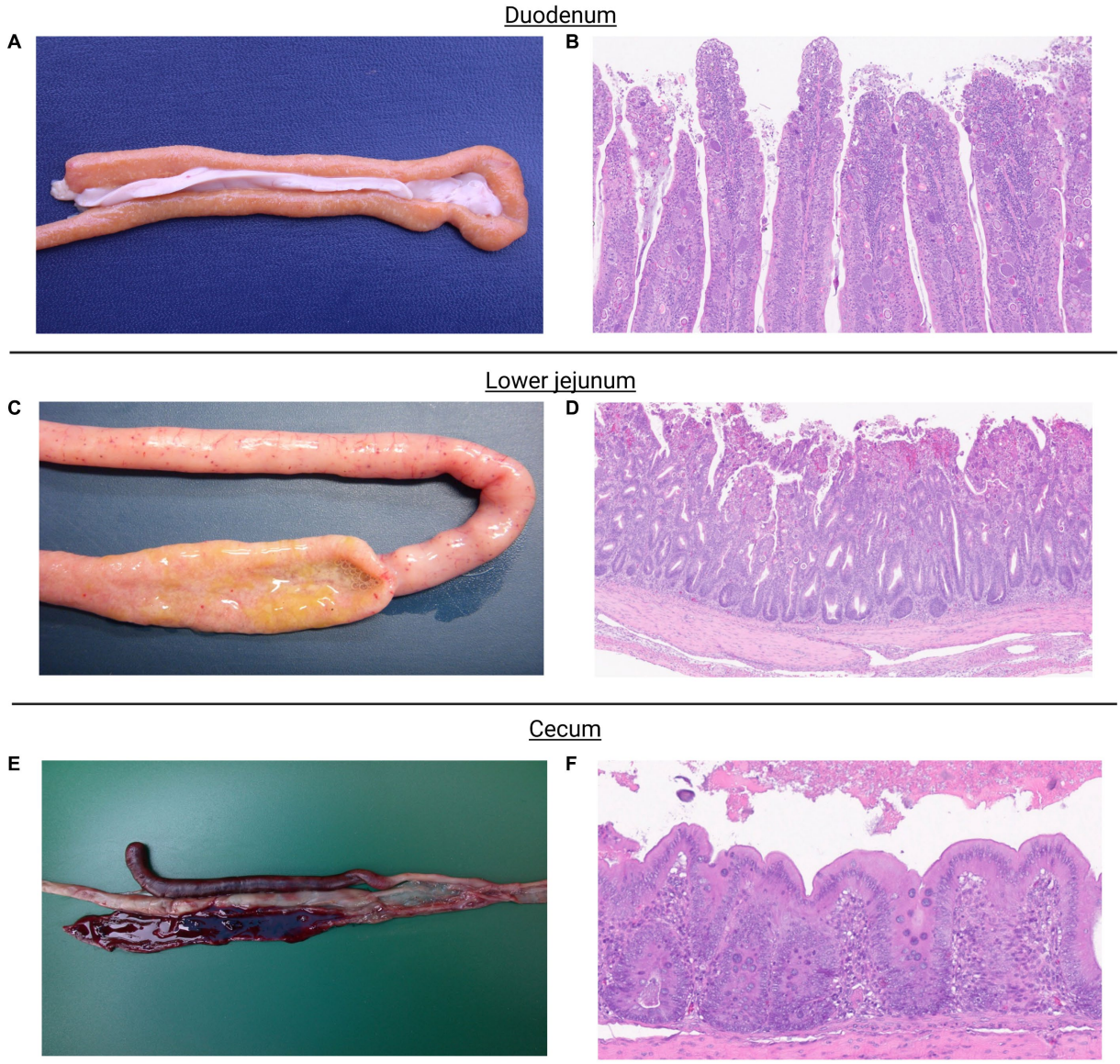

FIGURE 1
Eimeria acervulina primarily affects the duodenum and upper small intestine. Macroscopic lesions include congestion, edema, and thickening of the intestinal mucosa **(A)**. Microscopic examination reveals partial to complete destruction of the duodenal and upper jejunal villi. The damaged villi may exhibit blunting or fusion. The mucosal epithelium may show sloughing or detachment, leading to erosion of the intestinal surface. Endogenous stages of parasite development such as schizonts, microgametes, macrogametes, and immature oocysts can be observed **(B)**. *Eimeria maxima*, the most noticeable macroscopic lesions occur in the small intestine. The affected intestinal segments, particularly the jejunum, appear thickened, congested, and edematous. They may exhibit hemorrhages and appear dark red or black due to the presence of blood. The mucosa may also show a velvety or grainy appearance **(C)**. Microscopic examination shows severe damage to the intestinal mucosa. The villi, which are finger-like projections on the mucosal surface, become shortened and broadened. The epithelial cells lining the villi may be detached, leading to denuded areas. Infiltration of inflammatory cells, such as lymphocytes and heterophils, may also be observed. Endogenous stages of parasite development such as schizonts, microgametes, macrogametes, and immature oocysts can be observed **(D)**. *Eimeria tenella* affects the ceca. Macroscopic lesions in infected chickens involve hemorrhages, congestion, and thickening of the cecal mucosa. The ceca may become distended and contain blood, fibrin, and a characteristic reddish mucoid exudate **(E)**. Microscopic lesions cause severe damage to the cecal mucosa. The cecal epithelium is destroyed, resulting in the formation of ulcers. The base of these ulcers often contains a reddish fibrin necrotic material. Inflammatory infiltrations, including heterophils, lymphocytes, and plasma cells, are commonly observed in the affected areas. Endogenous stages of parasite development, primarily schizonts, can be observed **(F)**.

of epithelial cells, inflammation, and alterations in tight junction proteins that maintain the integrity of the intestinal barrier. This damage can lead to various consequences, including nutrient malabsorption, impaired immune responses, and secondary bacterial infections (22). In the current study, elevated levels of serum FITC-d associated with mixed *Eimeria* spp. infection and marked macroscopic lesion scores indicate the presence of intestinal damage and increased permeability associated with challenge. This was also reflected by performance which was negatively impacted during the d16–31 for PC compared to NC. It is worth noting that while increased levels of serum FITC-d are indicative of intestinal damage during a coccidia challenge, other factors and biomarkers

TABLE 5 Evaluation of serum levels of fluorescein isothiocyanate dextran (FITC-d) and relative mRNA expression level of tight and gap junction genes in the ileum of broiler chickens at 6-day post-challenge with mixed *Eimeria* spp.

Item	Non-challenged	Challenged
Serum FITC-d [ng/mL]	34.22 ± 70.43 [b]	229.50 ± 176.08 [a]
CDH1	1.00 ± 0.11 [b]	2.99 ± 0.49 [a]
CALPR	1.00 ± 0.41 [b]	255.11 ± 48.75 [a]
Cx45	1.00 ± 0.17 [b]	2.91 ± 0.45 [a]
VIL1	1.00 ± 0.08 [b]	0.44 ± 0.09 [a]
ZO-1	1.00 ± 0.10	0.88 ± 0.11
ZO-2	1.00 ± 0.05	1.19 ± 0.16
ZO-3	1.00 ± 0.11	1.43 ± 0.21
OCLN	1.00 ± 0.09	0.88 ± 0.16
LCN2	1.00 ± 0.24	1.36 ± 0.22
GJA1	1.00 ± 0.09	1.26 ± 0.19
PATJ	1.00 ± 0.07	0.88 ± 0.13
JAMA	1.00 ± 0.08	1.10 ± 0.12

[a,b]Non-matching superscripts within rows indicates significant difference at $p < 0.05$. FITC-d, fluorescein isothiocyanate dextran; CDH1, cadherin 1; CALPR, calprotectin; Cx45, connexin 45; VIL1, villin 1; ZO-1-3, zonula occludens; OCLN, occludin; LCN2, lipocalin 2; GJA1, gap junction protein alpha 1; PATJ, PALS1-associated tight junction protein; and JAMA, junctional adhesion molecule A.

may also be considered to comprehensively evaluate the host response and the progression of the disease.

Adherent junctions are cell adhesion complexes that help maintain the integrity and stability of epithelial tissues by promoting cell–cell adhesion. Cadherin, also known as CDH1 is crucial in regulating cell-to-cell adhesion and is a critical component of adherent junctions in various tissues, including enterocytes lining the intestinal epithelium (23). This adhesive interaction is essential for forming and maintaining the epithelial barrier. In the gut, CDH1-mediated adhesion between enterocytes is crucial for several processes, including regulating paracellular permeability, establishing apical-basal polarity, and maintaining tissue architecture. Loss or dysfunction of CDH1 can lead to compromised barrier function and increased intestinal permeability, which may contribute to various pathological conditions such as inflammation, intestinal injury, and cancer metastasis (24). In the current study, mixed *Eimeria* spp. challenge upregulated CDH1 mRNA expression in the ileum at 6 days post-challenge. Although CDH1 expression in the cecum was not evaluated in the present study, it has been shown to be downregulated post-challenge with *E. tenella* (25). The gene regulation of CDH1 can vary in different gut locations, although the core regulatory mechanisms remain largely similar. During an *Eimeria* infection, calprotectin (CALPR) expression can also be upregulated as part of the host immune response (26). CALPR has antimicrobial properties and can inhibit the growth and survival of pathogens, including coccidia (27). CALPR can also modulate the host inflammatory response by regulating the production of pro-inflammatory cytokines and chemokines, preventing excessive inflammation while promoting an effective defense against coccidia (28, 29). In the current study, CALPR expression in the ileum, where *E. maxima* invades and develops in the host, was upregulated as a result of challenge. Additionally, in the current study, challenged chickens showed an increase in the expression of Cx45 (connexin 45) gap junction proteins. This upregulation is part of the host's immune response to the infection and serves several important functions. Connexins are a family of proteins that form gap junctions, allowing for direct cell-to-cell communication and the exchange of small molecules between adjacent cells. Overall, the upregulation of CALPR and Cx45 post-challenge with mixed *Eimeria* spp. in the current study reflects the host's attempt to mount an effective immune response, including antigen presentation, modulation of cytokines, stress response, immune cell communication, tissue repair, and metabolite exchange. These protein expressions contribute to the overall defense against coccidian parasites and the restoration of tissue integrity.

Gap junctions are specialized intercellular channels formed by connexin proteins, which allow for direct communication and exchange of molecules between adjacent cells. VIL1 is a protein primarily found in the microvilli of intestinal epithelial cells. It has been implicated in forming and maintaining these structures (30). Several mechanisms can contribute to the downregulation of VIL1 gap junction proteins during a coccidia challenge model, including the release of pro-inflammatory cytokines (31–33). In addition, coccidia infection can cause cytoskeletal rearrangements in the infected intestinal epithelial cells, affecting the localization and stability of VIL1 and other junctional proteins, leading to their downregulation (34). Interestingly, chickens challenged with mix *Eimeria* spp. showed downregulation of VIL1 gap junction proteins in the ileum, which may be associated with the reduction in the expression of these proteins in response to coccidia infection. It is important to note that the downregulation of VIL1 during *Eimeria* spp. challenge might be a protective response aimed at limiting parasite replication and spread. By downregulating VIL1 and potentially disrupting gap junction communication, the host may limit the movement of the parasite between infected and uninfected cells. Further research is needed to understand better the specific mechanisms involved and their impact on host–parasite interactions.

In contrast to other studies that have evaluated the expression of other tight junctions (35, 36), there were no significant differences in tight junction expression for junctional adhesion molecule A (JAMA), occludin (OCLN), lipcalin 2 (LCN2), gap junction protein alpha 1 (GJA1), PALS1-associated tight junction protein (PATJ), and zonula occludens 1–3 (ZO-1, ZO-2, and ZO-3) in chickens challenged with *Eimeria maxima*, *Eimeria acervulina*, and *Eimeria tenella* compared with the non-challenged chickens in the current study. Tight junction proteins such as JAMA, OCLN, and ZO1 play a crucial role in maintaining the integrity and function of epithelial cell barriers, including those found in the intestines of chickens. These proteins are involved in forming tight seals between adjacent cells, regulating the passage of molecules and ions, and preventing the entry of pathogens into the underlying tissues (37). However, in the present study, chickens were challenged with *E. maxima*, *E. acervulina*, and *E. tenella*, and the significance of tight junction expression of these proteins appears to be lacking in the ileum at the specific time point evaluated. Despite the crucial role of tight junction proteins in maintaining the integrity of the intestinal barrier, some studies have also suggested that their expression may not be significantly altered during *Eimeria* infection in chickens. One possible explanation for this lack of significance is that the downregulation of tight junction proteins may occur at a post-transcriptional level rather than through changes in gene expression or perhaps sampling at other timepoints would have provided different results. In other words, the expression of JAMA,

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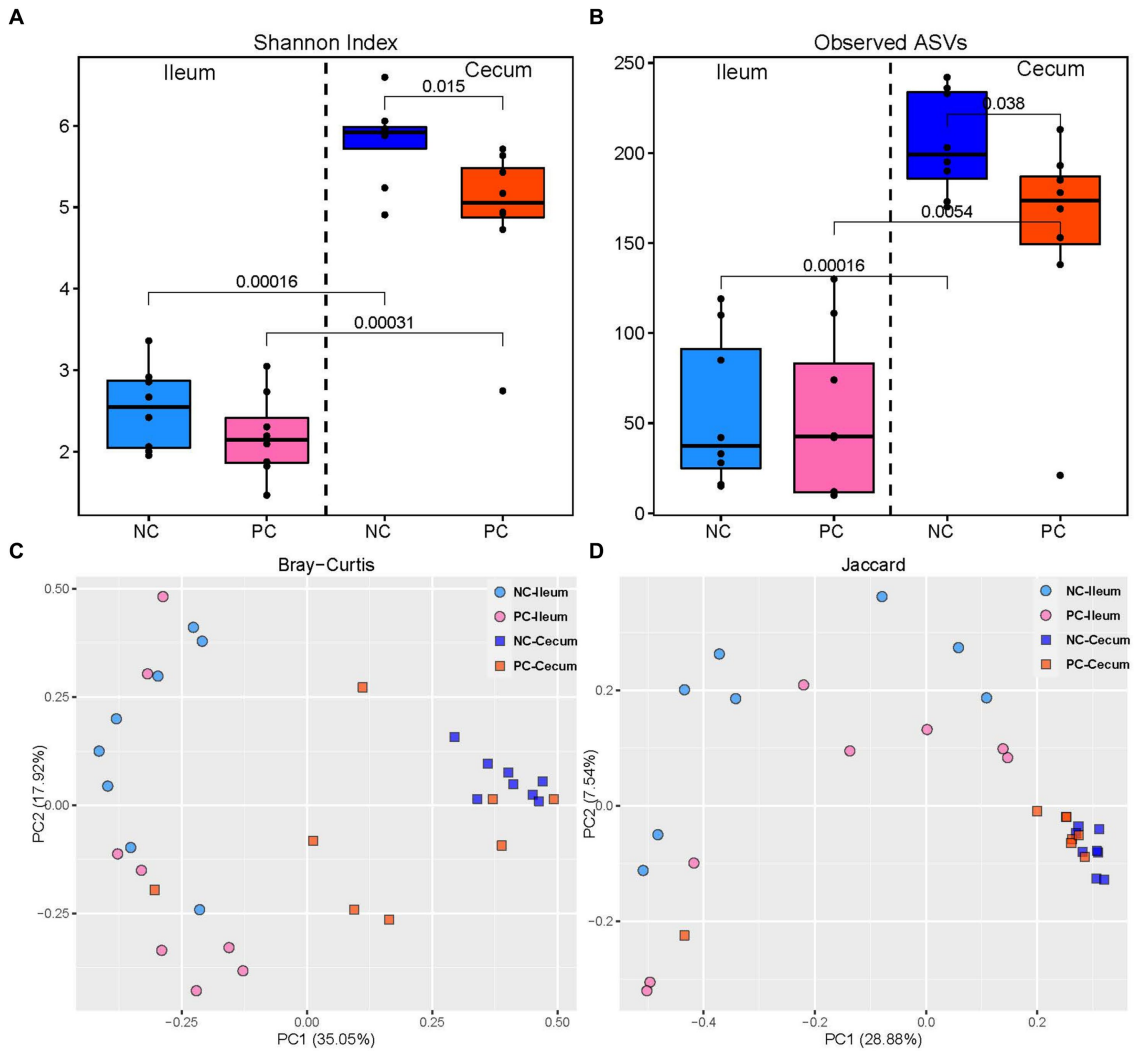

FIGURE 2
Alpha and beta diversity in ileal and cecal contents; alpha diversity including Shannon Index **(A)** and the number of observed ASVs **(B)** in the ileal and cecal luminal content of broiler chickens. Numbers over bars represent the *p* value. The principal coordinate analysis (PCoA) is based on the Bray—Curtis **(C)** and Jaccard **(D)** distances. Each point represents a unique sample.

OCLN, and ZO1 genes may remain relatively constant, but the proteins themselves could be modified or degraded, leading to decreased functional tight junctions. Additionally, it is important to consider that tight junction expression might not be the only factor determining the severity of coccidiosis in chickens.

The host inflammatory response and enteric microbiome can also impact disease progression. The microbiome composition in broiler chickens is influenced by various factors, including genetics. Each chicken's genetic makeup contributes to the selection of specific microbial communities within the gut and other body sites (38). Genetic variations among individual chickens can impact the diversity, stability, and functionality of their microbiomes. These variations may influence the host's susceptibility or resistance to diseases, such as *Eimeria* spp. infection (39). Several genetic factors can influence the composition and diversity of the microbiome in broiler chickens. These factors include host genetics related to immune function, mucosal integrity, metabolic pathways, and other physiological traits (40). Genetic variations in immune-related genes, such as pattern recognition receptors (PRRs), cytokines, and antimicrobial peptides, can affect the host's ability to recognize and respond to *Eimeria* spp. infection. These genetic differences may also influence the composition of the microbiome by altering the availability of nutrients or modulating the immune response toward specific microbial taxa (41). Moreover, the presence of specific commensal bacteria, such as *Lactobacillus* and *Bifidobacterium*, has been associated with improved gut health and resistance to coccidiosis. Genetic variations in the host can shape the microbiome composition and, in turn, impact the host's susceptibility to *Eimeria* spp. infection (42).

In the present study, challenge with *E. acervulina*, *E. maxima*, and *E. tenella* lowered alpha diversity in the cecal contents but did not affect alpha diversity in the ileal contents suggesting that the presence of *Eimeria* did not significantly alter the overall species richness or evenness in the ileal lumen. Thus, the diversity of microbial species remained relatively stable despite the *Eimeria* infection. There could be several reasons for this observation. The microbial community in the ileal lumen may possess inherent resilience, allowing it to withstand or adapt to the presence of *Eimeria* without experiencing significant alterations in alpha diversity. The microbial community might maintain

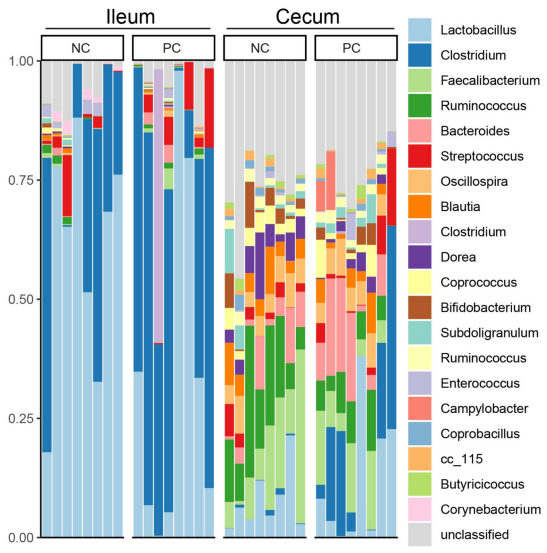

FIGURE 3
Microbial composition of ileum and cecal content at phylum level. Stacked bar charts demonstrate temporal changes of the top 20 common phyla or genera. Each column represents one sample.

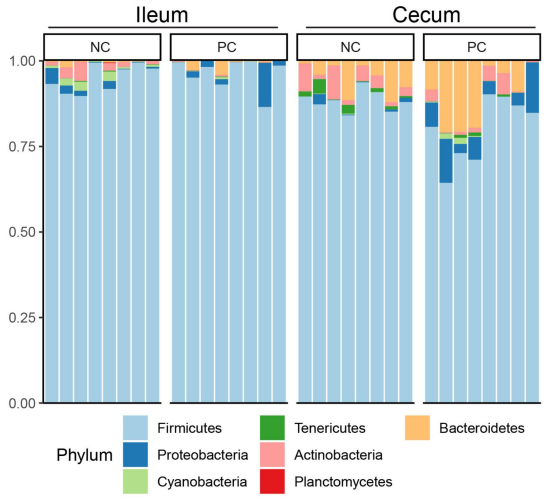

FIGURE 4
Microbial composition of ileum and cecal content at genus level. Stacked bar charts demonstrate temporal changes of the top 21 common phyla or genera. Each column represents one sample.

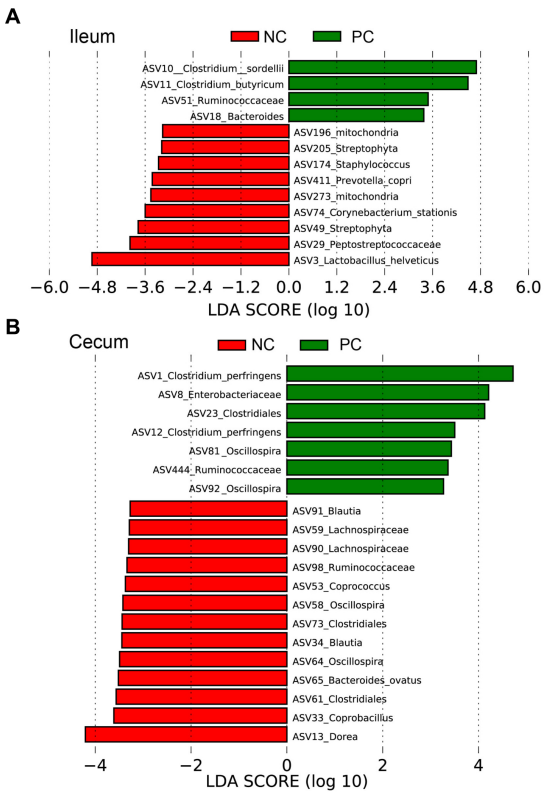

FIGURE 5
The signature bacteria within each group identified by Linear discriminant analysis Effect Size (LEfSe) to identify bacterial biomarkers for each group at the ASV level. Differentially abundant ASVs between ileum **(A)** and cecum **(B)** contents within the negative control group (NC) and the positive control group (PC). The ASVs in this graph were statistically significant ($p < 0.05$) and had an LDA Score > 2.

a dynamic equilibrium despite the temporary disturbance caused by the *Eimeria* infection. Additionally, the immune response of the chickens to *Eimeria* infection could play a role in preserving the alpha diversity of the microbial community. The immune system might modulate the interactions between *Eimeria* and the microbial community, preventing drastic changes in the overall composition and diversity. Furthermore, the experimental conditions and study duration could influence the results. If the study period was relatively short, the effects of *Eimeria* on the microbial community might have yet to fully manifest or reach a point where they could impact alpha diversity. Alternatively, the experimental design may not have been sensitive enough to detect subtle changes in the microbial community. It is important to note that although the alpha diversity remained unaffected, the presence of *Eimeria* species could still lead to other changes in the microbial community, such as alterations in beta diversity (the composition of species between samples) or changes in specific microbial taxa. Therefore, further research is needed to comprehensively understand the interactions between *Eimeria* infection and the microbial community in the ileal lumen of chickens.

In conclusion, administration of a mixed *Eimeria* spp. challenge can be used to evaluate alternative strategies to mitigate the effects of coccidiosis on performance and gut health in broiler chickens. This approach also allows researchers to simulate a more realistic and complex scenario, mimicking the infection with multiple *Eimeria* spp. commonly found in commercial poultry operations. This research contributes to the ongoing efforts to develop sustainable and effective strategies for coccidiosis control, which should be evaluated under controlled, challenged conditions.

Data availability statement

The raw data reads of 16S rRNA gene sequencing of microbiota are deposited in the National Center for

Biotechnology Information (NCBI) repository, accession number PRJNA918528.

Ethics statement

The animal study was approved by Animal handling and experimental procedures were approved by the University of Arkansas Division of Agriculture Institutional Animal Care and Use Committee (#21134). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DG, GT-I, and BH conceptualized the study. MC, JL, SS, JC, and JZ handled the methodology. VP-G, MJ-E, AF, RS, LL, and KM were in charge of the software. DG and GT-I validated the study and prepared and wrote the original draft. CT-P, IL, LG, and DG performed the formal analysis. XH-V, DG, and GT-I contributed to the writing, reviewing, and editing of the final manuscript. BH and GT-I oversaw the project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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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. Mesa-Pineda C, Navarro-Ruíz JL, López-Osorio S, Chaparro-Gutiérrez JJ, Gómez-Osorio LM. Chicken coccidiosis: from the parasite lifecycle to control of the disease. *Front Vet Sci*. (2021) 8:787653. doi: 10.3389/fvets.2021.787653

2. Blake DP, Knox J, Dehaeck B, Huntington B, Rathinam T, Ravipati V, et al. Re-calculating the cost of coccidiosis in chickens. *Vet Res*. (2020) 51:115. doi: 10.1186/s13567-020-00837-2

3. Taylor J, Walk C, Misiura M, Sorbara JB, Giannenas I, Kyriazakis I. Quantifying the effect of coccidiosis on broiler performance and infection outcomes in the presence and absence of control methods. *Poult Sci*. (2022) 101:101746. doi: 10.1016/j.psj.2022.101746

4. Martins RR, Silva LJG, Pereira AMPT, Esteves A, Duarte SC, Pena A. Coccidiostats and poultry: a comprehensive review and current legislation. *Foods*. (2022) 11:2738. doi: 10.3390/foods11182738

5. Başaran E, Doğru NH. "Antibiotic resistant profile of enteric bacteria isolated from chicken meats" in *ICSAR: Proceedings of the 2nd International Conference on Scientific and Academic Research*. (ed.) Özkaya U Konya, Turkey: ICSAR (2023). March 14–16, 2023; p. 91–94.

6. Barbour EK, Ayyash DB, Iyer A, Harakeh STKA, Kumosani T. A review of approaches targeting the replacement of coccidiostat application in poultry production. *Br J Poult Sci*. (2015) 17:405–18. doi: 10.1590/1516-635x1704405-418

7. National Research Council. *Nutrient Requirements of Poultry*. Washington, DC: National Academy Press (1994). 176 p.

8. Cobb-Vantress Inc. Cobb 500 broiler performance and nutrition supplement. (2022). Available at: https://www.cobb-vantress.com/assets/Cobb-Files/product-guides/5502e86566/2022-Cobb500-Broiler-Performance-Nutrition-Supplement.pdf (Accessed February 20, 2023).

9. Johnson J, Reid WM. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. *Exp Parasitol*. (1970) 28:30–6. doi: 10.1016/0014-4894(70)90063-9

10. Baxter MFA, Merino-Guzman R, Latorre JD, Mahaffey BD, Yang Y, Teague KD, et al. Optimizing fluorescein isothiocyanate dextran measurement as a biomarker in a 24-h feed restriction model to induce gut permeability in broiler chickens. *Front Vet Sci*. (2017) 4:56. doi: 10.3389/fvets.2017.00056

11. Martin AG, Danforth HD, Barta JR, Fernando MA. Analysis of immunological cross-protection and sensitivities to anticoccidial drugs among five geographical and temporal strains of *Eimeria maxima*. *Int J Parasitol*. (1997) 27:527–33. doi: 10.1016/s0020-7519(97)00027-1

12. Latorre JD, Adhikari B, Park SH, Teague KD, Graham LE, Mahaffey BD, et al. Evaluation of the epithelial barrier function and ileal microbiome in an established necrotic enteritis challenge model in broiler chickens. *Front Vet Sci*. (2018) 5:199. doi: 10.3389/fvets.2018.00199

13. Tabler TW, Greene ES, Orlowski SK, Hiltz JZ, Anthony NB, Dridi S. Intestinal barrier integrity in heat-stressed modern broilers and their ancestor wild jungle fowl. *Front Vet Sci*. (2020) 7:249. doi: 10.3389/fvets.2020.00249

14. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. (2008) 3:1101–8. doi: 10.1038/nprot.2008.73

15. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. (2019) 37:852–7. doi: 10.1038/s41587-019-0209-9

16. Amir A, McDonald D, Navas-Molina JA, Kopylova E, Morton JT, Zech XZ, et al. Deblur rapidly resolves single-nucleotide community sequence patterns. *mSystems*. (2017) 2:e00191-16. doi: 10.1128/mSystems.00191-16

17. SAS Institute Inc. *SAS/Share: 9.4 User's Guide*. Cary, NC: SAS Publishing (2002). 112 p.

18. Segata N, Abubucker S, Goll J, Schubert AM, Izard J, Cantarel BL, et al. Microbial community function and biomarker discovery in the human microbiome. *Genome Biol*. (2011) 12:P47–55. doi: 10.1186/gb-2011-12-s1-p47

19. Giannenas I, Tsalie E, Triantafillou E, Hessenberger S, Teichmann K, Mohnl M, et al. Assessment of probiotics supplementation via feed or water on the growth performance, intestinal morphology and microflora of chickens after experimental infection with *Eimeria acervulina*, *Eimeria maxima* and *Eimeria tenella*. *Avian Pathol*. (2014) 43:209–16. doi: 10.1080/03079457.2014.899430

20. Baxter MFA, Latorre JD, Dridi S, Merino-Guzman R, Hernandez-Velasco X, Hargis BM, et al. Identification of serum biomarkers for intestinal integrity in a broiler chicken malabsorption model. *Front Vet Sci*. (2019) 6:144. doi: 10.3389/fvets.2019.00144

21. Liu G, Ajao AM, Shanmugasundaram R, Taylor J, Ball E, Applegate TJ, et al. The effects of arginine and branched-chain amino acid supplementation to reduced-protein diet on intestinal health, cecal short-chain fatty acid profiles, and immune response in broiler chickens challenged with *Eimeria* spp. *Poult Sci*. (2023) 102:102773. doi: 10.1016/j.psj.2023.102773

22. Teng PY, Liu G, Choi J, Yadav S, Wei F, Kim WK. Effects of levels of methionine supplementations in forms of L- or DL-methionine on the performance, intestinal development, immune response, and antioxidant system in broilers challenged with *Eimeria* spp. *Poult Sci*. (2023) 102:102586. doi: 10.1016/j.psj.2023.102586

23. Wu A, Gao Y, Kan R, Ren P, Xue C, Kong B, et al. Alginate oligosaccharides prevent dextran-sulfate-sodium-induced ulcerative colitis via enhancing intestinal barrier function and modulating gut microbiota. *Foods*. (2023) 12:220. doi: 10.3390/foods12010220

24. Yang S, Xiong Z, Xu T, Peng C, Hu A, Jiang W, et al. Compound probiotics alleviate cadmium-induced intestinal dysfunction and microbiota disorders in broilers. *Ecotoxicol Environ Saf*. (2022) 234:113374. doi: 10.1016/j.ecoenv.2022.113374

25. Antoszczak M, Huczyński A. Salinomycin and its derivatives—a new class of multiple-targeted "magic bullets". *Eur J Med Chem*. (2019) 176:208–27. doi: 10.1016/j.ejmech.2019.05.031

26. Tellez-Isaias G, Eisenreich W, Petrone-Garcia VM, Hernandez-Velasco X, Castellanos-Huerta I, Tellez G Jr, et al. Effects of chronic stress and intestinal inflammation on commercial poultry health and performance: a review. *Ger J Vet Res*. (2023) 3:38–57. doi: 10.51585/gjvr.2023.1.0051

27. De Meyer F, Eeckhaut V, Ducatelle R, Dhaenens M, Daled S, Dedeurwaerder A, et al. Host intestinal biomarker identification in a gut leakage model in broilers. *Vet Res*. (2019) 50:46. doi: 10.1186/s13567-019-0663-x

28. Lail NU, Sattar A, Omer MO, Hafeez MA, Khalid AR, Mahmood S, et al. Biosynthesis and characterization of zinc oxide nanoparticles using *Nigella sativa* against coccidiosis in commercial poultry. *Sci Rep*. (2023) 13:6568. doi: 10.1038/s41598-023-33416-4

29. Dal Pont GC, Belote BL, Lee A, Bortoluzzi C, Eyng C, Sevastiyanova M, et al. Novel models for chronic intestinal inflammation in chickens: intestinal inflammation pattern and biomarkers. *Front Immunol*. (2021) 12:676628. doi: 10.3389/fimmu.2021.676628

30. Xu Y, Zhang B, Zhao R, Gao K, Liang S, Wu Y, et al. Effect of riboflavin deficiency on intestinal morphology, jejunum mucosa proteomics, and cecal microbiota of Pekin ducks. *Anim Nutr*. (2022) 12:215–26. doi: 10.1016/j.aninu.2022.09.013

31. Nash TJ, Morris KM, Mabbott NA, Vervelde L. Inside-out chicken enteroids with leukocyte component as a model to study host-pathogen interactions. *Commun Biol*. (2021) 4:377. doi: 10.1038/s42003-021-01901-z

32. Al-Quraishy S, Metwaly MS, Dkhil MA, Abdel-Baki AA, Wunderlich F. Liver response of rabbits to *Eimeria coecicola* infections. *Parasitol Res*. (2012) 110:901–11. doi: 10.1007/s00436-011-2574-2

33. Erf GF. Cell-mediated immunity in poultry. *Poult Sci*. (2004) 83:580–90. doi: 10.1093/ps/83.4.580

34. Dkhil MA, Khalil M, Al-Quraishy S. Profiling of jejunum inflammatory gene expression during murine eimeriosis. *Microb Pathog*. (2017) 104:196–201. doi: 10.1016/j.micpath.2017.01.038

35. Al-Otaibi T, Hawsah MA, Alojayri G, Mares MM, Aljawdah HMA, Maodaa SN, et al. In vivo anticoccidial, antioxidant, and anti-inflammatory activities of avocado fruit, *Persea americana* (Lauraceae), against *Eimeria papillata* infection. *Parasitol Int*. (2023) 95:102741. doi: 10.1016/j.parint.2023.102741

36. Wickramasuriya SS, Park I, Lee Y, Lillehoj HS. Effect of dietary organic selenium on growth performance, gut health, and coccidiosis response in broiler chickens. *Animals*. (2023) 13:1560. doi: 10.3390/ani13091560

37. Murray JW, Spray DC. Gap and tight junctions in liver: structure, function, and pathology In: IM Arias, HJ Alter, JL Boyer, DE Cohen, AD Shafritz and SS Thorgeirsson, editors. *The Liver: Biology and Pathobiology*. Oxford, UK: Wiley-Blackwell (2020). 160–73.

38. Zhao L, Wang G, Siegel P, He C, Wang H, Zhao W, et al. Quantitative genetic background of the host influences gut microbiomes in chickens. *Sci Rep*. (2013) 3:1163. doi: 10.1038/srep01163

39. Yu H, Wang Q, Tang J, Dong L, Dai G, Zhang T, et al. Comprehensive analysis of gut microbiome and host transcriptome in chickens after *Eimeria tenella* infection. *Front Cell Infect Microbiol*. (2023) 13:1191939. doi: 10.3389/fcimb.2023.1191939

40. Ding J, Dai R, Yang L, He C, Xu K, Liu S, et al. Inheritance and establishment of gut microbiota in chickens. *Front Microbiol*. (2017) 8:1967. doi: 10.3389/fmicb.2017.01967

41. Madlala T, Okpeku M, Adeleke MA. Understanding the interactions between *Eimeria* infection and gut microbiota, towards the control of chicken coccidiosis: a review. *Parasite*. (2021) 28:48. doi: 10.1051/parasite/2021047

42. Tang X, Suo J, Li C, Du M, Wang C, Hu D, et al. Transgenic *Eimeria tenella* expressing profilin of *Eimeria maxima* elicits enhanced protective immunity and alters gut microbiome of chickens. *Infect Immun*. (2018) 86:10–1128. doi: 10.1128/IAI.00888-17

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The roles of soybean lecithin in aquafeed: a crucial need and update

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Soybean lecithin is extensively used as the dietary supplementation of phospholipids in animal production. Soybean lecithin plays significant roles in aquafeed as growth promoter, feed enhancer, immunity modulator and antioxidant activity stimulator for aquaculture species. Besides, soybean lecithin is also reported to help aquaculture species being resilient to physical and chemical stressors. In this review, common sources, chemical structure and mode of action of lecithin, with highlight on soybean lecithin application in aquaculture over four-decadal studies published between 1983 and 2023, were evaluated and summarized. By far, soybean lecithin is best-known for its beneficial effects, availability yet cost-effective for aquafeed formulation. Findings from this review also demonstrate that although nutritional profile of long-chain polyunsaturated fatty acids and phosphatidylcholine from egg yolk and marine sources are superior to those from plant sources such as soybean, it is rather costly for sustainable application in aquafeed formulation. Moreover, commercially available products that incorporate soybean lecithin with other feed additives are promising to boost aquaculture production. Overall, effects of soybean lecithin supplementation are well-recognized on larval and juvenile of aquaculture species which having limited ability to biosynthesis phospholipids *de novo*, and correspondingly attribute to phospholipid, a primary component of soybean lecithin, that is essential for rapid growth during early stages development. In addition, soybean lecithin supplementation plays a distinguish role in stimulating maturation of gonadal development in the adults, especially for crustaceans.

KEYWORDS

lecithin, growth performance, feed utilization, immunity, antioxidant, stressor, sustainable aquaculture

1. Introduction

Aquafeed is the main production expense of an aquaculture operation, accounting for about 50 to 70% of the total aquaculture operation cost. According to a recent study, aquafeed costs about 65% of the total aquaculture production cost (1). The feed cost is expected to further increase due to excessive reliance on conventional raw materials, especially fish oil and fish meal in aquafeed formulation (2). Sustainability of the aquaculture industry can be greatly impacted by the future shortages of fish oil and fish meal (3). Hence, it is vital to have some animal origin materials gradually replaced by substances derived from plant origin such as soybean lecithin, soybean oil, and soybean meal in the aquafeed formulation.

As a byproduct of the oilseed industry, lecithin is widely introduced into food, cosmetic, pharmaceutical and other non-food industries as the emulsifier and liposomes producer, along with their great nutritional value (4). In the aquaculture industry, lecithin becomes one of the important raw materials to meet essential fatty acid requirements of the targeted species (5). Lecithin production became well-established in the United States in 1940 and expanded rapidly upon the commercial introduction of genetically modified (GMO) soybeans in 1996. On the other hand, lecithin from non–GMO sources like sunflower, rapeseed, and rice bran are favored by the European market (6, 7). Soybean lecithin is currently the primary source available in the worldwide market and offers a comparatively affordable price compared to lecithin from other sources, especially the marine lecithin (8). Commercial soybean lecithin claimed to consist high concentration of phospholipid, 65–75% (9) while some other studies showed that concentration of phospholipids in soybean lecithin ranged between 28–44% (10), 55–57% (11), 47% (12), and 38–45% (13). These differences were probably due to the discrepancies in the purity of soybean lecithin and approaches used to determine the concentration of phospholipid in soybean lecithin.

Aquafeed formulation that offer phospholipid composition resembles to the fish egg is deemed to be an ideal diet for fish larvae to ensure maintenance and functionality of cellular membrane structure, and as a source of energy to the fish (14). Therefore, phospholipid is supplemented in the larval diet of the aquatic animals owing to their limited biosynthetic capacity in the initial stages (15–17). For most of the aquaculture species, a supplementation of 8 to 12% dietary phospholipid is regarded to be optimal to promote growth and survival (18). Nonetheless, requirements for dietary phospholipids vary among species, life stage of the species, and the source and purity of the phospholipids. Phospholipid from different sources of lecithin such as soybean, milk, egg and krill, differs from one source to another in terms of phospholipid classes and fatty acids nature (19). Remarkably, soybean lecithin predominated by phosphatidylcholine, followed by phosphatidylethanolamine, and phosphatidylinositol provides an excellent source of phospholipids and fatty acids which are essential to aquatic animals during their early life stages (20). In this review, common sources, chemical structure and mode of action of lecithin are presented with the examples on how soybean lecithin promotes aquaculture production by improving feed utilization, growth performance, intestinal health, antioxidant capacity and resilience against stressors.

2. Lecithin sources and chemical structure

Literally means egg yolk in Greek word *lekithos*, lecithin was first extracted from the egg yolk by a French chemist, Theodore Nicolas Gobley in 1850. Subsequently, soybean lecithin came into worldwide commercial availability in 1921, almost a decade after the introduction of soybean from China into Europe and North America (21, 22). Global lecithin market size has exceeded USD 2 billion in 2021 and is forecasted to surpass USD 3.4 billion by 2030 (23). Lecithin is a complex mixture of phosphatide fraction available in both plants and animals, especially in the soybean and egg yolk (19). As a result of the esterification of choline, ethanolamine, serine, and inositol to a phosphatidic acid backbone, phospholipids are formed and classified into respective phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) with other constituents of fatty acids, triglycerides, and carbohydrates (4). Despite that the term for lecithin specifically refers to phosphatidylcholines in some earlier literatures, it is often used interchangeably with phospholipids since the commercial lecithin is largely made up of phospholipids and vegetable oils (24, 25).

Lecithin can be isolated and characterized from various sources, both from the terrestrial and aquatic (Table 1). These including soybean (48), sunflower (29), rapeseed (26), corn (34), camelina seed (32), canola (31), rice bran (35), egg yolk (37), dairy products (38, 39), and marine products (41, 42) that displaying assorted profile of phospholipids due to variations of two ester-bonded fatty acids at the *sn*-1 and *sn*-2 positions of the glycerol backbone. The *sn*-1 position chiefly carries a saturated fatty acid such as stearic acid or palmitic acid, whereas the *sn*-2 position carries an unsaturated fatty acid such as oleic acid, linoleic acid, α-linolenic acid, arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (49). Lecithin from different sources differs substantially on structural and functional roles depending on its origin, either plant or animal origin, and the extraction process (27, 50). An example of chemical structure and model of soybean lecithin is presented in Figure 1.

Lecithin is commonly extracted by degumming crude vegetable oils and drying the hydrated gums. Soybean oil, for example, contains approximately 60% acetone-insoluble which corresponds to 2–3% of the commercial lecithin and high amounts of phosphorus (12). Compared to sunflower and rapeseed oils, crude soybean oil has the greatest phospholipid concentration that is still present in vegetable oils after extraction (51). Therefore, soybean oil is the main source for the production of commercial lecithin. A typical crude soybean lecithin is reported to contain 18% PC, 14% PE, 9% PI, 5% phosphatidic acid, 2% minor phospholipids, 11% glycolipids, 5% complex sugars and 37% neutral oil (20). However, these properties are subjected to alteration by deoiling the crude lecithin into high-purity refined lecithin products, or by chemical and enzymatic modification of the phospholipids (52). For examples, the refined soybean lecithin has a greater purity of phospholipid (97.6%) than those from the crude soybean lecithin (60%) (53). Similarly, corn lecithin deoiled by supercritical carbon dioxide is claimed to contain 96% of phospholipids (34).

Animal lecithin derived from eggs, milk and bovine brain contains sphingomyelin on top of the major phospholipid classes such as PC, PE, PS and PI. Egg yolk lecithin is distinguished from soybean lecithin by having relatively higher proportion of saturated fatty acids, *n*-6 and *n*-3 polyunsaturated fatty acids (PUFAs) such as arachidonic acid and DHA. It is even more oxidatively stable than soybean lecithin (54). However, the application of egg yolk lecithin in aquafeed is not a viable option due its cost and commercial availability (37). On the other hand, marine lecithin derived from cold-water fishes is predominated by PC and PE and characterized by the presence of *n*-3 PUFAs such as EPA [20:5(n-3)] and DHA [22:6(n-3)] (55). For instance, marine lecithin extracted from salmon head was found to be rich in phospholipids, especially the PC (44). Nonetheless, supplies of the marine lecithin are not sustainable and therefore much more costly than other sources of lecithin (41, 56). Unlike the animal lecithin, plant lecithin derived from oilseeds such as soybean, rapeseed and sunflower contain mainly of PC, PE and PI and characterized by the availability of mono- and poly- unsaturated fatty acids, namely the oleic acid (18:1), linoleic acid [18:2 (n-6)], and α-linolenic acid [18:3(n-3)] (26). In particular, soybean lecithin has been the most widely used phospholipid source in aquaculture feed formulation due to its market availability and the beneficial effects of promoting growth performance and enhancing survival on fish (57).

TABLE 1 Phospholipid content of various lecithin sources.

Lecithin source	Phospholipid content (%)	References
Soybean	45.8–81.9	(26–28)
Sunflower	42.0–64.4	(28–30)
Rapeseed	44.6–77	(26, 28, 31)
Camelina seed	33.8–55.7	(32)
Peony seed	67	(33)
Corn	96	(34)
Rice bran	42.5	(6, 35, 36)
Egg yolk	10–54.3	(27, 37)
Milk and dairy products	0.2–48.4	(38–40)
Krill	30–80	(24, 41)
Mackerel	68.6–84.1	(42)
Anchovy	65	(43)
Salmon	61.1	(26, 44)
Fish roe	64.8–77.9	(27, 45, 46)
Fish by-product	68.9–79.8	(27, 45, 46)
Squid viscera	91.6	(47)
Fish meal	40	(41)

3. Mode of action

Soybean lecithin is an essential component of aquafeed that exhibits multi-faceted mode of action and involves diverse mechanisms. Primarily used in aquafeed as an emulsifying agent, phospholipid components of soybean lecithin form a protective layer surrounding the lipid droplets in the feed, thus improves the stability of aquafeed and reduces the leaching rate of water-soluble vitamins and minerals (58, 59). By increasing the surface area of the lipid droplets in the aquafeed, soybean lecithin serves to increase the digestibility of aquafeed by making it more easily accessed by digestive enzymes to break down the lipids into smaller molecules. This is particularly important in carnivorous fish species that require a high level of dietary lipids for optimal growth (60). Phospholipids are important for the maintenance of bio-membrane

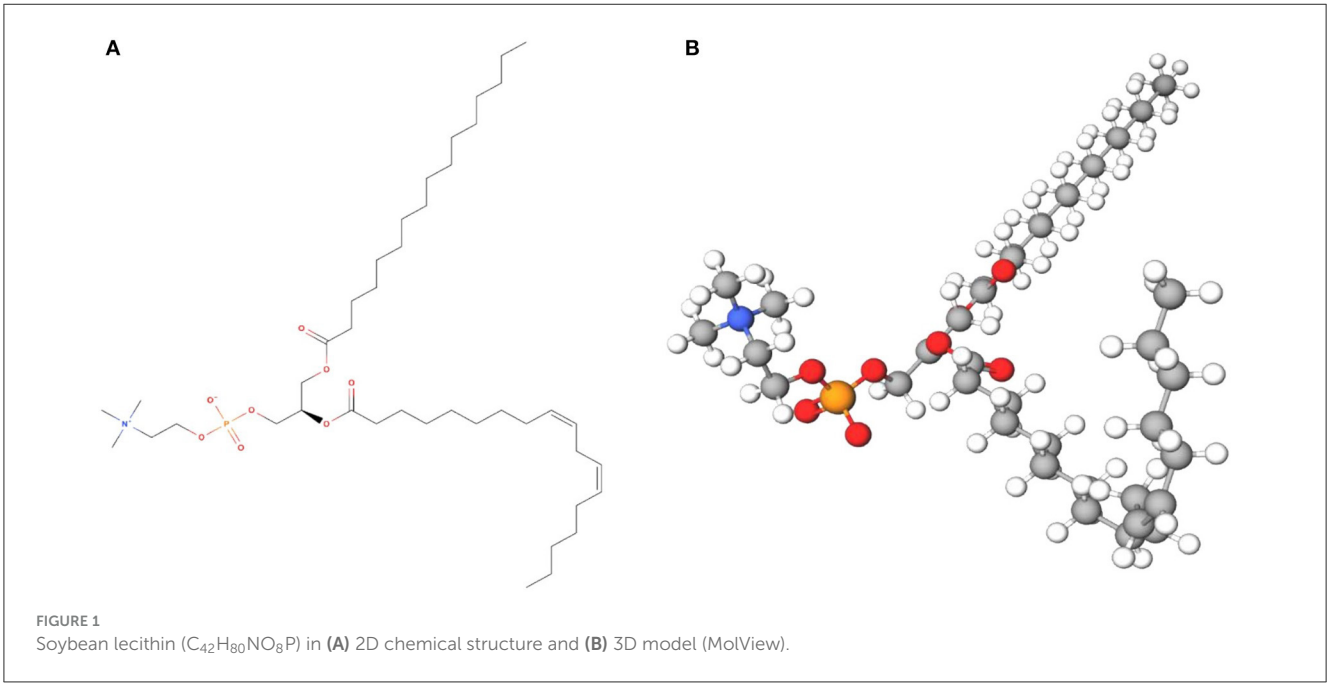

FIGURE 1
Soybean lecithin ($C_{42}H_{80}NO_8P$) in **(A)** 2D chemical structure and **(B)** 3D model (MolView).

structure, formation of cell organelles and superior to neutral lipids as the main energy source during early life history stages of some fish species (8, 18, 61). Phospholipid deficiency in fish larval diets has been reported to develop aberrant lipid deposition in intestinal enterocytes due to insufficient chylomicron synthesis and reduced specific activity of lipase (62, 63). Besides, phospholipids are reported to suppress cholesterol absorption in the animal digestive system (64) while facilitate the transport of lipids released from the hepatopancreas into hemolymph and enhance the availability of dietary cholesterol in the prawn *Penaeus japonicus* (65).

To ensure that a nutritionally complete and balanced diet is delivered, the mechanism of metabolism for soybean lecithin in aquafeed primarily involves the breakdown of its phospholipid components by digestive enzymes, followed by the absorption of fatty acids and nutrients, and the conversion of choline to betaine. Based on the chemical structure of soybean lecithin, free fatty acids are readily to be absorbed by the fish gut and transported to other tissues for storage, energy production, or used as precursors for various metabolic pathways upon the hydrolysis of ester bonds that link the fatty acid chains to the glycerol backbone of phospholipid (8, 66). Unsaturated fatty acids of soybean lecithin, particularly the omega-3 and omega-6 fatty acids, are crucial for fish growth and health (55). However, choline which typically forms the polar head group of soybean lecithin, is metabolized differently depending on fish species and its nutritional requirements. The conversion of choline to betaine is one of the common pathways that facilitate osmoregulation in fish and profoundly affects a series of metabolic processes including DNA methylation, protein synthesis, lipid metabolism, and energy production (67, 68).

Betaine, the oxidized form of choline, usually serves as a methyl group donor that transfers methyl groups (CH_3) to metabolic compounds such as DNA, RNA, protein and lipid (69). With the addition of methyl group from betaine and alteration to the structure of DNA molecule, betaine regulates gene expression for growth development and immune responses (70). Furthermore, methylation process is essential for the synthesis of structural proteins and enzymes in aquaculture species. Lacking methyl groups in an organism likely to trigger a condition of under–methylation of DNA and concomitant activation of oncogenes (58, 71). Yet, these methyl groups cannot be synthesized by animals and can only be derived from diet (72). By regulating the activity of enzymes involved in the synthesis and breakdown of fatty acids, betaine impacts on fish lipid metabolism, which modifies the composition of body fat and cell membrane consequently (73). On top of that, betaine is also known to serve as an osmoprotectant in the energy production in fish by lowering the energy demands to regulate ions and cell volume, and supporting the conversion of homocysteine into methionine, an essential amino acid required for protein synthesis (70). Hence, the availability of methyl groups in the diet and functionality of soybean lecithin imparts a number of advantages to aquaculture species.

In addition to its role as the source of fatty acids and choline, soybean lecithin also delivers other important nutrients, such as phosphorus and vitamin E. Phosphorus is an essential mineral that is required for bone formation and other metabolic processes (74), while vitamin E is a powerful antioxidant that can protect cell membranes from oxidative damage (75). By providing a balanced and nutritionally complete diet, soybean lecithin is well-documented to enhance growth performance, survival, proper bone skeletal formation and stress mitigation (76, 77).

Overall, mode of action of soybean lecithin in aquafeed highly relies on its emulsifying properties, as well as its abilities to enhance digestibility and nutritional quality of the aquafeed. Depending on the composition of phospholipid which includes the fatty acids chains, glycerol backbone, phosphate group, and the polar head group, soybean lecithin contributes crucial roles to the growth performance, lipid and carbohydrate metabolisms, nutrient utilization, antioxidant activities and stress resistance in farmed species (78).

4. Effects of soybean lecithin on the feed utilization and growth performance of aquaculture species

Extensive studies have been conducted to evaluate the effect of dietary phospholipid in different aquaculture species at different stages by using egg yolk, soybean and marine lecithin in the aquafeed formulation. Of major interest in aquafeed supplementation are the PC, PE and PI (79, 80). Soybean lecithin has been receiving considerable attention in aquafeed formulation due to their implication in numerous metabolic pathways and regulation processes that improve the feed utilization and growth performance of larval and juvenile fish species such as red sea bream, *Pagrus major* (81), knifejaw, *Oplegnathus fasciatus* (81), ayu, *Plecoglossus altivelis* (82), rainbow trout, *Oncorhynchus mykiss* (83), Atlantic salmon, *Salmo salar* (84–86), red drum, *Sciaenops ocellatus* (87), goldfish, *Carassius auratus* (88), common carp, *Cyprinus carpio* (89), seabass, *Dicentrarchus labrax* (74), Japanese flounder, *Paralichthys olivaceus* (90), cobia, *Rachycentron canadum* (91), amberjack *Seriola dumerili*(92), rohu, *Labeo rohita* (68), gilthead seabream, *Sparus aurata* (93, 94), silvery-black porgy, *Sparidentex hasta* (95), large yellow croaker, *Lamichthys crocea* (57), Nile tilapia, *Oreochromis niloticus* (96), hybrid grouper, *Epinephelus fuscoguttatus* x *E. lancolatus* (60), and giant grouper, *E. lanceolatus* (97) (Supplementary Table 1). There are numerous explanations on how the inclusion of soybean lecithin possibly promotes feed utilization and growth performance of the larval aquaculture species. Dietary soybean lecithin, notably phospholipids, has been showing stimulating effects on larval growth and survival when *de novo* synthesis of phospholipids in larval fish was insufficient to meet the requirements of the fish during their early development (98, 99). High phospholipids content in the dietary soybean lecithin not only serves as a superior energy source to the larvae (8), but also promotes the conversion of phospholipids to other lipids, including diacylglycerol, diphosphatidylglycerol, PUFAs, and cholesterol (100). Comparatively, larval stages are more vulnerable to dietary phospholipid deficiency and require more dietary phospholipids than the juveniles. Phospholipids requirements for larval fish varied from 2 to 12% with higher requirements among the marine larval fish (8, 100). As larval stages are highly sensitive to phospholipid deficiency, inclusion of soybean lecithin in microparticulate diet serves an essential role to satisfy phospholipid requirement for ontogenetic development at the expense of fish oil, fish meal and live food. For instance,

linoleic acid, a polyunsaturated fatty acid that fish require but cannot be produced on their own, is provided by soybean lecithin (101). Soybean lecithin is known to be easily digested by fish into lysophosphatidylcholine form and absorbed directly by fish (102). The distribution and digestion of ingested free oleic acid (18:1n-9) in fish larvae were significantly influenced by dietary phosphatidylcholine (103, 104). Furthermore, supplemental emulsifier such as soybean lecithin can stimulate the production of lipoprotein in fish digestive system to enhance feed digestibility (105). High PC composition in soybean lecithin functions as an age-dependent feed attractant in enhancing feeding activity to fish larvae and therefore displaying a notable impact on fish development (106, 107).

Besides finfish species, soybean lecithin also demonstrated significant role in supporting good growth performance in a number of larval and juvenile penaeid species, including *Penaeus japonicus* (65, 108), *Penaeus merguiensis* (109), *Penaeus monodon* (66, 110), *Litopenaeus vannamei* (53, 111–113), and *Macrobrachium rosenbergii* (114, 115). The dietary phospholipid is essential for promoting greater rates of cholesterol turnover from the gut to the circulatory system in crustaceans (116). In recent studies, larval mud crab, *Scylla serrata* (117), juvenile swimming crab, *Portunus trituberculatus* (118, 119) and Chinese mitten crab, *Eriocheir sinensis* (120, 121) also benefited from the inclusion of soybean lecithin that enhances feed utilization, improves survival rate, promotes growth performance and molting frequency. Besides growth performance, positive effect of dietary soybean lecithin is gaining attention on gonadal development of the brood stock such as Chinese mitten crab, *Eriocheir sinensis* (122), swimming crab, *Portunus trituberculatus* (123, 124), red claw crayfish, *Cherax quadricarinatus* (125), and adult sea urchin, *Strongylocentrotus intermedius* (126). Nevertheless, juvenile sea urchin (127) is observed to have reduced weight gain when increased dietary phospholipid levels are provided and these excessive phospholipids eventually converted to neutral lipid in the gut and gonad (Supplementary Table 1).

In feed formulations for juvenile black seabream, *Acanthopagrus schlegeli*, soybean oil may be used up to 60 to 80% in place of fish oil. However, fully replacement of fish oil with soybean oil in the feed formulation is not recommended as it can reduce growth performance in juvenile black seabream (128). This was supported by the studies of Seiliez et al. (129) that growth performance of larval gilthead seabream, *Sparus aurata* was affected when soybean lecithin was utilized as a total replacement of live feed whereas overdose of soybean lecithin in the feed formulation (> 35.6 g/ kg diet) lead to decline in growth performance of early juvenile milkfish, *Chanos chanos* (130). On the other hand, some aquaculture species were found to perform better when marine lecithin was administered instead of soybean lecithin (93, 131). Similar finding was also observed in the study of Salini et al. (132) where juvenile barramundi, *Lates calcarifer* received marine lecithin from krill showed better growth performance than those received soybean lecithin. This may be attributed to the presence of high concentration of PUFAs mainly the EPA and DHA in marine lecithin which are important in promoting growth performance of aquatic animals in the early stage of life (27). As demonstrated by Liu et al. (133), EPA is needed to improve larval growth and survival when DHA level is high but arachidonic acid (ARA) level is low. Although marine lecithin tends to outperform as growth promoter for aquaculture species, soybean lecithin is favored as an alternative growth promoter because it is more viable and economically wise compared to marine lecithin from krill and fish meal. Moreover, study by Jaxion-Harm (56) established that phospholipids derived from soybean lecithin are not significantly different from marine sources such as krill and fish meal in promoting growth performance of Atlantic salmon, *Salmo salar* fry (Supplementary Table 1).

5. Effects of soybean lecithin on the abiotic stressors of aquaculture species

Global warming and climate change are issues being highlighted in recent years. These issues have an impact on world aquaculture production in which increased water temperature is one of the abiotic stressors to aquatic animals (134). Sensitivity of fish to xenobiotics is also indirectly influenced by the fluctuations of temperature. Presence of organochlorine pesticides such as endosulfan in the water was found to aggravate the situation by causing a 2.6 to 6°C reduction in thermal tolerance of freshwater fishes (135). Pesticides can seep into the aquatic environment as a result of extensive usage in agricultural activities and it is highly toxic to aquatic animals (136, 137). Thus, the nutritional approach is employed as one of the strategies to enhance the immune system and allow aquatic animals to be resistant to different stressors, especially to thermal stress.

It has been observed that inclusion of dietary soybean lecithin has a beneficial effect on thermal tolerance in aquaculture species which allows the aquaculture species to be more resilient to the fluctuation of water temperature. For instance, Kumar et al. (58) postulated that 1.5 to 2% of soybean lecithin helps milkfish, *Chanos chanos* coping stress from high temperature up to 46.4°C via protection of antioxidative status and neurotransmitter enzymes. Besides, soybean lecithin was observed to allow fish being resistant to stress caused by low temperature (138). Thermal tolerance of aquatic animals is influenced by many factors such as the presence of toxic in the water (139), species of aquatic animals (140), size of aquatic animals (141) and acclimation temperature (142). In this case, antioxidant defense system of fish may be fortified by soybean lecithin and thereby less susceptible to fluctuations in water temperature. Not only serves as the raw material in the repair of cell damage caused by thermal stress, soybean lecithin also promotes the expression of heat shock protein, which offers protection to the cells against the accumulation of altered proteins caused by high or low temperature stress (143–145).

Other than thermal stress, soybean lecithin is found to be effective in helping aquaculture species to cope with stress caused by hypoxic condition (78, 100), changes in water salinity (57), and the presence of pesticide in the water (68). Therefore, supplementation of lecithin from soybean in the feed formulation for aquaculture species is noteworthy to enhance stress tolerance of aquaculture species toward abiotic stressors (Supplementary Table 1).

6. Effects of soybean lecithin on the intestinal health, whole body total lipid content, antioxidant capacity and immunity of aquaculture species

Despite those phospholipid and fatty acid components of soybean lecithin are playing important roles in the health maintenance of various aquaculture species, documentation on the effects of soybean lecithin on intestinal health, whole body total lipid content, antioxidant capacity and immunity of aquaculture species are relatively limited in the past. Supplementation of soybean lecithin in the micro diet has been observed to prevent intestinal steatosis in the larval common carp, *Cyprinus carpio* (146), and promotes intestinal health of juvenile red drum, *Sciaenops ocellatus* (87), larval largemouth bass, *Micropterus salmoides* (147), yellow drum, *Nibea albiflora* (148), and adult Nile tilapia, *Oreochromis niloticus* (105). In addition, phospholipid in the soybean lecithin helps to regulate lipid metabolism and increases body lipid content of juvenile amberjack, *Seriola dumerili* (92), large yellow croaker, *Larmichthys crocea* (63), silvery-black porgy, *Sparidentex hasta* (95), hybrid grouper, *Epinephelus fuscoguttatus* × *E. lancolatus* (149). Higher body lipid content is obtained when fatty acids delivery and uptake in the fish are improved (Supplementary Table 1).

In many organisms, antioxidant system functions to mitigate the effects of reactive oxygen species (ROS) by protecting and repairing cells from oxidative damage. Dietary supplementation of soybean lecithin is capable to trigger antioxidant responses in aquaculture species when dealing with oxidative stress, or the elevation of ROS at intracellular level (150, 151). Activities of radical scavenging enzymes such as the superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione-S-transferase (GST) and glutathione reductase (GR) have been used as effective biomarkers to examine the effects of dietary phospholipid on enzymatic function and biochemical pathways in several studies involving larval Dojo loach, *Misgurnus anguillicaudatus* (152), common carp, *Cyprinus carpio* (153), golden mahseer, *Tor putitora* (138), stellate sturgeon, *Acipenser stellatus* (75), gilthead seabream, *Sparus aurata* (94), and hybrid snakehead, *Channa argus x C. maculata* (154). As a consequence of antioxidant capacity-promoting effect of dietary phospholipid, lipid peroxidation is reduced and survival rate is increased in the fish (63).

Dietary supplementation of soybean lecithin is also associated with the enhancement of fish systemic immunity against bacterial infection. According to Adel et al. (153), better immunostimulatory effect was reflected by common carp, *Cyprinus carpio* that received 3% soybean lecithin-enriched diet with an increase of mucosal immune parameters including alkaline phosphatase, lysozyme, protease, and esterase activity. These enzymes are involved in the regulatory secretion of antimicrobial peptides to suppress bacterial pathogens such as *Aeromonas hydrophila*, *Streptococcus iniae*, *Yersinia ruckeri*, and *Lactococcus garviea*. The enhancement on immunity and phagocytic activity is further supported by Jafari et al. (155) with an inclusion of 3.3% soybean lecithin for juvenile stellate sturgeon, *Acipenser stellatus*, 6–9% of soybean lecithin for pre-spawning Caspian brown trout, *Salmo trutta caspius* (156), and 0.3 g of soybean lecithin-containing bioemulsifier Lysomax® per kg of diet for the adult Nile tilapia, *Oreochromis niloticus* (105). In contrast, there is almost no observable impact on immune genes expression in golden mahseer fry when diet is supplemented with soybean lecithin (138). Similarly, diet supplemented with 4% soybean lecithin showed limited effects on the innate immune system and whole-body composition of juvenile channel catfish, *Ictalurus punctatus*, despite an improvement on feed conversion (157). Further studies need to be carried on different aquaculture species in order to be conclusive on the effect of dietary soybean lecithin on immunity enhancement.

7. Conclusion and future perspectives

Lecithin is obtainable from a wide variety of sources, including terrestrial and aquatic animals and plants. Different sources of lecithin have different compositions of phospholipids and fatty acids which determine the structural and functional roles of lecithin. Despite the fact that marine lecithin contains a high concentration of PUFAs, especially EPA and DHA, soybean lecithin is leading the role as growth promoter in the production of major aquaculture species because it is more practical and cost-effective for aquafeed formulation. Many studies have been included in this review to evaluate the potential of soybean lecithin supplementation and substitution for marine lecithin and live feed in the micro diet of aquaculture species in their early life stages. As concluded from the literature, proper supplementation of phospholipids from soybean lecithin in the larval and juvenile diet have significantly improves growth, survival, feed utilization, lipid metabolism, skeletal formation, stress tolerance, antioxidant capacity, and immune response in a number of finfish and shellfish species from larval to juvenile stage. However, research on the effect of dietary soybean lecithin on pre-reproductive phase and brood stock of crustacean species are relatively lacking. In this regard, future work that address the application of soybean lecithin in the diet formulation for pre-reproductive and brood stock of crustacean species is strongly recommended, with emphasis on enhancing lipid utilization, gonadal development and seed quality.

Author contributions

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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.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1188659/full#supplementary-material

References

1. Ekmekci H, Gül M. Economic structure and problems of trout enterprises: a case of Fethiye. *Turkish J Agric Food Sci Technol.* (2017) 5:33–42. doi: 10.24925/turjaf.v5i1.33-42.806

2. Aponte FR, Tveterås S. On the drivers of cost changes in the Norwegian salmon aquaculture sector: a decomposition of a flexible cost function from 2001 to 2014. *Aquac Econ Manag.* (2019) 23:276–91. doi: 10.1080/13657305.2018.1551438

3. Turchini GM, Trushenski JT, Glencross BD. Thoughts for the future of aquaculture nutrition: realigning perspectives to reflect contemporary issues related to judicious use of marine resources in aquafeeds. *N Am J Aquac.* (2019) 81:13–39. doi: 10.1002/naaq.10067

4. Johnson W, Bergfeld WF, Belsito D V, Hill RA, Klaassen CD, Liebler DC, et al. Safety assessment of lecithin and other phosphoglycerides as used in cosmetics. *Int J Toxicol.* (2020) 39:5S–25S. doi: 10.1177/1091581820953123

5. Hardy RW, Brezas A. "Diet formulation and manufacture," In: Hardy RW, Kaushik SJ, editors. *Fish Nutrition*. Amsterdam: Elsevier (2022). p. 643–708.

6. Jala RCR, Prasad RBN. "Rice bran lecithin: compositional, nutritional, and functional characteristics," In: Moghis UA, Xu X, editors. *Polar Lipids: Biology, Chemistry, and Technology*. Amsterdam: Elsevier (2015). p. 35–55.

7. List GR. "Soybean lecithin: food, industrial uses, and other applications," In: Moghis UA, Xu X, editors. *Polar Lipids: Biology, Chemistry, and Technology*. Amsterdam: Elsevier (2015). p. 1–33.

8. Tocher DR, Bendiksen EÅ, Campbell PJ, Bell JG. The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture.* (2008) 280:21–34. doi: 10.1016/j.aquaculture.2008.04.034

9. Scholfield CR. Composition of soybean lecithin. *J Am Oil Chem Soc.* (1981) 58:889–92. doi: 10.1007/BF02659652

10. Hurst WJ, Martin RA. The analysis of phospholipids in soy lecithin by HPLC. *J Am Oil Chem Soc.* (1984) 61:1462–3. doi: 10.1007/BF02636365

11. Helmerich G, Koehler P. Comparison of methods for the quantitative determination of phospholipids in lecithins and flour improvers. *J Agric Food Chem.* (2003) 51:6645–51. doi: 10.1021/jf0345088

12. van Nieuwenhuyzen W, Tomás MC. Update on vegetable lecithin and phospholipid technologies. *Eur J Lipid Sci.* (2008) 110:472–86. doi: 10.1002/ejlt.200800041

13. Pires LN, Brandão GC, Teixeira LSG. Determination of phospholipids in soybean lecithin samples via the phosphorus monoxide molecule by high-resolution continuum source graphite furnace molecular absorption spectrometry. *Food Chem.* (2017) 225:162–6. doi: 10.1016/j.foodchem.2017.01.019

14. Sargent J, McEvoy L, Estevez A, Bell G, Bell M, Henderson J, et al. Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture.* (1999) 179:217–29. doi: 10.1016/S0044-8486(99)00191-X

15. D'Abramo LR, Bordner CE, Conklin DE, Baum NA. Essentiality of dietary phosphatidylcholine for the survival of juvenile lobsters. *J Nutr.* (1981) 111:425–31. doi: 10.1093/jn/111.3.425

16. Akiyama DM, Dominy WG, Lawrence AL. "Penaeid shrimp nutrition," In: Fast AW, James Lester L, editors. *Marine Shrimp Culture: Principles and Practices*. Amsterdam, The Netherlands: Elsevier Science (1992). p. 535–568 doi: 10.1016/B978-0-444-88606-4.50031-X

17. Carmona-Antoñanzas G, Taylor JF, Martinez-Rubio L, Tocher DR. Molecular mechanism of dietary phospholipid requirement of Atlantic salmon, *Salmo salar*, fry. *BBA Mol Cell Biol Lipids.* (2015) 1851:1428–41. doi: 10.1016/j.bbalip.2015.08.006

18. Cahu CL, Gisbert E, Villeneuve LAN, Morais S, Hamza N, Wold PA, et al. Influence of dietary phospholipids on early ontogenesis of fish. *Aquac Res.* (2009) 40:989–99. doi: 10.1111/j.1365-2109.2009.02190.x

19. Rossi M. "Use of lecithin and lecithin fractions," In: Huopalahti R, López-Fandiño R, Anton M, Schade R, editors. *Bioactive Egg Compounds*. Berlin Heidelberg: Springer-Verlag (2007). p. 229–239.

20. Wu Y, Wang T. Soybean lecithin fractionation and functionality. *J Am Oil Chem Soc.* (2003) 80:319–26. doi: 10.1007/s11746-003-0697-x

21. Hertrampf JW, Piedad-Pascual F. "Soya lecithin," In: *Handbook on Ingredients for Aquaculture Feeds*. Dordrecht: Springer (2000). p. 383–395.

22. Shurtleff W, Aoyagi A. *History of Lecithin and Phospholipids (1850-2016).* Lafayette, CA: Soyinfo Center (2016), 954.

23. Ahuja K, Malkani T. *Lecithin Market Share, Size and Statistics Report 2030.* (2022). Available online at: https://www.gminsights.com/industry-analysis/lecithin-market (accessed December 12, 2022).

24. Yu HY, Park SE, Chun HS, Rho JR, Ahn S. Phospholipid composition analysis of krill oil through HPLC with ELSD: development, validation, and comparison with 31P NMR spectroscopy. *J Food Compost Anal.* (2022) 107:104408. doi: 10.1016/j.jfca.2022.104408

25. Fernandes GD, Alberici RM, Pereira GG, Cabral EC, Eberlin MN, Barrera-Arellano D. Direct characterization of commercial lecithins by easy ambient sonic-spray ionization mass spectrometry. *Food Chem.* (2012) 135:1855–60. doi: 10.1016/j.foodchem.2012.06.072

26. Arab Tehrany E, Kahn CJF, Baravian C, Maherani B, Belhaj N, Wang X, et al. Elaboration and characterization of nanoliposome made of soya; rapeseed and salmon lecithins: application to cell culture. *Colloids Surf B Biointerfaces.* (2012) 95:75–81. doi: 10.1016/j.colsurfb.2012.02.024

27. Topuz OK, Aygün T, Alp AC, Yatmaz HA, Torun M, Yerlikaya P. Characterization and emulsifying properties of aquatic lecithins isolated from processing discard of rainbow trout fish and its eggs. *Food Chem.* (2021) 339:128103. doi: 10.1016/j.foodchem.2020.128103

28. Lončarević I, Pajin B, Petrović J, Zarić D, Sakač M, Torbica A, et al. The impact of sunflower and rapeseed lecithin on the rheological properties of spreadable cocoa cream. *J Food Eng.* (2016) 171:67–77. doi: 10.1016/j.jfoodeng.2015.10.001

29. Cabezas DM, Madoery R, Diehl BWK, Tomás MC. Emulsifying properties of different modified sunflower lecithins. *J Am Oil Chem Soc.* (2012) 89:355–61. doi: 10.1007/s11746-011-1915-8

30. Bot F, Cossuta D, O'Mahony JA. Inter-relationships between composition, physicochemical properties and functionality of lecithin ingredients. *Trends Food Sci Technol.* (2021) 111:261–70. doi: 10.1016/j.tifs.2021.02.028

31. Xie M, Dunford NT. Fractionating of canola lecithin from acid degumming and its effect. *Food Chem.* (2019) 300:125217. doi: 10.1016/j.foodchem.2019.125217

32. Belayneh HD, Wehling RL, Cahoon E, Ciftci ON. Lipid composition and emulsifying properties of *Camelina sativa* seed lecithin. *Food Chem.* (2018) 242:139–46. doi: 10.1016/j.foodchem.2017.08.082

33. Xia ZW, Zhang JG, Ni ZJ, Zhang F, Thakur K, Hu F, Wei ZJ. Functional and emulsification characteristics of phospholipids and derived o/w emulsions from peony seed meal. *Food Chem.* (2022) 389:133112. doi: 10.1016/j.foodchem.2022.133112

34. Liu H, Liu T, Fan H, Gou M, Li G, Ren H, et al. Corn lecithin for injection from deoiled corn germ: extraction, composition, and emulsifying properties. *Eur J Lipid Sci.* (2018) 120:1700288. doi: 10.1002/ejlt.201700288

35. Sun X, Zhang L, Tian S, Yang K, Xie J. Phospholipid composition and emulsifying properties of rice bran lecithin from enzymatic degumming. *LWT.* (2020) 117:108588. doi: 10.1016/j.lwt.2019.108588

36. Pragasam A, Indira TN, Gopala Krishna AG. Preparation and physico chemical characteristics evaluation of rice bran lecithin in relation to soya lecithin. *Beverage Food World.* (2002) 12:19–22.

37. Palacios LE, Wang T. Egg-yolk lipid fractionation and lecithin characterization. *J Am Oil Chem Soc.* (2005) 82:571–8. doi: 10.1007/s11746-005-1111-4

38. Rombaut R, Dewettinck K. Properties, analysis and purification of milk polar lipids. *Int Dairy J.* (2006) 16:1362–73. doi: 10.1016/j.idairyj.2006.06.011

39. Price N, Fei T, Clark S, Wang T. Extraction of phospholipids from a dairy by-product (whey protein phospholipid concentrate) using ethanol. *J Dairy Sci.* (2018) 101:8778–87. doi: 10.3168/jds.2018-14950

40. Burling H, Graverholt G. Milk - a new source for bioactive phospholipids for use in food formulations. *Lipid Technol.* (2008) 20:229–31. doi: 10.1002/lite.200800058

41. Schneider M. "Marine phospholipids and their applications: next-generation omega-3 lipids," In: De Meester F, Watson R, Zibadi S, editors. *Omega-6/3 Fatty Acids: Functions, Sustainability Strategies and Perspectives*. New Jersey, NJ: Humana Press Inc. (2013), p. 297–308.

42. Asaduzzaman AKM, Chun BS. Quality characteristics of lecithin isolated from deoiled mackerel (*Scomber japonicus*) muscle using different methods. *J Ind Eng Chem.* (2015) 21:620–6. doi: 10.1016/j.jiec.2014.03.029

43. Lee S-M, Asaduzzaman AKM, Chun B-S. Characterization of lecithin isolated from anchovy (*Engraulis japonica*) residues deoiled by supercritical carbon dioxide and organic solvent extraction. *J Food Sci.* (2012) 77:C773–8. doi: 10.1111/j.1750-3841.2012.02764.x

44. Belhaj N, Arab-Tehrany E, Linder M. Oxidative kinetics of salmon oil in bulk and in nanoemulsion stabilized by marine lecithin. *Process Biochemistry.* (2010) 45:187–95. doi: 10.1016/j.procbio.2009.09.005

45. Ahmmed MK, Carne A, Ahmmed F, Stewart I. (Sabrina) Tian H, Bekhit AEDA. Positional distribution of fatty acids and phospholipid composition in King salmon (*Oncorhynchus tshawytscha*) head, roe and skin using nuclear magnetic resonance spectroscopy. *Food Chem.* (2021) 363:130302. doi: 10.1016/j.foodchem.2021.130302

46. Ahmmed MK, Ahmmed F, Stewart I, Carne A, Tian H (Sabrina), Bekhit AEDA. Omega-3 phospholipids in Pacific blue mackerel (*Scomber australasicus*) processing by-products. *Food Chem.* (2021) 353:451. doi: 10.1016/j.foodchem.2021.129451

47. Uddin MS, Kishimura H, Chun BS. Isolation and characterization of lecithin from squid (*Todarodes pacificus*) viscera deoiled by supercritical carbon dioxide extraction. *J Food Sci.* (2011) 76:C350–4. doi: 10.1111/j.1750-3841.2010.02039.x

48. Yang B, Zhou R, Yang JG, Wang YH, Wang WF. Insight into the enzymatic degumming process of soybean oil. *J Am Oil Chem Soc.* (2008) 85:421–5. doi: 10.1007/s11746-008-1225-y

49. Lordan R, Tsoupras A, Zabetakis I. Phospholipids of animal and marine origin: structure, function, and anti-inflammatory properties. *Molecules.* (2017) 22:1964. doi: 10.3390/molecules22111964

50. Szuhaj BF. "Phospholipids: properties and occurrence," In: Caballero B, Finglas PM, Toldra F, editors. *Encyclopedia of Food and Health*. Kidlington, United Kingdom: Academic Press (2016), p. 360–364.

51. Nguyen MT, Van De Walle D, Petit C, Beheydt B, Depypere F, Dewettinck K. Mapping the chemical variability of vegetable lecithins. *J Am Oil Chem Soc.* (2014) 91:1093–101. doi: 10.1007/s11746-014-2455-9

52. Pokorný J. "Production, separation and modification of phospholipids for use in food." In: Gunstone FD, editor. *Modifying Lipids for Use in Food*. Cambridge: Woodhead Publishing (2006), p. 369–390.

53. Gong H, Lawrence AL, Jiang DH, Castille FL, Gatlin DM. Lipid nutrition of juvenile *Litopenaeus vannamei*: I. Dietary cholesterol and de-oiled soy lecithin requirements and their interaction. *Aquaculture.* (2000) 190:305–24. doi: 10.1016/S0044-8486(00)00414-2

54. Sun N, Chen J, Wang D, Lin S. Advance in food-derived phospholipids: Sources, molecular species and structure as well as their biological activities. *Trends Food Sci Technol.* (2018) 80:199–211. doi: 10.1016/j.tifs.2018.08.010

55. Turchini GM, Francis DS, Du Z-Y, Olsen RE, Ringø E, Tocher DR. "The lipids," In: Hardy RW, Kaushik SJ, editors. *Fish Nutrition*. Amsterdam: Elsevier (2022). p. 303–467.

56. Jaxion-Harm J. Effects of dietary phospholipids on early stage Atlantic Salmon (*Salmo salar*) performance: a comparison among phospholipid sources. *Aquaculture.* (2021) 544:737055. doi: 10.1016/j.aquaculture.2021.737055

57. Zhao J, Ai Q, Mai K, Zuo R, Luo Y. Effects of dietary phospholipids on survival, growth, digestive enzymes and stress resistance of large yellow croaker, *Larmichthys crocea* larvae. *Aquaculture.* (2013) 411:122–8. doi: 10.1016/j.aquaculture.2013.05.018

58. Kumar N, Minhas PS, Ambasankar K, Krishnani KK, Rana RS. Dietary lecithin potentiates thermal tolerance and cellular stress protection of milk fish (*Chanos chanos*) reared under low dose endosulfan-induced stress. *J Therm Biol.* (2014) 46:40–6. doi: 10.1016/j.jtherbio.2014.10.004

59. Li Y, Gao J, Huang S. Effects of different dietary phospholipid levels on growth performance, fatty acid composition, PPAR gene expressions and antioxidant responses of blunt snout bream *Megalobrama amblycephala* fingerlings. *Fish Physiol Biochem.* (2015) 41:423–36. doi: 10.1007/s10695-014-9994-8

60. Huang Y, Xu J, Sheng Z, Chen N, Li S. Integrated response of growth performance, fatty acid composition, antioxidant responses and lipid metabolism to dietary phospholipids in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) larvae. *Aquaculture.* (2021) 541:736728. doi: 10.1016/j.aquaculture.2021.736728

61. Gisbert E, Villeneuve L, Zambonino-Infante JL, Quazuguel P, Cahu CL. Dietary phospholipids are more efficient than neutral lipids for long-chain polyunsaturated fatty acid supply in European sea bass *Dicentrarchus labrax* larval development. *Lipids.* (2005) 40:609–18. doi: 10.1007/s11745-005-1422-0

62. Daprà F, Geurden I, Corraze G, Bazin D, Zambonino-Infante JL, Fontagné-Dicharry S. Physiological and molecular responses to dietary phospholipids vary between fry and early juvenile stages of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture.* (2011) 319:377–84. doi: 10.1016/j.aquaculture.2011.07.016

63. Cai Z, Feng S, Xiang X, Mai K, Ai Q. Effects of dietary phospholipid on lipase activity, antioxidant capacity and lipid metabolism-related gene expression in large yellow croaker larvae (*Larimichthys crocea*). *Comp Biochem Physiol B Biochem Mol Biol.* (2016) 201:46–52. doi: 10.1016/j.cbpb.2016.06.007

64. Rampone AJ, Machida CM. Mode of action of lecithin in suppressing cholesterol absorption. *J Lipid Res.* (1981) 22:744–52. doi: 10.1016/S0022-2275(20)37345-4

65. Teshima S, Kanazawa A, Kakuta Y. Growth, survival, and body lipid composition of the prawn larvae receiving several dietary phospholipids. *Memoir Faculty Fisheries Kagoshima Univ.* (1986) 35:17–27.

66. Paibulkichakul C, Piyatiratitivorakul S, Kittakoop P, Viyakarn V, Fast AW, Menasveta P. Optimal dietary levels of lecithin and cholesterol for black tiger prawn *Penaeus monodon* larvae and postlarvae. *Aquaculture.* (1998) 167:273–81. doi: 10.1016/S0044-8486(98)00327-5

67. Ghosh TK, Chauhan YH, Mandal RN. Growth performance of *Labeo bata* (Hamilton, 1822) in freshwater and its acclimatization in brackish water with betaine as feed additive. *Aquaculture.* (2019) 501:128–34. doi: 10.1016/j.aquaculture.2018.11.020

68. Kumar N, Jadhao SB, Chandan NK, Kumar K, Jha AK, Bhushan S, et al. Dietary choline, betaine and lecithin mitigates endosulfan-induced stress in *Labeo rohita* fingerlings. *Fish Physiol Biochem.* (2012) 38:989–1000. doi: 10.1007/s10695-011-9584-y

69. Frontiera MS, Stabler SP, Kolhouse JF, Allen RH. Regulation of methionine metabolism: Effects of nitrous oxide and excess dietary methionine. *J Nutr Biochem.* (1994) 5:28–38. doi: 10.1016/0955-2863(94)90006-X

70. Geng H, Yang P, Chen Y, Qin Y, Li X, He C, et al. Dietary choline can partially spare methionine to improve the feeds utilization and immune response in juvenile largemouth bass (*Micropterus salmoides*): Based on phenotypic response to gene expression. *Aquac Rep.* (2023) 30:101546. doi: 10.1016/j.aqrep.2023.101546

71. Newberne PM. The methyl-deficiency model: History characteristics and research directions. *J Nutr Biochem.* (1993) 4:618–24. doi: 10.1016/0955-2863(93)90032-R

72. Kidd MT, Ferket PR, Garlich JD. Nutritional and osmoregulatory functions of betaine. *Worlds Poult Sci J.* (1997) 53:125–39. doi: 10.1079/WPS19970013

73. Hung SSO, Berge GM, Storebakken T. Growth and digestibility effects of soya lecithin and choline chloride on juvenile Atlantic salmon. *Aquac Nutr.* (1997) 3:141–4. doi: 10.1046/j.1365-2095.1997.00080.x

74. Cahu CL, Infante JLZ, Barbosa V. Effect of dietary phospholipid level and phospholipid: neutral lipid value on the development of sea bass (*Dicentrarchus labrax*) larvae fed a compound diet. *Br J Nutr.* (2003) 90:21–8. doi: 10.1079/BJN2003880

75. Jafari F, Noori F, Agh N, Estevez A, Ghasemi A, Alcaraz C, et al. Phospholipids improve the performance, physiological, antioxidative responses and, lpl and igf1 gene expressions in juvenile stellate sturgeon (*Acipenser stellatus*). *Aquaculture.* (2021) 541:736809. doi: 10.1016/j.aquaculture.2021.736809

76. Torfi Mozanzadeh M, Marammazi JG, Yaghoubi M, Agh N, Pagheh E, Gisbert E. Macronutrient requirements of Silvery-Black porgy (*Sparidentex hasta*): a

comparison with other farmed Sparid species. *Fishes.* (2017) 2:5. doi: 10.3390/fishes 2020005

77. Sivaramakrishnan T, Ambasankar K, Kumaraguru Vasagam KP, Syama Dayal J, Sandeep KP, Bera A, et al. Effect of dietary soy lecithin inclusion levels on growth, feed utilization, fatty acid profile, deformity and survival of milkfish (*Chanos chanos*) larvae. *Aquac Res.* (2021) 52:5366–74. doi: 10.1111/are.15406

78. Maleki Moghaddam MR, Agh N, Sarvi Moghanlou K, Noori F, Taghizadeh A, Gisbert E. Dietary conjugated linoleic acid (CLA) and lecithin affects levels of serum cholesterol, triglyceride, lipoprotein and hypoxic stress resistance in rainbow trout (*Oncorhynchus mykiss*). *Int J Aquat Biol.* (2021) 9:124–33. doi: 10.22034/ijab.v9i2.544

79. Azarm HM, Kenari AA, Hedayati M. Effect of dietary phospholipid sources and levels on growth performance, enzymes activity, cholecystokinin and lipoprotein fractions of rainbow trout (*Oncorhynchus mykiss*) fry. *Aquac Res.* (2013) 44:634–44. doi: 10.1111/j.1365-2109.2011.03068.x

80. Taylor JF, Martinez-Rubio L, Del Pozo J, Walton JM, Tinch AE, Migaud H, et al. Influence of dietary phospholipid on early development and performance of Atlantic salmon (*Salmo salar*). *Aquaculture.* (2015) 448:262–72. doi: 10.1016/j.aquaculture.2015.06.012

81. Kanazawa A, Teshima S, Inamori S, Matsubara H. Effects of dietary phospholipids on growth of the larval red sea bream and knife jaw. *Memoir Faculty Fish Kagoshima Univ.* (1983) 32:109–114.

82. Kanazawa A, Teshima S, Kobayashi T, Takae M, Iwashita T, Uehara R. Necessity of dietary phospholipids for growth of the larval ayu. *Memoir Faculty Fish Kagoshima Univ.* (1983) 32:115–120.

83. Poston HA. Performance of rainbow trout fry fed supplemental soy lecithin and choline. *The Progr Fish-Culturist.* (1990) 52:218–25.

84. Poston HA. Effect of body size on growth, survival, and chemical composition of Atlantic salmon fed soy lecithin and choline. *The Prog Fish-Culturist.* (1990) 52:226–30.

85. Poston HA. Response of Atlantic salmon fry to feed-grade lecithin and choline. *The Prog Fish-Culturist.* (1991) 53:224–8. doi: 10.1577/1548-8640(1991)053<0224:ROASFT>2.3.CO;2

86. De Santis C, Taylor JF, Martinez-Rubio L, Boltana S, Tocher DR. Influence of development and dietary phospholipid content and composition on intestinal transcriptome of Atlantic salmon (*Salmo salar*). *PLoS ONE.* (2015) 10:e0140964. doi: 10.1371/journal.pone.0140964

87. Craig SR, Gatlin DM. Growth and body composition of juvenile red drum (*Sciaenops ocellatus*) fed diets containing lecithin and supplemental choline. *Aquaculture.* (1997) 151:259–67. doi: 10.1016/S0044-8486(96)01479-2

88. Lochmann R, Brown R. Soybean-lecithin supplementation of practical diets for juvenile goldfish (*Carassius auratus*). *J Am Oil Chem Soc.* (1997) 74:149–52. doi: 10.1007/s11746-997-0160-7

89. Geurden I, Charlon N, Marion D, Bergot P. Influence of purified soybean phospholipids on early development of carp. *Aquac Int.* (1997) 5:137–149.

90. Uyan O, Koshio S, Ishikawa M, Uyan S, Ren T, Yokoyama S, et al. Effects of dietary phosphorus and phospholipid level on growth, and phosphorus deficiency signs in juvenile Japanese flounder, *Paralichthys olivaceus*. *Aquaculture.* (2007) 267:44–54. doi: 10.1016/j.aquaculture.2007.01.020

91. Niu J, Liu YJ, Tian LX, Mai KS, Yang HJ, Ye CX, et al. Effects of dietary phospholipid level in cobia (*Rachycentron canadum*) larvae: growth, survival, plasma lipids and enzymes of lipid metabolism. *Fish Physiol Biochem.* (2008) 34:9–17. doi: 10.1007/s10695-007-9140-y

92. Uyan O, Koshio S, Ishikawa M, Yokoyama S, Uyan S, Ren T, et al. The influence of dietary phospholipid level on the performances of juvenile amberjack, *Seriola dumerili*, fed non-fishmeal diets. *Aquac Nutr.* (2009) 15:550–7. doi: 10.1111/j.1365-2095.2008.00621.x

93. Saleh R, Betancor MB, Roo J, Benítez-Dorta V, Zamorano MJ, Bell JG, et al. Effect of krill phospholipids versus soybean lecithin in microdiets for gilthead seabream (*Sparus aurata*) larvae on molecular markers of antioxidative metabolism and bone development. *Aquac Nutr.* (2015) 21:474–88. doi: 10.1111/anu.12177

94. Saleh NE, Wassef EA, Kamel MA, El-Haroun ER, El-Tahan RA. Beneficial effects of soybean lecithin and vitamin C combination in fingerlings gilthead seabream (*Sparus aurata*) diets on; fish performance, oxidation status and genes expression responses. *Aquaculture.* (2022) 546:737345. doi: 10.1016/j.aquaculture.2021.737345

95. Pagheh E, Agh N, Marammazi JG, Nouri F, Sepahdari A, Gisbert E, et al. Dietary soybean lecithin affects growth performance, fillet biochemical composition and digestive enzyme activity in *Sparidentex hasta* juvenile. *J Appl Anim Res.* (2019) 47:24–33. doi: 10.1080/09712119.2018.1557663

96. El-Naggar K, Mohamed R. El-katcha MI, Abdo SE, Soltan MA. Plant ingredient diet supplemented with lecithin as fish meal and fish oil alternative affects growth performance, serum biochemical, lipid metabolism and growth-related gene expression in Nile tilapia. *Aquac Res.* (2021) 52:6308–21. doi: 10.1111/are.15494

97. Lin Y-H, Chen K-H, Wu P-C. Effects of the diet based on soybean meal supplemented with soy lecithin on growth, biochemical parameters and digestibility of nutrients in grouper, *Epinephelus lanceolatus*. *Aquac Res.* (2022) 53:700–6. doi: 10.1111/are.15593

98. Coutteau P, Geurden I, Camara MR, Bergot P, Sorgeloos P. Review on the dietary effects of phospholipids in fish and crustacean larviculture. *Aquaculture.* (1997) 155:149–64. doi: 10.1016/S0044-8486(97)00125-7

99. Sargent JR, Tocher DR, Bell JG. "The lipids," In: Halver JE, Hardy RW, editors. *Fish Nutrition*. San Diego, CA: Elsevier (Academic Press) (2011). p. 181–257.

100. Tan P, Zhang P, Zhang L, Zhu W, Wang L, Chen R, et al. Effects of soybean lecithin on growth performance, intestine morphology, and liver tissue metabolism in rock bream (*Oplegnathus fasciatus*) larvae. *Front Mar Sci.* (2022) 9:942259. doi: 10.3389/fmars.2022.942259

101. Hamza N, Mhetli M, Khemis I. Ben, Cahu C, Kestemont P. Effect of dietary phospholipid levels on performance, enzyme activities and fatty acid composition of pikeperch (*Sander lucioperca*) larvae. *Aquaculture.* (2008) 275:274–82. doi: 10.1016/j.aquaculture.2008.01.014

102. Llorente I, Fernández-Polanco J, Baraibar-Diez E, Odriozola MD, Bjørndal T, Asche F, et al. Assessment of the economic performance of the seabream and seabass aquaculture industry in the European Union. *Mar Policy.* (2020) 117:103876. doi: 10.1016/j.marpol.2020.103876

103. Hadas E, Koven W, Sklan D, Tandler A. The effect of dietary phosphatidylcholine on the assimilation and distribution of ingested free oleic acid (18:1n–9) in gilthead seabream (*Sparus aurata*) larvae. *Aquaculture.* (2003) 217:577–88. doi: 10.1016/S0044-8486(02)00431-3

104. Olsen RE, Myklebust R, Kaino T. Ringø E. Lipid digestibility and ultrastructural changes in the enterocytes of Arctic char (*Salvelinus alpinus* L) fed linseed oil and soybean lecithin. *Fish Physiol Biochem.* (1999) 21:35–44. doi: 10.1023/A:1007726615889

105. El-Sayed AFM, Tammam MS, Makled SO. Lecithin-containing bioemulsifier boosts growth performance, feed digestion and absorption and immune response of adult Nile tilapia (*Oreochromis niloticus*). *Aquac Nutr.* (2021) 27:757–70. doi: 10.1111/anu.13221

106. Koven WM, Parra G, Kolkovski S, Tandler A. The effect of dietary phosphatidylcholine and its constituent fatty acids on microdiet ingestion and fatty acid absorption rate in gilthead sea bream, *Sparus auratus*, larvae. *Aquac Nutr.* (1998) 4:39–45. doi: 10.1046/j.1365-2095.1998.00101.x

107. Koven WM, Kolkovski S, Tandler A, Kissil GW, Sklan D. The effect of dietary lecithin and lipase, as a function of age, on n-9 fatty acid incorporation in the tissue lipids of *Sparus aurata* larvae. *Fish Physiol Biochem.* (1993) 10:357–64. doi: 10.1007/BF00004502

108. Kanazawa A, Teshima SI, Sakamoto M. Effects of dietary lipids, fatty acids, and phospholipids on growth and survival of prawn (*Penaeus japonicus*) larvae. *Aquaculture.* (1985) 50:39–49. doi: 10.1016/0044-8486(85)90151-6

109. Thongrod S, Boonyaratpalin M. Cholesterol and lecithin requirement of juvenile banana shrimp, *Penaeus merguiensis*. *Aquaculture.* (1998) 161:315–21. doi: 10.1016/S0044-8486(97)00278-0

110. Kumaraguru Vasagam KP, Ramesh S, Balasubramanian T. Dietary value of different vegetable oil in black tiger shrimp *Penaeus monodon* in the presence and absence of soy lecithin supplementation: effect on growth, nutrient digestibility and body composition. *Aquaculture.* (2005) 250:317–27. doi: 10.1016/j.aquaculture.2005.02.035

111. Coutteau P, Camara MR, Sorgeloos P. The effect of different levels and sources of dietary phosphatidylcholine on the growth, survival, stress resistance, and fatty acid composition of postlarval *Penaeus vannamei*. *Aquaculture.* (1996) 147:261–73. doi: 10.1016/S0044-8486(96)01387-7

112. Hu Y, Tan B, Mai K, Ai Q, Zhang L, Zheng S. Effects of dietary menhaden oil, soybean oil and soybean lecithin oil at different ratios on growth, body composition and blood chemistry of juvenile *Litopenaeus vannamei*. *Aquac Int.* (2011) 19:459–73. doi: 10.1007/s10499-010-9361-4

113. Liou C-H, To V-A, Zhang Z-F, Lin Y-H. The effect of dietary lecithin and lipid levels on the growth performance, body composition, hemolymph parameters, immune responses, body texture, and gene expression of juvenile white shrimp (*Litopenaeus vannamei*). *Aquaculture.* (2023) 567:739260. doi: 10.1016/j.aquaculture.2023.739260

114. Briggs MRP, Jauncey K, Brown JH. The cholesterol and lecithin requirements of juvenile prawn (*Macrobrachium rosenbergii*) fed semi-purified diets. *Aquaculture.* (1988) 70:121–9. doi: 10.1016/0044-8486(88)90011-7

115. Hien TTT, Hai TN, Phuong NT, Ogata HY, Wilder MN. The effects of dietary lipid sources and lecithin on the production of giant freshwater prawn *Macrobrachium rosenbergii* larvae in the Mekong Delta region of Vietnam. *Fisheries Sci.* (2005) 71:279–86. doi: 10.1111/j.1444-2906.2005.00961.x

116. Teshima S, Kanazawa A, Kakuta Y. Effects of dietary phospholipids on growth and body composition of the juvenile prawn. *Bullet Soc Sci Fish.* (1986) 52:155–8. doi: 10.2331/suisan.52.155

117. Holme MH, Southgate PC, Zeng C. Assessment of dietary lecithin and cholesterol requirements of mud crab, *Scylla serrata*, megalopa using semi-purified microbound diets. *Aquac Nutr.* (2007) 13:413–23. doi: 10.1111/j.1365-2095.2007.00492.x

118. Li X, Wang J, Han T, Hu S, Jiang Y, Wang C. Effect of dietary phospholipids levels and sources on growth performance, fatty acid composition of

the juvenile swimming crab, *Portunus trituberculatus*. *Aquaculture.* (2014) 430:166–72. doi: 10.1016/j.aquaculture.2014.03.037

119. Wang JT, Han T, Li XY, Hu SX, Jiang YD, Wang CL. Effects of dietary phosphatidylcholine (PC) levels on the growth, molt performance and fatty acid composition of juvenile swimming crab, *Portunus trituberculatus*. *Anim Feed Sci Technol.* (2016) 216:225–33. doi: 10.1016/j.anifeedsci.2016.03.023

120. Lin Z, Han F, Lu J, Guo J, Qi C, Wang C, et al. Influence of dietary phospholipid on growth performance, body composition, antioxidant capacity and lipid metabolism of Chinese mitten crab, *Eriocheir sinensis*. *Aquaculture.* (2020) 516:734653. doi: 10.1016/j.aquaculture.2019.734653

121. Lin Z, Bu X, Wang N, Lei Y, Liu S, Wang X, et al. Dietary phospholipid alleviates the adverse effects of high-lipid diet in Chinese mitten crab (*Eriocheir sinensis*). *Aquaculture.* (2021) 531:735899. doi: 10.1016/j.aquaculture.2020.735899

122. Sui LY, Wu XG, Wille M, Cheng YX, Sorgeloos P. Effect of dietary soybean lecithin on reproductive performance of Chinese mitten crab *Eriocheir sinensis* (H. Milne-Edwards) broodstock. *Aquac Int.* (2009) 17:45–56. doi: 10.1007/s10499-008-9178-6

123. Ding LY, Jin M, Sun P, Lu Y, Ma HN, Yuan Y, et al. Cloning, tissue expression of the fatty acid-binding protein (Pt-FABP1) gene, and effects of dietary phospholipid levels on fabp and vitellogenin gene expression in the female swimming crab *Portunus trituberculatus*. *Aquaculture.* (2017) 474:57–65. doi: 10.1016/j.aquaculture.2017.03.029

124. Song D, Shi B, Ding L, Jin M, Sun P, Jiao L, et al. Regulation of dietary phospholipids on growth performance, antioxidant activities, phospholipid metabolism and vitellogenesis in pre reproductive phase of female swimming crabs, *Portunus trituberculatus*. *Aquaculture.* (2019) 511:734230. doi: 10.1016/j.aquaculture.2019.734230

125. Wang L, Zuo D, Lv W, Li J, Wang Q, Zhao Y. Effects of dietary soybean lecithin on gonadal development and vitellogenin mRNA expression in the female redclaw crayfish *Cherax quadricarinatus* (von Martens) at first maturation. *Aquac Res.* (2013) 44:1167–76. doi: 10.1111/j.1365-2109.2012.03128.x

126. Zhang F, Ning Y, Yuan R, Ding J, Chang Y, Zuo R. Effects of soya lecithin addition on the growth, antioxidant capacity, gonad development and nutritional quality of adult sea urchin (*Strongylocentrotus intermedius*). *Aquac Rep.* (2022) 22:100990. doi: 10.1016/j.aqrep.2021.100990

127. Gibbs VK, Watts SA, Lawrence AL, Lawrence JM. Dietary phospholipids affect growth and production of juvenile sea urchin *Lytechinus variegatus*. *Aquaculture.* (2009) 292:95–103. doi: 10.1016/j.aquaculture.2009.03.046

128. Peng S, Chen L, Qin JG, Hou J, Yu N, Long Z, et al. Effects of replacement of dietary fish oil by soybean oil on growth performance and liver biochemical composition in juvenile black seabream, *Acanthopagrus schlegeli*. *Aquaculture.* (2008) 276:154–61. doi: 10.1016/j.aquaculture.2008.01.035

129. Seiliez I, Bruant JS, Zambonino Infante JL, Kaushik S, Bergot P. Effect of dietary phospholipid level on the development of gilthead sea bream (*Sparus aurata*) larvae fed a compound diet. *Aquac Nutr.* (2006) 12:372–8. doi: 10.1111/j.1365-2095.2006.00436.x

130. Balito-Liboon JS, Ferdinand R, Traifalgar M, Pagapulan MJBB, Mameloco EJG, Temario EE. Dietary soybean lecithin enhances growth performance, feed utilization efficiency and body composition of early juvenile milkfish, *Chanos chanos*. *Israeli J Aquac Bamidgeh.* (2018) 70:20297. doi: 10.46989/001c.20927

131. Alves Martins D, Estévez A, Stickland NC, Simbi BH, Yúfera M. Dietary lecithin source affects growth potential and gene expression in *Sparus aurata* larvae. *Lipids.* (2010) 45:1011–23. doi: 10.1007/s11745-010-3471-7

132. Salini MJ, Wade N, Bourne N, Turchini GM, Glencross BD. The effect of marine and non-marine phospholipid rich oils when fed to juvenile barramundi (*Lates calcarifer*). *Aquaculture.* (2016) 455:125–35. doi: 10.1016/j.aquaculture.2016.01.013

133. Liu J, Caballero MJ, Izquierdo M, El-Sayed Ali T, Hernández-Cruz CM, Valencia A, et al. Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead sea bream *Sparus aurata*. *Fisheries Sci.* (2002) 68:1165–72. doi: 10.1046/j.1444-2906.2002.00551.x

134. Joshi KD, Das SCS, Pathak RK, Khan A, Sarkar UK, Roy K. Pattern of reproductive biology of the endangered golden mahseer *Tor putitora* (Hamilton 1822) with special reference to regional climate change implications on breeding phenology from lesser Himalayan region, India. *J Appl Anim Res.* (2018) 46:1289–95. doi: 10.1080/09712119.2018.1497493

135. Patra RW, Chapman JC, Lim RP, Gehrke PC. The effects of three organic chemicals on the upper thermal tolerances of four freshwater fishes. *Environ Toxicol Chem.* (2007) 26:1454–9. doi: 10.1897/06-156R1.1

136. Cengiz EI, Unlu E. Histopathological changes in the gills of mosquitofish, *Gambusia affinis* exposed to endosulfan. *Bull Environ Contam Toxicol.* (2002) 68:290–6. doi: 10.1007/s001280251

137. Pawar AP, Sanaye SV, Shyama S, Sreepada RA, Dake AS. Effects of salinity and temperature on the acute toxicity of the pesticides, dimethoate and chlorpyrifos in post-larvae and juveniles of the whiteleg shrimp. *Aquac Rep.* (2020) 16:100240. doi: 10.1016/j.aqrep.2019.100240

138. Ciji A, Akhtar MS, Tripathi PH, Pandey A, Rajesh M, Kamalam BS. Dietary soy lecithin augments antioxidative defense and thermal tolerance but fails to modulate non-specific immune genes in endangered golden mahseer (*Tor putitora*) fry. *Fish Shellfish Immunol.* (2021) 109:34–40. doi: 10.1016/j.fsi.2020.11.031

139. Beitinger TL, Bennett WA, McCauley RW. Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environ Biol Fishes.* (2000) 58:237–75. doi: 10.1023/A:1007676325825

140. Das T, Pal AK, Chakraborty SK, Manush SM, Chatterjee N, Mukherjee SC. Thermal tolerance and oxygen consumption of Indian Major Carps acclimated to four temperatures. *J Therm Biol.* (2004) 29:157–63. doi: 10.1016/j.jtherbio.2004.02.001

141. Díaz F, Re AD, González RA, Sánchez LN, Leyva G, Valenzuela F. Temperature preference and oxygen consumption of the largemouth bass *Micropterus salmoides* (Lacépède) acclimated to different temperatures. *Aquac Res.* (2007) 38:1387–94. doi: 10.1111/j.1365-2109.2007.01817.x

142. Sarma K, Pal AK, Ayyappan S, Das T, Manush SM, Debnath D, et al. Acclimation of *Anabas testudineus* (Bloch) to three test temperatures influences thermal tolerance and oxygen consumption. *Fish Physiol Biochem.* (2010) 36:85–90. doi: 10.1007/s10695-008-9293-3

143. Nakano K, Iwama GK. The 70-kDa heat shock protein response in two intertidal sculpins, Oligocottus *maculosus* and *O. snyderi*: relationship of hsp70 and thermal tolerance. *Comp Biochem Physiol Mol Integr Physiol.* (2002) 133:79–94. doi: 10.1016/S1095-6433(02)00115-0

144. Werner I, Smith TB, Feliciano J, Johnson ML. Heat shock proteins in juvenile steelhead reflect thermal conditions in the Navarro River watershed, California. *Trans Am Fish Soc.* (2005) 134:399–410. doi: 10.1577/T03-181.1

145. Das T, Pal AK, Chakraborty SK, Manush SM, Chatterjee N, Apte SK. Metabolic elasticity and induction of heat shock protein 70 in *Labeo rohita* acclimated to three temperatures. *Asian-Australas J Anim Sci.* (2006) 19:1033–9. doi: 10.5713/ajas.2006.1033

146. Fontagné S, Geurden I, Escaffre AM. Bergot P. Histological changes induced by dietary phospholipids in intestine and liver of common carp *(Cyprinus carpio* L). larvae *Aquaculture.* (1998) 161:213–23. doi: 10.1016/S0044-8486(97)00271-8

147. Wang S, Han Z, Turchini GM, Wang X, Fang Z, Chen N, et al. Effects of dietary phospholipids on growth performance, digestive enzymes activity and intestinal health of largemouth bass (*Micropterus salmoides*) larvae. *Front Immunol.* (2021) 12:827946. doi: 10.3389/fimmu.2021.827946

148. Tan P, Zhu W, Zhang P, Wang L, Chen R, Xu D. Dietary soybean lecithin inclusion promotes growth, development, and intestinal morphology of yellow drum (*Nibea albiflora*) larvae. *Aquaculture.* (2022) 559:738446. doi: 10.1016/j.aquaculture.2022.738446

149. Huang Y, Xu J, Sheng Z, Xie R, Zhang H, Chen N, Li S. Effects of dietary phospholipids on growth performance, fatty acid composition, and expression of lipid metabolism related genes of juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lancolatus* ♂). *Aquac Rep.* (2022) 22:100993. doi: 10.1016/j.aqrep.2021.100993

150. Zhang H, Mu Z, Xu L, Xu G, Liu M, Shan A. Dietary lipid level induced antioxidant response in Manchurian trout, *Brachymystax lenok* (Pallas) larvae. *Lipids.* (2009) 44:643–54. doi: 10.1007/s11745-009-3313-7

151. Mourente G, Diaz-Salvago E, Bell JG. Tocher DR. Increased activities of hepatic antioxidant defence enzymes in juvenile gilthead sea bream (*Sparus aurata* L) fed dietary oxidised oil: attenuation by dietary vitamin E. *Aquaculture.* (2002) 214:343–61. doi: 10.1016/S0044-8486(02)00064-9

152. Gao J, Koshio S, Wang W, Li Y, Huang S, Cao X. Effects of dietary phospholipid levels on growth performance, fatty acid composition and antioxidant responses of Dojo loach *Misgurnus anguillicaudatus* larvae. *Aquaculture.* (2014) 427:304–9. doi: 10.1016/j.aquaculture.2014.02.022

153. Adel M, Gholaghaie M, Khanjany P, Citarasu T. Effect of dietary soybean lecithin on growth parameters, digestive enzyme activity, antioxidative status and mucosal immune responses of common carp (*Cyprinus carpio*). *Aquac Nutr.* (2017) 23:1145–52. doi: 10.1111/anu.12483

154. Lin SM Li FJ, Yuangsoi B, Doolgindachbaporn S. Effect of dietary phospholipid levels on growth, lipid metabolism, and antioxidative status of juvenile hybrid snakehead (*Channa argus* × *Channa maculata*). *Fish Physiol Biochem.* (2018) 44:401–10. doi: 10.1007/s10695-017-0443-3

155. Jafari F, Agh N, Noori F, Tokmachi A, Gisbert E. Effects of dietary soybean lecithin on growth performance, blood chemistry and immunity in juvenile stellate sturgeon (*Acipenser stellatus*). *Fish Shellfish Immunol.* (2018) 80:487–96. doi: 10.1016/j.fsi.2018.06.023

156. Jenabi Haghparast R, Sarvi Moghanlou K, Mohseni M, Imani A. Effect of dietary soybean lecithin on fish performance, hemato-immunological parameters, lipid biochemistry, antioxidant status, digestive enzymes activity and intestinal histomorphometry of pre-spawning Caspian brown trout (*Salmo trutta caspius*). *Fish Shellfish Immunol.* (2019) 91:50–7. doi: 10.1016/j.fsi.2019.05.022

157. Sink TD, Lochmann RT. The effects of soybean lecithin supplementation to a practical diet formulation on juvenile channel catfish, *Ictalurus punctatus*: growth, survival, hematology, innate immune activity, and lipid biochemistry. *J World Aquac Soc.* (2014) 45:163–72. doi: 10.1111/jwas.12108

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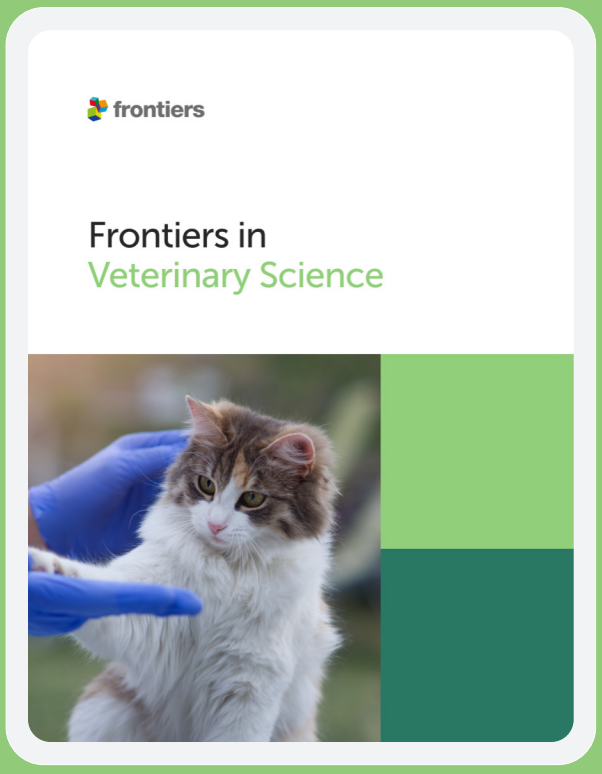

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