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Rising stars in comparative and clinical medicine 2022

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Rising stars in comparative and clinical medicine: 2022

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Editorial: Rising stars in comparative and clinical medicine: 2022

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Editorial on the Research Topic
Rising stars in comparative and clinical medicine: 2022

Introduction

The main aim of this Research Topic is to establish a platform for the sharing of research findings in the field of comparative and clinical medicine. Many research queries and recommendations have been offered as components of this Research Topic by promising talents in the field of clinical medicine. In this special e-collection, there are 28 papers covering the various aspects of clinical and comparative medicine. Most of the studies featured in this compilation can be classified into the following research domains: (1) Animal diseases and their management; (2) Drug resistance and alternative strategy; (3) Microbiota in the context of health and diseases; and (4) Poultry diseases and toxicological studies.

Animal diseases and their management

Abdullah et al. carried out a study on molecular detection of *Theileria* spp., a significant prevalence of *Theileria* infection was revealed in native sheep, affirming the endemic nature of the disease in the investigated region. In traditional husbandry practices, animals that were sub-clinically infected might continue to serve as a source for infecting ticks. The impact of theileriosis on metabolic disruptions of different systems and organs of the host body is evidenced by alterations in specific biochemical and hematological profiles in affected sheep (1). The disease is economically important because of its association with the elevated level of morbidity and mortality in sheep and attributing to significant economic losses in the livestock sector (2).

Naeem et al. studied the molecular prevalence of *Anaplasma ovis* in sheep. Blood samples tested from sheep in Punjab province of Pakistan revealed a moderate prevalence of *A. ovis*. Specific susceptibility to *A. ovis* was not observed in any of the enrolled sheep breeds in

the study. Data generated in this study might support the development of prophylactic detection methods and integrated control strategies against tick-borne diseases in sheep.

Qamar and Alsayeqh conducted a review on foodborne *Toxoplasma gondii* infections. Developed nations exhibit a lower prevalence of food-related disorders compared to third-world countries (3). According to their review, it was observed that animals and pets served as both parasite reservoirs and source of *T. gondii* infections in humans. Most of the diagnostic techniques employed in the studies under observation primarily included serological tests, with only a limited number of studies utilizing molecular tools. To gain a precise understanding of the disease pattern and to control the disease efficiently, it is advisable to adopt advanced molecular diagnostic tools, given their greater reliability.

Alvi and Alsayeqh performed a review on echinococcosis, a zoonotic disease transmitted through food, with a particular emphasis on its epidemiology. Echinococcosis is a neglected parasitic disease listed by the WHO, and it is caused by various species within the *Echinococcus* genus (4). Worldwide prevalence characterizes this disease, with significant economic losses incurred among the farmers and the development of cystic disease in unintentional human hosts (5). This review article provides a concise overview of taxonomy, a brief historical account, the extent of economic losses, the range of hosts and life cycle, factors contributing to risk, and clinical manifestations of echinococcosis. Additionally, the review compiles the prevalence of echinococcosis across various continents using copro- and sero-ELISA-based methods. Existence of genetic variations within species and their correlation with the virulence of the parasite demands the identification of specific markers to be used for clinical applications toward the diagnosis and control of the disease in the future.

Rashid et al. evaluated the cost-effectiveness of anthelmintic treatment and its comparative efficacy in commercial dairy farms. Intestinal parasitic infections posed significant challenges in achieving optimal production and ensuring the health and welfare of animals, including cattle and buffaloes. Utilizing anti-parasitic treatments seemed to be a dependable approach (6). Nevertheless, the efficacy and choice of suitable anthelmintics must be assessed based on local conditions. Overall, this enhanced production efficiency results in a favorable and direct cost-benefit ratio for dairy farmers.

Avberšek et al. studied the identification of pathogenic skin bacteria in dogs and cats through visual means. The diagnostic cytology was complemented by the use of chromogenic culture media. Skin infections caused by bacteria, such as otitis externa and pyoderma, were frequently observed in dogs and cats brought to the veterinary clinic. The chromogenic media incorporation in animal hospitals could substantially enhance the diagnostic capabilities for identifying skin problem by the species of pathogens in ∼80% of cases.

Teoh et al. reported that single-nucleotide polymorphisms (SNPs) associated with inflammatory bowel disease (IBD) leads to an elevation in local thyroglobulin expression, contributing to the development of inflammation in miniature dachshunds (MDs). In MDs, Inflammatory Colorectal Polyp (ICRP) represents a chronic form of IBD. It is distinguished by granulomatous inflammation involving neutrophil infiltration and hyperplasia of goblet cells in the colon. As a result, TG emerges as a potential diagnostic target for ICRP in MDs, and the targeting of TG-mediated activation of the IL-6 amplifier in the colon is potentially a viable therapeutic approach for ICRP.

Pila et al. studied assessing of B-cell lymphoma 2 (Bcl-2) as a predictor for chronic kidney disease (CKD) in cats where they found lowered Bcl-2 levels in CKD cats compared to age-matched healthy cats. These lower levels were linked to higher BUN, creatinine, and greater CKD severity. The study suggested that Bcl-2 might be helpful in differentiating CKD-afflicted cats from healthy ones.

Ahmed et al. studied the effects of adding β-mercaptoethanol (βME) and epidermal growth factor (EGF) to buffalo embryos produced *in vitro*. Their findings showed that adding EGF improved buffalo embryo growth and development, while βME had a lesser effect. βME stimulated embryo growth when added during maturation and fertilization. However, the combination of EGF (20 ng/mL) and βME (50 μM) did not significantly enhance buffalo embryos compared to using each one separately.

Zeeshan et al. performed a sero-epidemiological study on abortion-causing bacterial agents in small ruminants. According to the study, fetal losses in small ruminants were primarily caused by *Brucella* spp., *Coxiella burnetiid*, and *Chlamydia abortus* (7). In the study area, the prevalence of brucellosis and coxiellosis was relatively higher compared to chlamydiosis. Binary logistic regression revealed a substantial link between ticks and brucellosis and coxiellosis.

Parvin et al. investigated respiratory diseases epidemics of poultry in Bangladesh using two real-time PCR-based simultaneous detection assays. Respiratory microorganisms posed a significant risk for poultry sector in Bangladesh, with Infectious Bronchitis Virus (IBV), Newcastle Disease Virus (NDV) and Avian Influenza viruses (LPAI H9N2 and HPAI H5N1) being common culprits among chicken flocks. The utilization of TaqMan and SYBR Green multitarget simultaneous RT-qPCR assays in screening outbreak samples (tissues and swabs) proved effective, offering accurate, swift, and reproducible results.

Drug resistance and alternative strategy

Alvi et al. uncovered new population diversity and benzimidazole resistance in *Echinococcus granulosus* from bovine sources using novel *cytb* and *nad5* genes. Cystic echinococcosis (CE) is an overlooked zoonotic illness induced by *Echinococcus granulosus* (8). The parasite impacts a broad spectrum of domesticated animals and wildlife (4). The research revealed that the *Echinococcus* species exhibited genetic diversity using NADH dehydrogenase subunit 5 (nad5) and mitochondrial cytochrome b (cytb) genes. This investigation provides insights into the prevalence of benzimidazole resistance in Pakistani *Echinococcus granulosus*.

Lee et al. studied analysis of fluoroquinolone usage and the identification of high-level ciprofloxacin resistance in *Enterococcus faecalis* isolated from integrated broiler operations in South Korea. The study confirmed the widespread presence

of HLCR *E. faecalis* in Korean broiler operations. As a result, it is essential to reduce the prevalence of resistant bacteria through regulatory measures such as farm environment cleaning and disinfection. Additionally, there is a need to minimize antimicrobial usage by prescribing them according to scientific proof of disease instead of solely action for disease prevention.

Aqib and Alsayeqh reviewed vancomycin resistance, a growing concern for both public health and animal (9). These resistant genes, collectively referred to as the "Van cluster", were harbored by a variety of microbes, which in turn exchanged them among themselves. Among animals, humans, and the environment, resistance transfer was widely observed, with the potential for both reverse zoonosis and zoonosis. Relevant cost-effective losses, as well as risks to public health, farm animal health, food security and food safety were posed by this situation.

Ul Haq et al. studied on the enhancement of β-lactam and fluoroquinolones antibiotics' effectiveness using artemisinin and its derivatives against multi-drug resistant (MDR) *Escherichia coli*. Time-kill assays revealed that these synergistic combinations exhibited significant effectiveness in the early hours of incubation, indicating their potential utility during outbreaks. The study suggests that, alongside the development of new antibiotics, improvements can be made to existing ones by incorporating enhancers such as artemisinin and its derivatives.

Altaf and Alkheraije showed that cell membrane-coated nanoparticles might be considered as an emerging antibacterial approach for pathogens in food animals. Traditional methods for fighting against infections primarily relied on antibiotics. However, the rise of drug-resistant bacteria and the increasing prevalence of deadly bacterial infections had underscored the need for new therapeutic agents against the infectious diseases. Recent progress in nanotechnology has led to the development of various nanoscale nanoparticles enclosed within cell membranes. Significant efforts have focused on enhancing nanoparticle functionality and protecting them during interactions with pathogens or exo/endotoxins.

Manan et al. made an investigation aiming to alter drug resistance in emerging milk-borne pathogens using antibiotics and nanoparticles based on sodium alginate. The study observed a rise in the drug resistance and occurrence of *S. agalactiae* and *K. pneumoniae*. It was noteworthy that nanoparticles and antibiotics stabilized with sodium alginate exhibited strong antibacterial properties against both the bacterial strains. Specifically, the formulation containing nanoparticles of MgO and antibiotics within a gel (sodium alginate) displayed an enhanced antibacterial effect. Notably, the cytotoxicity of the nanoparticles of MgO used in this investigation was considerably less than the positive control.

Abdel Ghfar et al. studied the impact of *Nigella sativa* and *Allium sativum* on the mitigation of aluminum toxicity in albino rats. It was concluded that aluminum had detrimental effects on the health of both animals and humans. Treatment with *Allium sativum* and *Nigella sativa* was found to alleviate the adverse effects of aluminum and facilitate the restoration of the liver, kidney, and testis to their normal state. Furthermore, the effectiveness of *Nigella sativa* and *Allium sativum* in combating aluminum toxicity was confirmed by the blood biochemical results in this study.

Zia and Alkheraije studied on recent developments in utilizing bacteriophages as substitutes for antimicrobials against pathogens in food animals. The foremost challenges in the global food industry continued to revolve around food safety and sustainability (10). Bacteriophages, natural antibacterial agents, had effectively worked on various established and emerging foodborne pathogens (11). While bacteriophages (phages) are widespread in the environment and pose no harm to humans or animals, they are well-suited for detecting and managing pathogens throughout the food supply chain. Their inherent capacity to infect and eliminate specific bacteria makes them a potent tool for both detection and control purposes. Instead of relying on chemical preservatives, biological approaches like bacteriophage biocontrol can be employed to address food contamination, even though their antimicrobial effectiveness in food systems may be diminished compared to lab conditions (12).

Guo et al. performed an *in-vitro* assessment of the immunomodulatory effects of sulphation-modified total ginsenosides derivative-3 (SMTG-d3). According to the study, TG and SMTG-d3 were found to enhance the activity of peritoneal macrophages, NK cells and T lymphocytes, along with promoting the production of TNF-α and IFN-γ during the anti-tumor process. Therefore, utilizing the active components of ginseng as a foundation and exploring the relationship between structural changes and activity (toxicity) could be used to develop novel therapeutic target molecules.

Microbiota in the context of health and diseases

Zhu et al. performed a comparative study on gut fungus structure and makeup in yaks fed on different feeding models. Specifically, the researchers compared and assessed the diversity of the intestinal fungus species in grazing domestic yaks (GYG), house-fed domestic yaks (HFG) and wild yaks (WYG). The findings showed that Ascomycota and Basidiomycota were the dominant phyla in the gut fungus spices, regardless of the feeding models. Fungal taxonomic analysis identified significant differences in 20 genera among GYG and WYG, as well as 16 genera between WYG and HFG. In summary, this research demonstrated that the structure and makeup of gut fungi varied significantly among yaks raised in different breeding groups.

Li et al. performed a study on *Bacillus subtilis* from yaks and its probiotic qualities through complete genome analysis. Probiotics offer various health benefits to hosts and are of interest. Yaks in the Tibetan plateau are known for their disease resistance and resilience, possibly due to their internal probiotics. The research pinpointed genes related to anti-oxidation and biological adhesion in the *Bacillus subtilis* genome. This study highlighted *Bacillus subtilis*' beneficial properties from a genomic angle, raising awareness of its potential and laying the groundwork for probiotic product development.

Lan et al. performed a study on the shifts in the intestinal fungus community in horses at different health conditions. The results revealed significant alterations in both the diversity and composition of the gut fungus spices in horses with diarrhea.

There were notable changes in the types and in diarrheal horses. Furthermore, the analysis identified 175 distinct fungal genera that differed between the intestinal fungus communities of infected and healthy horses, with four fungal genera increasing significantly and 171 bacterial genera decreasing significantly during diarrhea. Previous researches also highlighted the value of comparing equine gut microbiota to gain insights into its vital role in animal health (13).

Poultry diseases and toxicological studies

Saeed and Alkheraije reported that botanicals offered a promising method for managing cecal coccidiosis in poultry. Prior research had indicated that plants contained various botanical compounds, which varied in their quantities and proportions (14). These compounds exhibited anticoccidial and antioxidant characteristics against avian cecal coccidiosis (15, 16). Anticoccidial effects have been demonstrated by sulfur compounds, essential oils (terpenes and derivatives), saponins, phenolics and other compounds through various mechanisms. However, research on their potential toxicity concerning coccidiosis is lacking. Evaluating their toxicological profiles is essential before considering their therapeutic use to understand their interactions within the body.

Wang et al. observed notable changes in liver metabolism in chickens exposed to thiram, resulting in significant alterations in specific metabolites and metabolic pathways. These findings enhance our understanding of how thiram impacted broiler liver metabolism and emphasized the potential role of hepatic metabolic disorder. In conclusion, this study highlights the significant influence of thiram exposure on chicken liver metabolism and provides a foundation for regulating thiram use and disposal to protect environmental quality and poultry health.

Naz et al. studied on the induction of histopathological, serum biochemical, oxidative stress and clinico-hematological changes in freshwater fish rohu (*Labeorohita*) by copper sulfate. Significant modification in serum biochemical and hematological parameters, along with alterations in histopathological conditions in *Labeorohita*, were observed in this study. As a result of these findings, further research is suggested to determine the optimal CuSO4 dose for regular use in the decontamination of fish ponds.

Mashkoor et al. showed that arsenic and chromium exposure in broiler chicks resulted in oxidative stress and toxicity, leading to increased gross and microscopic lesion severity compared to other treatment groups. These contaminants negatively impacted on hematological and biochemical parameters. However, the administration of vitamin E and bentonite can mitigate the toxicity and oxidative stress induced by arsenic and chromium.

Conclusion

Taken together, the articles published in our Research Topic make important contributions to understand that how livestock production can be improved with the application of various diagnostic and treatment options in clinical and comparative veterinary medicine. However, further research on these topics is required to develop an improved understanding of the use of alternative strategies for the treatment of various pathological disorders. The authors would like to thank all the contributors who participated in this Research Topic.

Author contributions

JC: Conceptualization, Formal analysis, Methodology, Visualization, Writing—original draft. YJ: Conceptualization, Investigation, Software, Writing—review and editing. KM: Conceptualization, Funding acquisition, Resources, Visualization, Writing—original draft, Writing—review and editing. MR: Formal analysis, Project administration, Validation, Writing—review and editing. IO: Data curation, Methodology, Supervision, Writing—review and editing. HZ: Writing—review and editing, Funding acquisition, Investigation, Resources, Supervision. KL: Conceptualization, Project administration, Software, Validation, Writing—review and editing.

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Dynamic changes of gut fungal community in horse at different health states

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Accumulating studies indicated that gut microbial changes played key roles in the progression of multiple diseases, which seriously threaten the host health. Gut microbial dysbiosis is closely associated with the development of diarrhea, but gut microbial composition and variability in diarrheic horses have not been well characterized. Here, we investigated gut fungal compositions and changes in healthy and diarrheic horses using amplicon sequencing. Results indicated that the alpha and beta diversities of gut fungal community in diarrheal horses changed significantly, accompanied by distinct changes in taxonomic compositions. The types of main fungal phyla (*Neocallimastigomycota*, *Ascomycota,* and *Basidiomycota*) in healthy and diarrheal horses were same but different in relative abundances. However, the species and abundances of dominant fungal genera in diarrheal horses changed significantly compared with healthy horses. Results of Metastats analysis indicated that all differential fungal phyla (*Blastocladiomycota*, *Kickxellomycota*, *Rozellomycota*, *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Mortierellomycota*, *Neocallimastigomycota*, *Glomeromycota,* and *Olpidiomycota*) showed a decreasing trend during diarrhea. Moreover, a total of 175 differential fungal genera were identified for the gut fungal community between healthy and diarrheal horses, where 4 fungal genera increased significantly, 171 bacterial genera decreased dramatically during diarrhea. Among these decreased bacteria, 74 fungal genera even completely disappeared from the intestine. Moreover, this is the first comparative analysis of equine gut fungal community in different health states, which is beneficial to understand the important role of gut fungal community in equine health.

KEYWORDS

horse, gut microbiota, fungal, diarrhea, amplicon sequencing

Introduction

As a forgotten or hidden organ, the role of the gut microbiota is increasingly recognized (1, 2). Studies showed that there are more than 100 trillion of microorganisms in the intestine including bacteria, fungi and protists, approximately 10 times the total number of host cells (3–5). Gut microbiota can not only synthesize nutrients

such as amino acids, vitamins, and short-chain fatty acids required for host growth, but also play key roles in digestion, absorption and metabolism (6, 7). Additionally, gut microbiota is also involved in the construction of the intestinal barrier and the maturation of the immune system, suggesting its vital role in disease resistance (8, 9). However, the gut microbial homeostasis is easily disrupted by several host- and environmental-related factors (10, 11). The execution of intestinal functions depends on the normal gut microbial structure, whereas gut microbial dysbiosis may cause aetiopathologic consequences (12, 13). For instance, it has been demonstrated that gut microbial dysbiosis is an important driver of diarrhea (14, 15). Moreover, disrupted gut microbiota may result in the development of other diseases such as obesity, diabetes and hypertension (16, 17).

Diarrhea is deemed as the major factor impeding the development of livestock industry because of it could cause reduced growth performance and a large number of deaths of farmed animals (18, 19). Increasing surveys demonstrated that diarrhea occurs in almost all animals, causing huge economic losses every year (20, 21). Diarrhea could cause the body to lose a great of water and nutrients, thus resulting in energy imbalances, weakness, starvation or even death (22, 23). Moreover, it may also cause inappetence, lassitude and weight loss, seriously affecting animal health and growth performance (24). Given the adverse effect of diarrhea on the livestock industry, investigating its treatment and etiology is of great significance. Research showed that gut microbial community is closely related to the development of diarrhea (14). For instance, early studies indicated that the compositions and structures of gut bacterial and fungal communities changed significantly in many diarrheal mammals (25, 26). Additionally, fecal microbiota transplantation was shown to alleviate diarrhea in some exploratory experiments, suggesting important roles of gut microbiota in diarrheal prevention and control (8).

Recently, metagenomics has developed into an effective tool for analyzing the gut microbiota (27, 28). By deep sequencing of intestinal contents or fecal samples, we can reveal the complex composition of the gut microbiota and explore gut microbial changes during disease, which contribute to understanding the pathological mechanism of disease and the role of gut microbiota (29, 30). Meanwhile, it also beneficial to diagnose and treat diseases from the microbiological perspective and decrease animal mortality and economic losses (31, 32). Horses (*Equus caballus*) are closely related to human life and development. In the past, the main uses of horses included meat, dairy, agricultural production, transportation, and the military. With the development of society, horses are mainly used for sports entertainment at this stage. However, horses are prone to diarrheal diseases due to stress response, excessive exercise and other factors. Currently, the characteristics of gut microbiota in many diarrheic animals such as giraffe, sheep and pigs have been detected by amplicon sequencing and revealed the changes in gut microbiota (14, 33). However, knowledge regarding diarrhea influence on gut microbiota in horses remains scarce. Here, we investigated the alterations of gut fungal community in diarrheic horses.

Materials and methods

Sample acquisition

In this research, 16 fecal sample collected from eight healthy and eight diarrheic horses were applied for amplicon sequencing. These horses were raised at the Wuhan Business University (Wuhan, China) and have similar age (∼2 years old) and breeding conditions. Prior to sample acquisition, the diarrheic horses were assessed and diagnosed by professional veterinarian. To collect clean samples, the sterile swabs were used for swabbing rectum in a rotating fashion. The collected fecal sample were immediately sub-sampled from the intermediate region to maximally decrease pollution and then snap-frozen utilizing liquid nitrogen and stored at −80°C for further study.

DNA extraction and illumine MiSeq sequencing

Fecal sample collected from different groups were thawed and then subjected to bacterial genome DNA extraction using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) based on the manufacturer's instructions. The gDNA were subjected to quality evaluation *via* using 0.8% (w/v) agarose gel electrophoresis, while its concentration was quantified by using UV-Vis spectrophotometer (NanoDrop 2000, United States). Subsequently, we amplified the V3/V4 regions of 16S rRNA using the primers (338F: ACTCCTACGGGAGGCAGCA and 806R: GGACTACHVGGGTWTCTAAT) synthesized from conserved regions. PCR amplification procedure was determined based on previous reports (34). The amplified products were conducted quality inspection, target fragment recovery, fluorescent quantitation and purification. The PacBio platform (Biomarker Technologies, China) was applied to construct sequencing libraries and qualified libraries were paired-end sequenced on MiSeq sequencing machine according to the standard protocols. The raw data generated by amplicon sequencing were filtered and identified to eliminate short sequences, mismatched primers and chimera. After quality assessment, the qualified sequences were clustered and OTUs partitioned based on 97% similarity. Moreover, we also calculated multiple alpha diversity indices and generated PCoA plots to further dissect changes in gut microbial abundance, diversity and principal components. Metastats analysis and LEfSe were used to distinguish differential taxa. *P*-values (means ± SD) <0.05 were considered statistically significant.

TABLE 1 Gut fungal sequence data of the samples.

Sample	Raw reads	Clean reads	Effective reads	AvgLen (bp)	GC (%)	Q20 (%)	Q30 (%)	Effective (%)
C1	80175	79788	76634	290	47.5	99.6	98.1	95.6
C2	79705	79297	78271	286	29.9	99.8	98.6	98.2
C3	79961	79540	78572	284	30.8	99.8	98.7	98.3
C4	80220	79801	78919	249	48.4	99.9	99.2	98.4
C5	79848	79414	78392	244	48.3	99.9	99.2	98.2
C6	79956	79514	78475	249	48.4	99.9	99.1	98.2
C7	80221	79823	78541	249	48.3	99.9	99.2	97.9
C8	79850	79402	78543	251	48	99.9	99.1	98.4
D1	79827	79369	78020	286	33.2	99.8	98.5	97.7
D2	79937	79577	78621	292	31.2	99.8	98.6	98.4
D3	80128	79714	78775	290	31.2	99.8	98.6	98.3
D4	79757	79355	77485	285	34.2	99.8	98.6	97.2
D5	79473	79074	78208	289	31.8	99.8	98.5	98.4
D6	79973	79582	78755	293	30.3	99.8	98.5	98.5
D7	79809	79455	78625	294	30.6	99.8	98.5	98.5
D8	79754	79353	78061	292	29.8	99.8	98.5	97.9

Results

Data acquisition and analysis

In this study, we amplified 16 fecal samples from healthy and diarrheic horses to assess changes in the gut fungal community during diarrhea. Results of amplicon sequencing indicated that 127,8594 (C = 639,936, D = 638,658) raw sequences were totally generated, with an average of 79,912 (varying from 79,473 to 80,221) sequences per sample (Table 1). After quality evaluation, a total of 1252,897 (C = 626,347, D = 626,550) qualified sequences were collected, with a qualification rate of over 60%. The rarefaction curve and species rank curve in each sample were wide and decreased slowly, showing the satisfactory sequencing evenness and richness (Figures 1A–C). According to 97% nucleotide-sequence similarity, the qualified sequences were clustered into 1341 OTUs, ranging from 329 to 562 OTUs per sample (Figures 1D,E). Among identified OTUs, 889 OTUs are common in both groups, accounting for approximately 66.29% of the total OTUs. Moreover, there are 433 and 19 unique OTUs in the healthy and diarrheic horses.

Diarrhea reduces the diversity of gut fungal community

To further investigate the influences of diarrhea in gut fungal community, we comparatively analyzed shifts in gut fungal diversity index between healthy and diarrheic horses. Good's coverage estimations in each sample ranged from 99.90 to 99.97%, covering nearly all fungal phenotypes. Statistical analysis of alpha diversity indicated that there were statistically distinct differences in the Chao1 (626.07 ± 54.84 vs. 422.88 ± 54.21, $P < 0.01$), ACE (763.48 ± 195.95 vs. 396.00 ± 54.65, $P < 0.01$), Simpson (0.93 ± 0.084 vs. 0.67 ± 0.094, $P < 0.01$) and Shannon (6.74 ± 1.94 vs. 3.06 ± 0.68, $P < 0.01$) indices between the control and diarrheic groups, indicating that diarrhea significantly reduced the gut fungal diversity and abundance (Figures 2A–D). Additionally, beta-diversity analysis showed that the dots in healthy and diarrheic group were separated, demonstrating that diarrhea dramatically changed the gut fungal main components (Figures 2E,F).

Comparative analysis of the gut fungal composition between healthy and diarrheic groups

We also visualized the composition and abundance of gut fungal community at different taxonomical levels through species distribution histograms and observed considerable variability. There were nine phyla and 119 genera detected in 16 samples, ranging from 5 to 7 phyla per sample. The phyla *Neocallimastigomycota* (21.30%, 85.59%), *Ascomycota* (55.01%, 10.87%), and *Basidiomycota* (12.49%, 1.89%) were abundantly present in the healthy and diarrheic groups, accounting for over 80% of total taxonomic groups recognized (Figure 3A). Other phyla such as *Chytridiomycota* (1.83%, 0.22%), *Glomeromycota* (0.16%, 0.018%), *Olpidiomycota* (0.060%, 0.0051%), *Blastocladiomycota* (0.031%, 0.00%), and

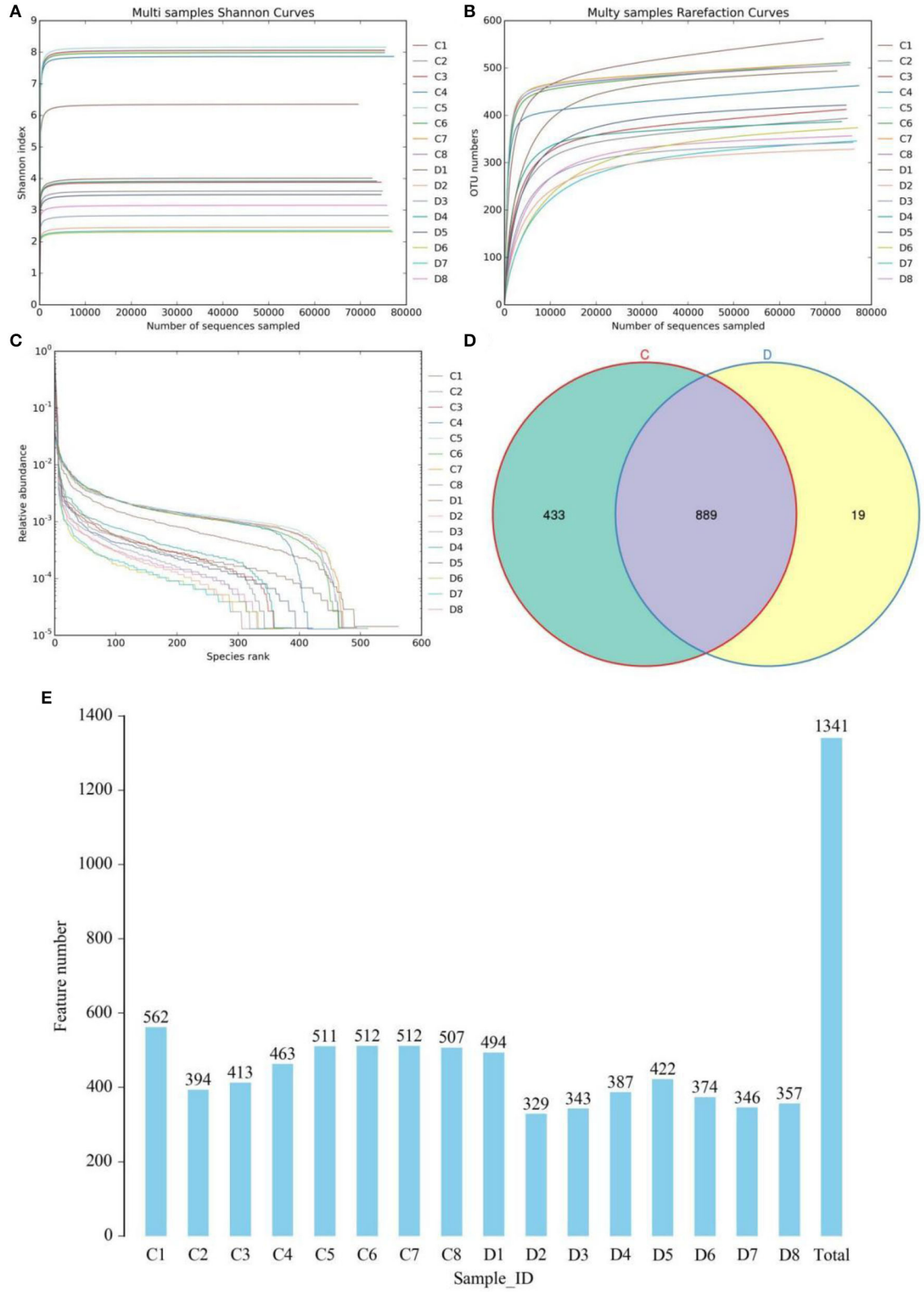

FIGURE 1
Gut fungal feasibility analysis and OTUs distribution. Rarefaction curves **(A,B)** and rank abundance curve **(C)** were used to evaluate the sequencing depth and evenness. **(D)** Venn diagrams for core and unique OTUs in the healthy and diarrheic groups. Histogram showing the number of OTUs in each sample. **(E)** Histogram showing the number of OTUs in each sample. C and D represent healthy and diarrheic groups, respectively.

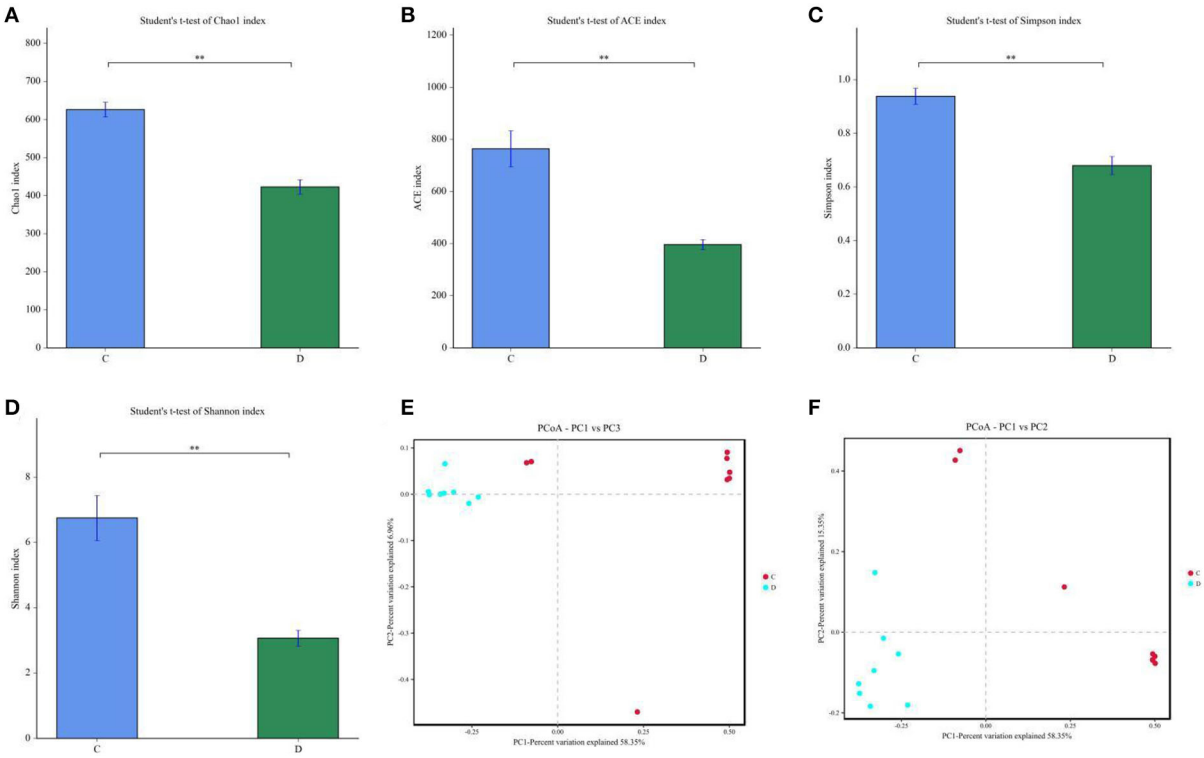

FIGURE 2
Effects of diarrhea on gut fungal diversity in horses. **(A–D)** represents the Chao1, ACE, Simpson and Shannon indices that can reflect the diversity of gut fungal community **(E,F)** Principal Coordinate Analysis (PCoA) of gut fungal community in healthy and diarrheic groups. C and D represent healthy and diarrheic groups, respectively. Data was presented as the mean ± SD. $^{**}P < 0.01$.

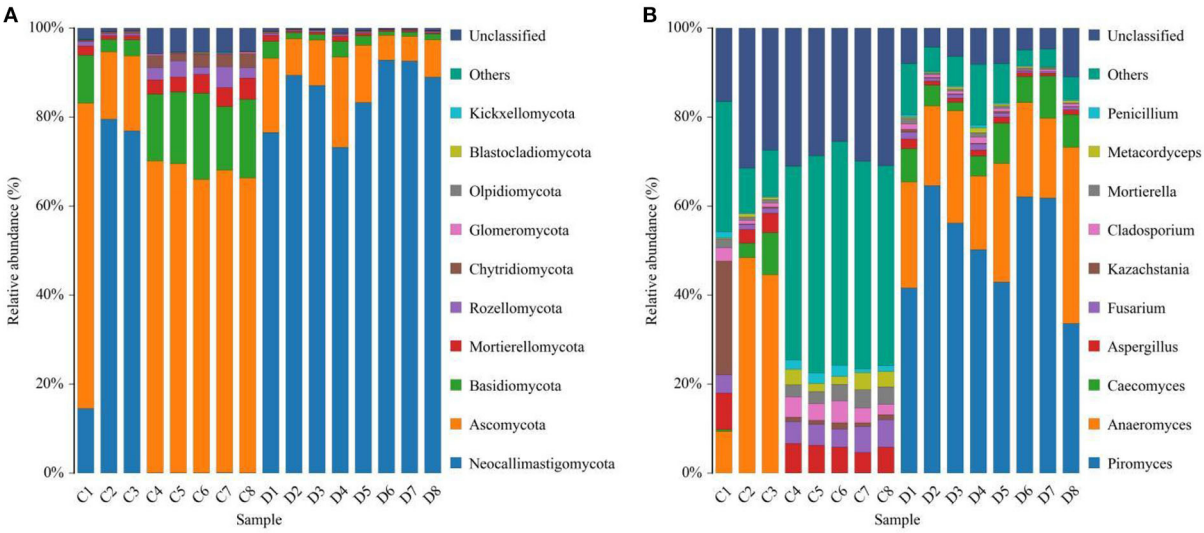

FIGURE 3
Effects of diarrhea on gut fungal composition in horses. **(A)** The composition of dominant fungi at the phylum level. **(B)** The composition of dominant fungi at the genus level. C and D represent healthy and diarrheic groups, respectively.

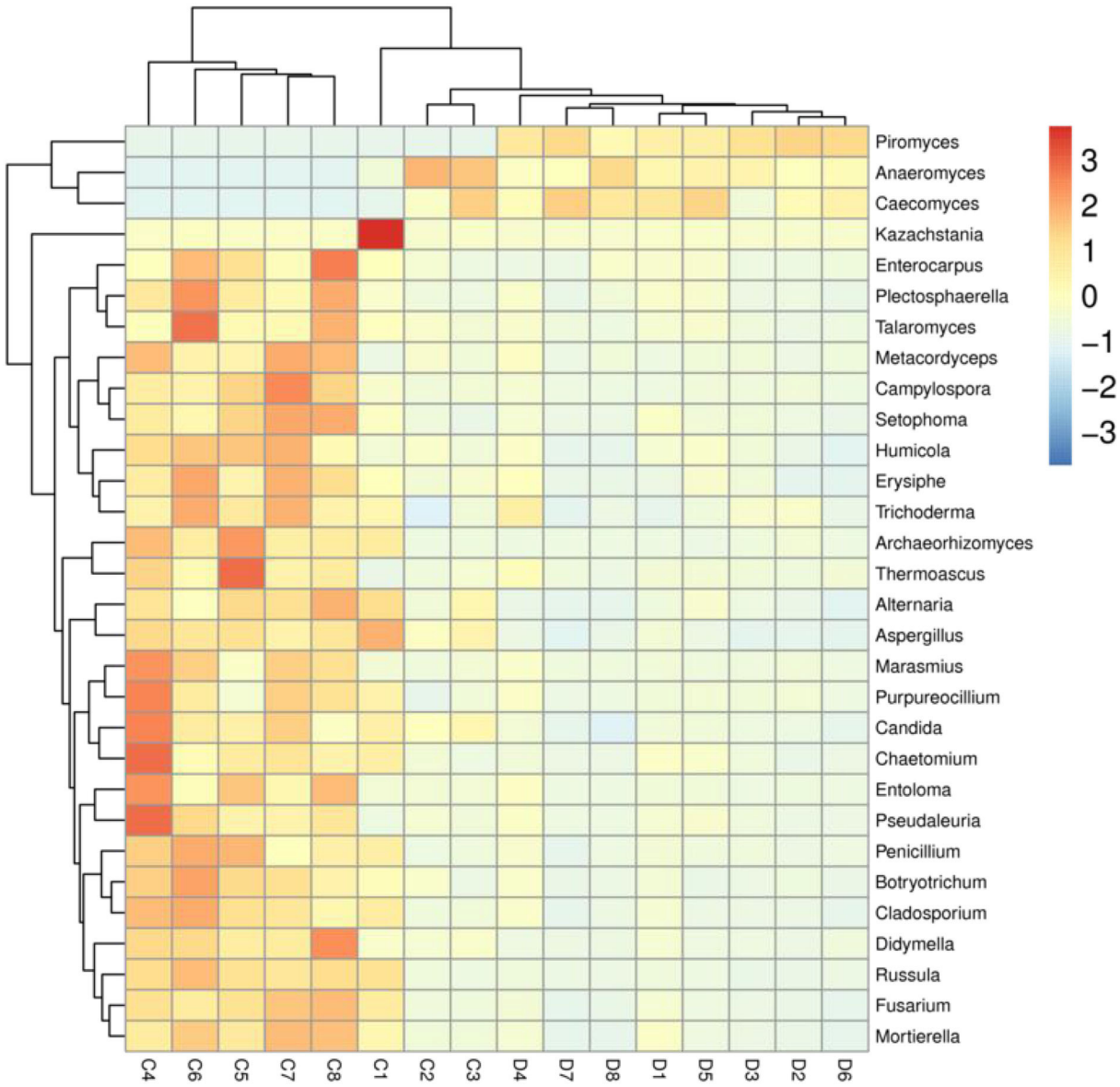

FIGURE 4
Heatmap of the most abundant fungal genera in both groups. The values of color in the heat map indicate the normalized relative richness of species. C and D represent healthy and diarrheic groups, respectively.

Kickxellomycota (0.023%, 0.00%) in healthy and diarrheic groups were recognized in low abundances. Among identified genera, *Anaeromyces* (12.74%), *Aspergillus* (5.56%), *Fusarium* (4.00%), and *Kazachstania* (3.68%) were the four predominant fungal genus in the control groups, accounting for more than 20.00% of the total composition (Figure 3B). Furthermore, the dominant fungal genus observed in gut fungal community in the diarrheic group were *Piromyces* (51.73%), *Anaeromyces* (23.61%), *Caecomyces* (6.29%), and *Aspergillus* (1.14%), accounting for over 80.00% of the total composition. Additionally, gut fungal distribution and variability between both groups could also be observed by the visualized clustering heatmap (Figure 4).

At the phylum level, *Blastocladiomycota*, *Kickxellomycota*, *Rozellomycota*, *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Mortierellomycota*, *Neocallimastigomycota*, *Glomeromycota*, and *Olpidiomycota* in the healthy group were significantly preponderant than diarrheic group (Table 2). Moreover, we also observed that 175 fungal genera were significantly different between the control and diarrheic groups

TABLE 2 Comparative analysis of differential fungal phyla between control and diarrheic groups.

Taxa	C (%)	D (%)	P
Blastocladiomycota	0.0313 ± 0.0213	0 ± 0	0
Kickxellomycota	0.0229 ± 0.0229	0 ± 0	0
Rozellomycota	2.13 ± 0.54	0.287 ± 0.0373	0
Ascomycota	55± 8.53	11 ± 1.87	0
Basidiomycota	12.5 ± 2.2	1.92 ± 0.409	0
Chytridiomycota	1.82 ± 0.437	0.226 ± 0.0446	0
Mortierellomycota	2.95 ± 0.543	0.578 ± 0.146	0
Neocallimastigomycota	21.4 ± 12.5	85.5 ± 2.57	0
Glomeromycota	0.165 ± 0.0509	0.0178 ± 0.00765	0.01
Olpidiomycota	0.0605 ± 0.0237	0.0052 ± 0.00218	0.01

C and D represent healthy and diarrheic groups, respectively.

(Supplementary Table 1). Among these differential taxa, the relative abundances of 171 fungal genera (*Achroiostachys*, *Acremoniopsis*, *Alatospora*, *Alternaria*, *Apodus*, *Arthrocladium*, *Aschersonia*, *Ascobolus*, *Aspergillus*, *Bartalinia*, *Berkleasmium*, *Brunneomyces*, *Butlerelfia*, *Candida*, *Coprinellus*, *Coryne*, *Craterellus*, *Cystodermella*, *Deconica*, *Didymella*, *Duddingtonia*, *Edenia*, *Entrophospora*, *Eremothecium*, *Erysiphe*, *Gamsia*, *Geminibasidium*, *Golovinomyces*, *Grammothele*, *Graphilbum*, *Gymnopilus*, *Hanseniaspora*, *Hypholoma*, *Kalmusia*, *Keissleriella*, *Leohumicola*, *Leucoagaricus*, *Leucosphaerina*, *Limnoperdon*, *Linderina*, *Microdochium*, *Microglossum*, *Minutisphaera*, *Monocillium*, *Myxospora*, *Panaeolus*, *Paraconiothyrium*, *Paracremonium*, *Phomatospora*, *Pleuroascus*, *Polyscytalum*, *Porodiplodia*, *Psathyrella*, *Pseudocoleophoma*, *Psilocybe*, *Pyrenochaeta*, *Ramichloridium*, *Ramularia*, *Rigidoporus*, *Roussoella*, *Scedosporium*, *Schizophyllum*, *Sclerostagonospora*, *Sebacina*, *Simplicillium*, *Spizellomyces*, *Sporormiella*, *Stellatospora*, *Strelitziana*, *Taifanglania*, *Talaromyces*, *Torula*, *Toxicocladosporium*, *Trichomerium*, *Trichometasphaeria*, *Trichomonascus*, *Trichophyton*, *Ustilaginoidea*, *Uwebraunia*, *Vishniacozyma*, *Wickerhamomyces*, *Wilcoxina*, *Xanthothecium*, *Archaeorhizomyces*, *Botryotrichum*, *Campylospora*, *Chaetomium*, *Fusarium*, *Kazachstania*, *Meyerozyma*, *Mortierella*, *Paecilomyces*, *Pichia*, *Russula*, *Saitozyma*, *Trichosporon*, *Cladosporium*, *Coniochaeta*, *Oidiodendron*, *Penicillium*, *Wallemia*, *Chaetomidium*, *Cladorrhinum*, *Hannaella*, *Humicola*, *Schizothecium*, *Trichocladium*, *Cephalotrichum*, *Entoloma*, *Ophiostoma*, *Setophoma*, *Acremonium*, *Enterocarpus*, *Sampaiozyma*, *Apiotrichum*, *Articulospora*, *Debaryomyces*, *Gibellulopsis*, *Lasiobolidium*, *Lecanicillium*, *Marquandomyces*, *Solicoccozyma*, *Staphylotrichum*, *Plectosphaerella*, *Pseudogymnoascus*, *Condenascus*, *Marasmius*, *Cladophialophora*, *Olpidium*, *Epicoccum*, *Pseudaleuria*, *Thelephora*, *Dactylonectria*, *Metacordyceps*, *Chrysosporium*, *Inosperma*, *Podospora*, *Rhodotorula*, *Pyrenochaetopsis*, *Monascus*, *Neomicrosphaeropsis*, *Tetracladium*, *Trechispora*, *Purpureocillium*, *Tausonia*, *Cercophora*, *Cercospora*, *Fusariella*, *Pseudeurotium*, *Thanatephorus*, *Preussia*, *Teunomyces*, *Exophiala*, *Amphinema*, *Cortinarius*, *Filobasidium*, *Zygosaccharomyces*, *Arxiella*, *Trichoderma*, *Thermoascus*, *Paraphaeosphaeria*, *Myriococcum*, *Triangularia*, *Cylindrobasidium*, *Knufia*, *Symmetrospora*, *Fusicolla*, *Byssochlamys*, *Gliocladiopsis*, *Stagonosporopsis,* and *Trametes*) dramatically decreased, whereas the relative richness of four fungal genera (*Claviceps*, *Piromyces*, *Geosmithia,* and *Caecomyces*) significantly increased during diarrhea. Notably, 74 genera even cannot be detected in the gut fungal community of diarrheic horses. Considering this discriminant analysis cannot found all the taxon, LEfSe combined with LDA scores were used to recognize the specific fungi associated with diarrhea (Figure 5).

Correlation network analysis

Erysiphe was positively associated with *Campylospora* (0.8706), *Russula* (0.8735), *Pseudaleuria* (0.8824), *Trichoderma* (0.7882), *Setophoma* (0.8471), *Marasmius* (0.8676), *Purpureocillium* (0.8324), *Alternaria* (0.8176), *Humicola* (0.8971), *Entoloma* (0.8500), *Plectosphaerella* (0.8941), *Enterocarpus* (0.6706), *Thermoascus* (0.6529), *Chaetomium* (0.8529), *Didymella* (0.8000), *Candida* (0.8735), *Archaeorhizomyces* (0.6412), *Saitozyma* (0.8971), *Meyerozyma* (0.7765), *Vishniacozyma* (0.8176), *Thelephora* (0.8029), *Rhodotorula* (0.7059), *Trichocladium* (0.6559), *Gibellulopsis* (0.8706), *Sebacina* (0.7640), *Debaryomyces* (0.5618), *Paecilomyces* (0.7206), *Acremonium* (0.7559), *Tetracladium* (0.6412), *Lecanicillium* (0.6785), *Pseudeurotium* (0.6500), *Ophiostoma* (0.7794), *Condenascus* (0.7176), *Podospora* (0.8971), *Solicoccozyma* (0.7108), *Preussia* (0.6176), *Schizophyllum* (0.8529), *Fusariella* (0.6882), *Monascus* (0.8647), *Trichosporon* (0.7647), *Cephalotrichum* (0.8176), *Tausonia* (0.6902), *Pyrenochaetopsis* (0.5765), *Cladorrhinum* (0.6941), *Trechispora* (0.6941), *Staphylotrichum* (0.6735), *Coniochaeta* (0.7029), *Oidiodendron* (0.7618), *Cercospora* (0.5471), *Filobasidium* (0.7882), *Zygosaccharomyces* (0.6896), and *Schizothecium* (0.7403) (Figure 6). *Podospora* was positively related to *Solicoccozyma* (7285), *Schizophyllum* (0.7588), *Fusariella* (0.8618), *Monascus* (0.7324), *Trichosporon* (0.7588), *Cephalotrichum* (0.7118), *Tausonia* (0.8859), *Pyrenochaetopsis* (0.7176), *Cladorrhinum* (0.7500), *Trechispora* (0.6882), *Staphylotrichum* (0.5853), *Coniochaeta* (0.6706), *Oidiodendron* (0.6588), *Cercospora* (0.5941), *Filobasidium* (0.8324), *Zygosaccharomyces* (0.8031) and *Schizothecium* (0.6652). *Fusarium* was positively associated with *Kazachstania* (0.8353), *Cladosporium* (0.9147) *Mortierella* (0.9794), *Metacordyceps* (0.6971), *Penicillium* (0.8824), *Botryotrichum* (0.8882), *Erysiphe* (0.8971), *Talaromyces* (0.8941), *Campylospora* (0.8382),

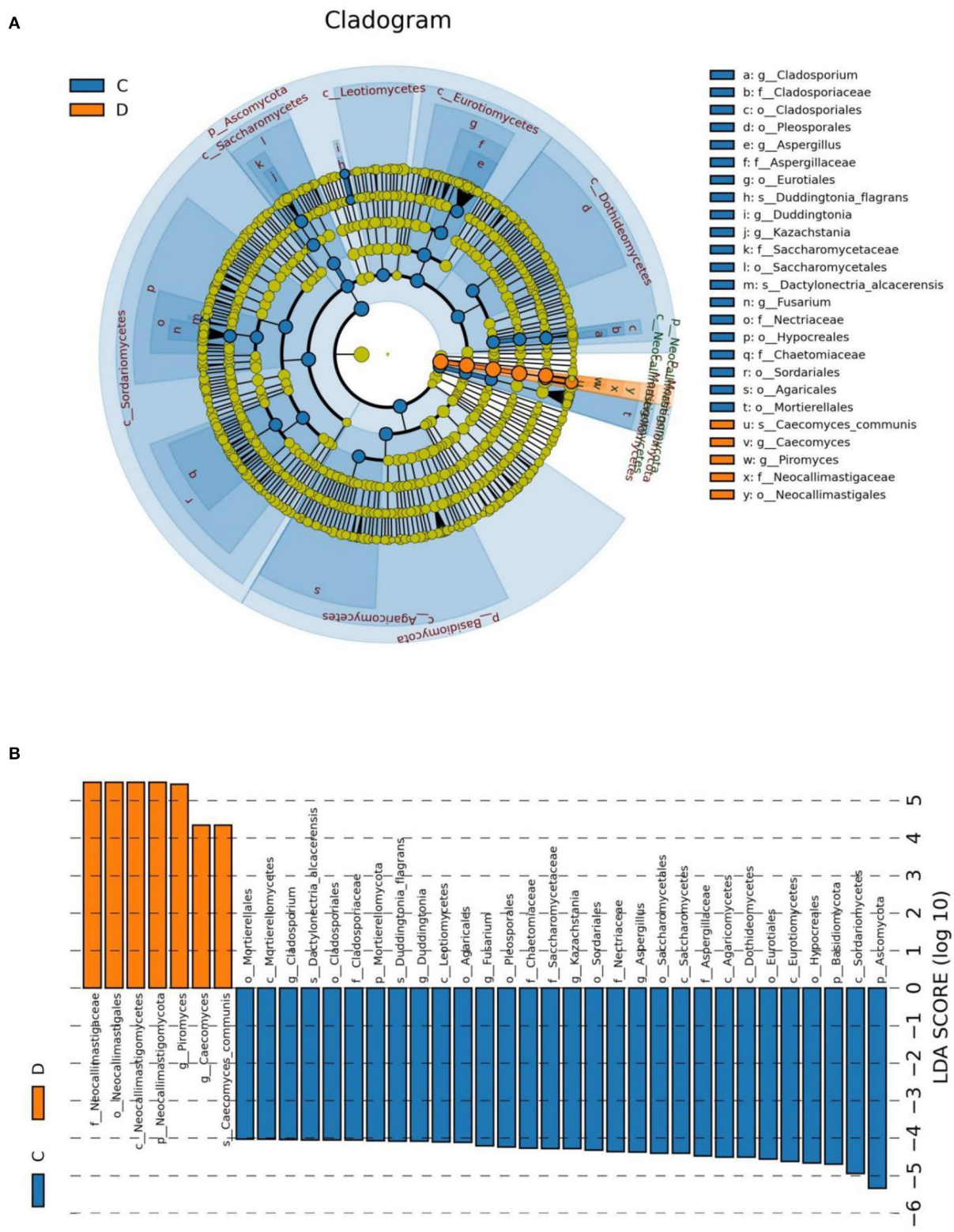

FIGURE 5
The identification of differential fungi associated with diarrhea. **(A)** Cladogram indicating the phylogenetic distribution of microbiota correlated with the healthy or diarrheic horses. **(B)** The differences in abundance between the healthy and diarrheic horses. C and D represent healthy and diarrheic groups, respectively.

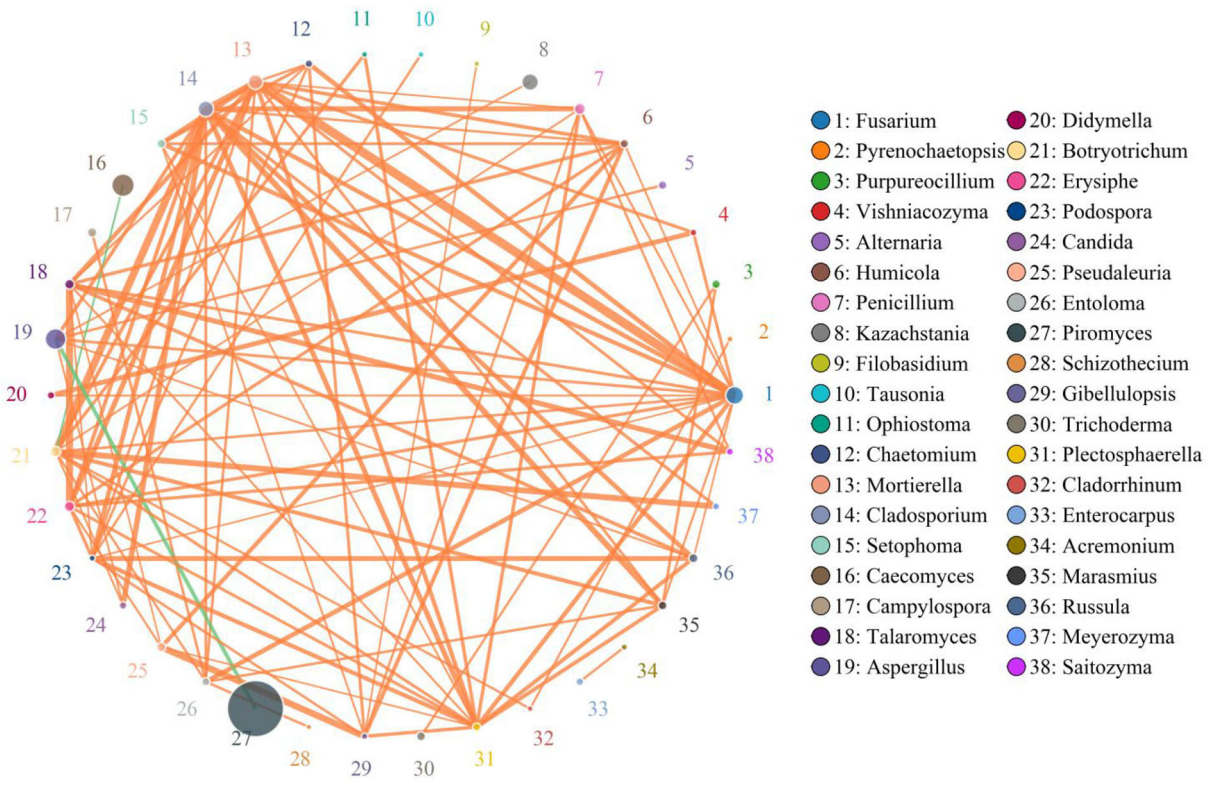

FIGURE 6
Correlation network analysis of gut fungal diversity. The correlation between fungi is represented by lines of different colors. The orange lines represent the positive correlation, whereas the green lines represent negative correlation. Detailed data were shown in the Supplementary Table 2.

Russula (0.8765), *Pseudaleuria* (0.8618), *Trichoderma* (0.7059), *Setophoma* (0.9118), *Marasmius* (0.8824), *Purpureocillium* (0.8059), *Alternaria* (0.8647), *Humicola* (0.8794), *Entoloma* (0.9176), *Plectosphaerella* (0.9147), *Enterocarpus* (0.7294), *Thermoascus* (0.7029), *Chaetomium* (0.9176), *Didymella* (0.8794), *Cadida* (0.8529), *Archaeorhizomyces* (0.7147), *Saitozyma* (0.8618), *Meyerozyma* (0.8441), *Vishniacozyma* (0.8941), *Thelephora* (0.7765), *Rhodotorula* (0.75), *Trichocladium* (0.7324), *Gibellulopsis* (0.8559), *Sebacina* (0.823), *Debaryomyces* (0.5176), *Paecilomyces* (0.7618), *Acremonium* (0.7941), *Tetracladium* (0.7647), *Lecanicillium* (0.4993), *Pseudeurotium* (0.5412), *Ophiostoma* (0.8294), *Condenascus* (0.7029), *Podospora* (0.8765), *Solicoccozyma* (0.7403) and *Preussia* (0.5529). *Schizophyllum* (0.7559), *Fusariella* (0.6912), *Monascus* (0.7294), *Trichosporon* (0.8324), *Cephalotrichum* (0.7676), *Tausonia* (0.727), *Pyrenochaetopsis* (0.8029), *Cladorrhinum* (0.8324), *Trechispora* (0.8088), *Staphylotrichum* (0.6059), *Epicoccum* (0.5882), *Coniochaeta* (0.7382), *Oidiodendron* (0.7353), *Filobasidium* (0.7235), *Zygosaccharomyces* (0.6866), *Leptobacillium* (0.6206), *Schizothecium* (0.8109), *Knufia* (0.5158) and *Thanatephorus* (0.6342).

Discussion

Numerous studies have indicated that gut microbiota played key roles in resisting the invasion of pathogenic bacteria and regulating the intestinal homeostasis, whereas gut microbial dysbiosis may cause many gastrointestinal disease or even systemic effects (35–37). Therefore, investigating thoroughly the changes of gut microbiota in different health status is of great significance for the disease prevention and treatment. Meanwhile, it also contributes to understanding the pathogenesis and developing novel methods for diagnosing the disease (38, 39). Diarrhea is a common gastrointestinal disease present in various animals, which results in a enormous threat to economic development, livestock production and animal welfare (20). Notably, the prevention and control of diarrhea-related diseases is difficult due to many factors including pathogenic bacteria, oxidative stress, environmental change and intoxication (40, 41). Diarrhea is often accompanied by intestinal damage, suggesting that the gut microbiota is inevitably affected by diarrhea (42, 43). Presently, many studies have been performed to explore gut microbial changes in sheep, yaks, dogs and chickens during diarrhea, but little is known

about the characteristics of gut microbiota in diarrheic horse. In this study, we first systematically explored the shifts of gut fungal community in diarrheic horse.

Gut microbiota is a complex and dynamically changing system that is susceptible to a many factors including elevation, temperature, age, diet and species (44–46). However, this normal fluctuation of gut microbiota does not affect the execution of complex intestinal functions (47, 48). However, strong stimulus such as various gastrointestinal diseases, antibiotics, heavy metal and microplastics could cause significant variation in gut microbial diversity and composition, causing gut microbial dysbiosis (49–51). Increasing investigations showed that the occurrence of diarrhea is often accompanied by significant changes in the gut microbiota. For instance, Li et al. showed that diarrhea can cause dramatic changes in gut microbial composition as well as obvious reduction in gut microbial diversity (14). In addition, Hong et al. the gut fungal diversity of patients with diarrhea-predominant irritable bowel syndrome was dramatically different from that of healthy population (52). Therefore, gut fungal community of diarrheal horses may also undergo significant changes. Consistent with previous investigations, this research demonstrated an significantly reduced alpha-diversity indices in the gut fungal community of diarrheal horses, indicating gut microbial dysbiosis. As an important component of the gut microbiota, gut fungal community also plays key roles in intestinal homeostasis and function (53). Early investigations indicated that the execution of intestinal function depended on normal gut microbiota, thus disordered gut microbiota inevitably affected the intestinal functions including energy utilization, nutrient absorption and metabolism (46, 53, 54). Moreover, disrupted gut microbial homeostasis also affects intestinal barrier function and mucosal immunity, which may increase disease susceptibility (7, 55, 56). Consequently, diarrheic horses may be infected with other diseases during gut microbial imbalance. PCoA was also conducted to evaluate the shifts in gut microbial main components to further explore the effects of diarrhea on the equine gut microbiota. We found that the individuals of the control group were clustered together but separated from the diarrheic group, suggesting obvious alternations in the gut microbial main components. The present research showed that despite of shared environment and diets, the diarrheic horses displayed obvious changes in the gut fungal community. Thus, we suspected that diarrhea was the primary driving force for shifts in gut fungal community of horses.

Our results revelaed that *Basidiomycota* and *Ascomycota* were detected to be abundantly presented in healthy and diarrheic horses. Notably, these fungal phyla were also the most abundant fungal phyla in other species such as giraffe, yak and sheep, showing their key roles in intestinal ecology and function (14). We further explored the changes of gut fungal abundance of diarrheic horses. The relationship between diarrhea and gut microbial community could be intuitively reflected by some specific bacteria and fungi. This study indicated obvious declines in the relative abundances of 10 fungal phyla during diarrhea in horses. Moreover, although the species of the dominant phyla did not alter, the relative abundances of *Basidiomycota* and *Ascomycota* were significantly decreased, suggesting gut fungal dysbiosis. Importantly, we also observed high variations in some fungal genera during diarrhea and these changed fungal genera may play important roles in intestinal homeostasis and functions. Among the altered fungi, more than 97% of the fungal genera were significantly decreased in abundances and 74 fungal genera even cannot be detected in the gut fungal community of diarrheic horses, suggesting that these fungal genera cannot adapt to the current intestinal environment. We suspected that intestinal environment was disrupted during diarrhea, which in turn limited the survival of these fungi.

Increasing evidence indicated that the disruption of gut microbial homeostasis was a pathological mediator of various gastrointestinal diseases (57–59). The interaction between gut microbes, including bacteria and fungi, is an important way to maintain intestinal homeostasis (37, 60, 61). Typically, these interreaction include synergy, antagonism and commensalism (61). Therefore, the shifts of some microorganisms could affect the other microbial functions, thereby further exaggerating the overall influence of gut microbiota on the host health and causing gut microbial dysbiosis (33, 62). In this study, we also observed significant correlations between some fungi by correlation network analysis, indicating that these altered fungi could affect other gut fungal functions. This research indicated that diarrhea not only directly destroyed the gut fungal composition and structure but also impaired the other fungal functions *via* interactions, which may further result in gut fungal dysbiosis in diarrheal horses.

In conclusion, this study compared and analyzed the differences in the gut fungal community of healthy and diarrheal horses. Results indicated that diarrhea dramatically altered gut fungal composition and structure, characterized by altered gut fungal diversity and composition. To our knowledge, this is the first study of gut fungal changes in diarrheal horses. This study filled a gap in the effect of diarrhea on the gut fungal community in horses and indicated that gut fungal dysbiosis may be one of the causes of diarrhea in horses. Meanwhile, the present results also provided a theoretical basis for the diagnosis and treatment of diarrhea from the gut microbial perspective. However, this study has some limitations including relatively small sample size and inability to control for potentially important variables such as individual variation and individual dietary habit.

Data availability statement

The data presented in the study are deposited in the Sequence Read Archive (SRA) (NCBI, USA) repository, accession number PRJNA881595.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee of the Wuhan Business University.

Author contributions

YLi and YLa conceived and designed the experiments. GY contributed sample collection and reagents preparation. ZZ analyzed the data. YLa wrote the manuscript. II revised the manuscript. All authors reviewed 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.

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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.2022.1047412/full#supplementary-material

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Vancomycin drug resistance, an emerging threat to animal and public health

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The need to supply quality food for the growing human population has led to the revolutionization of food production and processing in recent years. Meanwhile, food production sources are at risk of microbial attack, while the use of antibiotics to counter them is posing another threat to food safety and security. Vancomycin was used as the first line of defense against multiple drug-resistant bacteria salient of which is methicillin-resistant *S. aureus.* The emergence of the vancomycin resistance gene in bacteria impairs the efficacy of antibiotics on the one hand while its harmful residues impart food safety concerns on the other. Currently, a novel set of resistance genes "Van cluster" is circulating in a wider range of bacteria. Considerable economic losses in terms of low production and food safety are associated with this emerging resistance. The current review focuses on the emergence of vancomycin resistance and its impact on food safety. The review proposes the need for further research on the probable routes, mechanisms, and implications of vancomycin resistance from animals to humans and vice versa.

KEYWORDS

food safety, vancomycin, resistance genes, bacteria, gram-positive, gram-negative

Introduction

The use of antimicrobials poses a significant threat to animal production and profitability. Although there are Maximum Residue Limits (MRLs) of the approved veterinary drugs for their use to improve animal health, their implementation requires stern legislation and subsequent actions. On the other hand, there are a variety of guidelines in different countries but some of the countries still need to develop these guidelines (1). Implementing a withdrawal period of antibiotics in animal food products is an additional requirement to avoid food safety concerns. At the farm level, withdrawal periods are kept in line with the expected time of consumption of food. It is commonly required that drug residues must be below MRLs. Extra label utilization, miscalculated doses, non-specific use, and failure in observing the prescribed course of antibiotics may result in the spread of antimicrobial resistance.

Antimicrobial consumption through food or any other sources continuously leads to the suppression of intestinal microbiota that guards against pathogenic bacteria. Moreover, their presence in the bloodstream leads to the malfunctioning of various organs (2, 3). Extensive use of antimicrobials in animals may lead to the production of

novel resistant bacteria in the environment. Some of the salient bacteria responsible for food safety and security include *Salmonella, E. coli*, and *S. aureus* isolated from various sources in humans and poultry (4–7), dairy milk (8–11), and farm animals' skin and nares (12, 13). The bacteria are developing resistance to multiple antibiotics and thus becoming a challenge to treating an infection with commonly used antibiotics. Among such antibiotics is vancomycin, a member of glycopeptides, and was the primary applied drug of choice against methicillin-resistant *S. aureus* (14). Recent studies showed *S. aureus* is a resistant pathogen against vancomycin (15, 16) thus increasing the burden of antimicrobial drug resistance.

Resistance against vancomycin is found to be due to a cluster of genes known as the "Van cluster" that is found in a number of bacteria such as S. *aureus, E. fecalis,* Actinomycetes spp., *E. faceium, Clostridium difficile*, and anaerobic bacteria. It is pertinent to note that *Enterococcus* spp. appeared to be a prominent reservoir of vancomycin resistance and its spread to other bacteria is horizontal using the conjugation method. It has been found that *Enterococci* contain Inc18 pSK41-like multiple drug-resistant conjugated plasmids while their presence in *S. aureus* has yet not been documented. The transfer to the latter is found to be by plasmids from *E. faecalis* to other bacteria. Worsening of this resistance is thought to be controlled by manipulation of Van cluster from the bacteria. Hence, it provides a better target for new drug development e.g., phosphinate, phosphonate, and hydroxyethylamines can inhibit Van clusters. These inhibitors are mostly used with vancomycin for the effective uptake of antibiotics inside the bacterial cell (17). Such discoveries are necessary for better control of vancomycin resistance. Vancomycin-resistant genes are extensively transferred from animal to human, animal to bacteria, human to bacteria, and bacteria to bacteria. There is a dire need to focus on novel genes in bacteria to address their resistance and impact on animal and public health. The current review thus focuses on vancomycin resistance in bacteria, its mode of spread, and its impact on food safety/security.

Background of vancomycin

Vancomycin was isolated by E.C Kornfield in year 1957 from *Streptomyces orientalis*, a fungus, located in the deep jungles of Borneo (18). The obtained substance was named "compound 05865" having activity against gram-positive as well as anaerobic bacteria in initial findings. Vancomycin is a tricyclic glycopeptide that acts as a bactericidal against the polymerization of peptidoglycans in the bacterial cell wall. This antibiotic is equally effective against a wider range of pathogens and in surgical procedures to avoid secondary infection. The FDA has approved the use of this drug against *Clostridium difficile*, *Staphylococcus enterocolitis*, *Pseudomembranous colitis*, Enterococcal, Staphylococcal, and Streptococcal spp, which are responsible for diarrheas, endocarditis, sepsis, soft skin infection, bone infections, lower respiratory tract infections, and colitis (19). In other studies, there was an emergence of *mecA* or *mecC* gene in *S. aureus* that codes enzymes to crosslink the peptidoglycans in bacterial cell walls which is now considered a potential threat to control *S. aureus*. The enzymes had low affinity for β-lactams and hence there was a development of resistance. The resistance of Staphylococci against antibiotics was on the rise when this drug was developed therefore the FDA approved vancomycin with number 05865 in 1958 to control infection of Staphylococci. In such a scenario, there was a concern that this product would maintain its efficacy/activity or face resistance. A series of *in-vitro* and lab animal experiments were conducted to rule out this concern (20). The off-label uses of this drug consist of catheter-related infections, neonatal prophylaxis, bacterial meningitis, peritonitis, and many more (21). More than half of regularly used antibacterial medications are no longer effective against Gram-negative Pathogens (GNPs) including *E. coli* and *K. pneumoniae*, according to the WHO Global Report on Surveillance of Antimicrobial Resistance (22–24). It is important to note that drugs like colistin, which were used as a last resort, are no more effective against a GNP such as New Delhi metallo-lactamase-1.

Development of resistance against vancomycin

Vancomycin resistance was first reported in *Enterococcus faecium* in 1986 (25) and the resistant genes were named VRE (Vancomycin-resistant *Enterococci*). Avoparcin was overused as a growth enhancer in animal production in European communities. In addition to *Enterococci*, there was also a development of novel strains of *S. aureus* against vancomycin (Figures 1, 2) that gave rise to three distinct categories (i) VSSA (*S. aureus* sensitive to vancomycin), (ii) VRSA (*S. aureus* resistant to vancomycin), and (iii) VISA (*S. aureus* intermediate susceptible to vancomycin).

Acquired resistance

This kind of resistance resulted in a synthesis of precursors of peptidoglycan which ends in D-Ala-D-Lac and D-Ala-D-Ser instead of D-Ala-D-Ala (Figure 3). These precursors are highly incompatible with vancomycin and thus give rise to a cluster of 11 genes found primarily in Enterococci to confer vancomycin resistance. The precursor ending with D-Ala-D-Lac is coded by vanA, B, D, F, I, and M clusters while that of D-Ala-DSer is coded by vanC, E, G, L, and N clusters. It was found that the vanA gene and vanB gene are frequently encountered genes when it comes to acquired resistance (26, 27). The ability of vancomycin to bind to

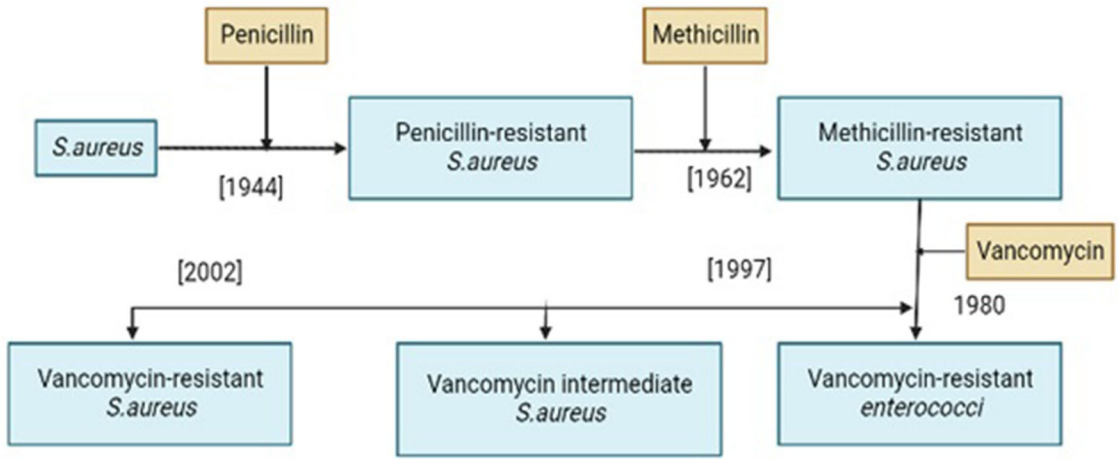

FIGURE 1
History of resistance development in *Staphylococcus aureus* against different antibiotics.

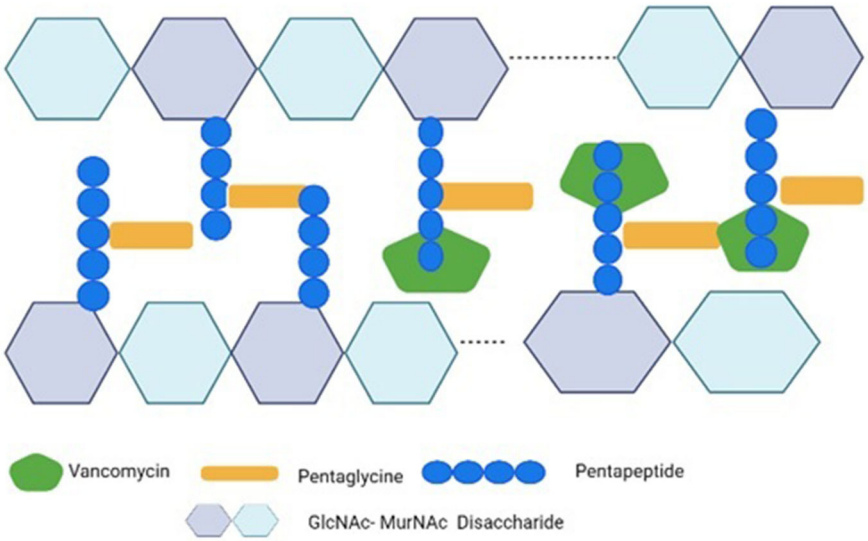

FIGURE 2
Vancomycin's mechanism of action against S. aureus. Vancomycin inhibits the cross-bridge formation between pentagen and pentaglycine by binding to the D-Ala-D-Ala residues of pentapeptide's C-terminal D-Ala-D-Ala. The GlcNAc stands for N-acetylglucosamine; the MurNAc stands for N-acetylmuramic acid.

targeted peptides is 1000 times less when these peptidoglycan precursors are produced with an association constant of 102 M-1 (28).

The gene clusters vanA and vanB consist of vanX, vanH, vanA, and other related homologous variant genes (29). Activating transcription and regulating cytoplasmic responses is achieved by phosphorylating the vanR protein. Among them are vanR, which upregulates the expression of vanR & S genes, and vanHAX. They bind to the core of vanHAX and vanH which encodes a D-lactate dehydrogenase converting pyruvate into D-lactate and vanX. Finally, the products (D-lactate and vanX) encode a D, D-dipeptidase that further cleaves D-Ala–D-Ala (30). The vanA and B clusters also contain vanY that encodes a D, D-carboxypeptidase. A late membrane-bound peptidoglycan precursor is cleaved at the C-terminus by this enzyme. Vancomycin intermediate susceptible *S. aureus* (VISA) also contains mutations in the RNA polymerase gene rpoB which regulates transcription of genes in cell wall biosynthesis but mutated genes in the walkR system (walkR system, yvqF/vraSR system) seem to be directly or indirectly

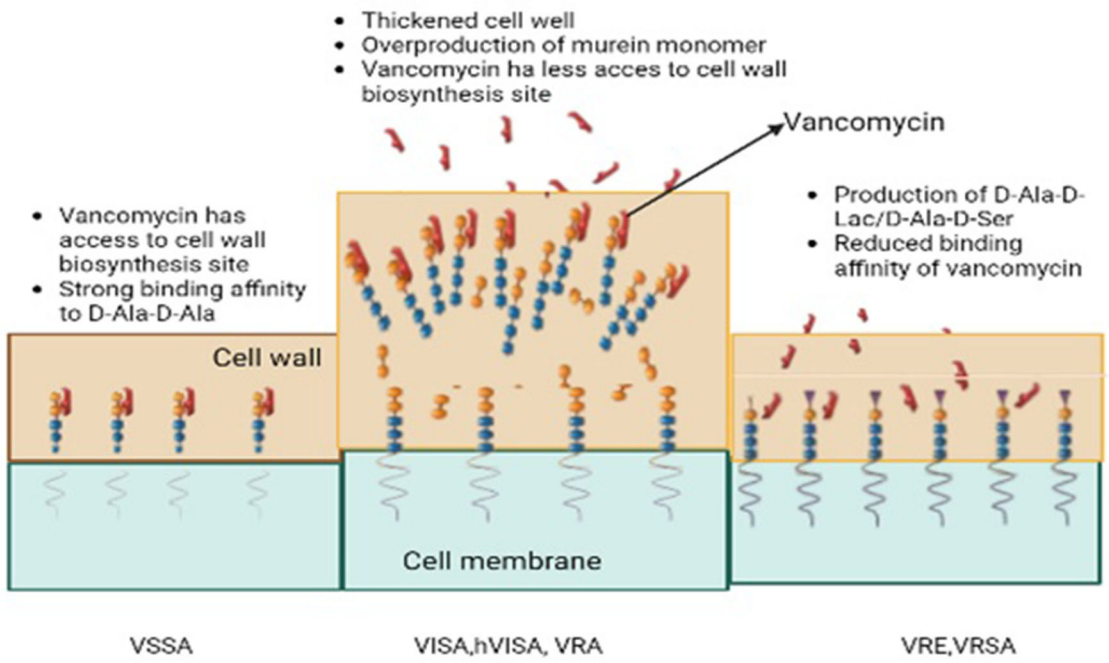

FIGURE 3
The development of acquired resistance; Vancomycin-resistant S. aureus (VRSA), Vancomycin-resistant Enterococci (VRE), Vancomycin susceptible S. aureus, Vancomycin intermediate S. aureus (VISA), Hetero-vancomycin intermediate S. aureus (hVISA).

related to the biosynthesis or metabolism of those two-component sensory regulatory systems (31). In addition to this, lactamase-encoding genes have also exhibited contributions to the expression of VISA (32). VISA isolates demonstrate several common characteristics such as excessive quantities in the cell wall, abnormal daughter cells, post-cellular division, and reduced rates of autolysis. The altered phenomena behind these molecular mechanisms are yet to be understood due to abnormally high amounts of synthesized peptidoglycan. The activation of the arginine catabolism pathway (*via* an activated ADI pathway) in VISA strains (Mu50 lineage) has been suggested to provide ATP molecules and ammonia to compensate for increased cell wall biosynthesis. There is a need for further research to investigate this phenomenon globally in different strains of VISA. There was also a decrease in the cross-linking of peptidoglycan strands in the thickened wall, leading to an increase in non-cross-linked D-alanyl-D-alanine side chains. In addition to the binding of vancomycin to DAla-D-Ala, the bound vancomycin also serves as an obstacle to the free molecules found deeper within the structure (33).

Intrinsic resistance

The gram-negative bacteria had an additional outer membrane that acted as a barrier for the entry of glycopeptides-like hydrophilic molecules. The higher molecular weight and size of these molecules prevent them from entering through the porins of the outer membrane to reach the target site within the cell wall region. This mechanism results in resistance of gram-negative bacteria against glycopeptides and is called 'intrinsic resistance'. The main contributor to this intrinsic resistance appears to be an extra outer membrane (OM) and numerous efflux pumps which successfully block the entry of several pharmacological compounds including glycopeptide antibiotics like vancomycin (34). According to Hancock and Brinkman (35) and Pagès et al. (36) hydrophobic medicines passively pass through the OM whereas hydrophilic antibiotics diffuse through porin channels. Glycopeptides have a complicated chemical structure, a large molecular weight, and are hydrophilic by nature (1,450–2,000 Da). They are hydrophilic but because of their enormous size and molecular weight, they cannot pass through the OM's porins to the cell wall region. GNPs inherently resist glycopeptides because they operate within cell walls.

Role of avoparcin in resistance development

Diseases were non-responsive to the antibiotics intended to be used as growth promotors for better FCR (feed conversion

ratio) and reduced morbidity/mortality amid clinical/subclinical infections (37). Avoparcin was mainly used to treat animals including broilers, turkeys, pigs, and veal calves (38). Data from Denmark shows the breadth of avoparcin use where >24,000 kg was used for growth promotion whereas 24 kg of the drug was utilized to treat humans. In Australia, < 600 kg of vancomycin and nearly 62,000 kg of avoparcin were purchased during 1992–996 in similar circumstances. Avoparcin provides cross-resistance to vancomycin, hence it is improperly used to treat VRE (38). The most clinically significant species is *Enterococcus faecalis* and the strains from animals may pose a risk to humans. For instance, it is believed that human VRE outbreaks in some countries were influenced by the rise of vancomycin-resistant enterococci (VRE). This factor is attributed to excessive usage of the vancomycin derivative avoparcin as a growth promoter in farm animals (39). Until it was forbidden in 1997, the widespread use of avoparcin as a growth enhancer in the agricultural sector was linked to the frequent isolation of VRE from farm animals (40). The first antibiotic feed additive used in food-producing animals banned in 1997 by the EU was avoparcin. This antibiotic was banned from use in food-producing animals because it belongs to the glycopeptide class, a critically important antibiotic used in human medicine. Resistant enterococci have also been isolated from the raw meat of animals fed avoparcin, creating a concern for the passage of resistant enterococci to people *via* the food chain (41). This is what led to the ban of avoparcin as an antibiotic feed additive in the EU.

Exploring resistance in different classes of bacteria

During the 1990s, *E. faecium* with the vanA genotype, or VRE, was prevalent in the intestinal flora of farmed animals in Europe (42, 43). There are several explanations for why VRE still exists in livestock animals. Because the genes for both resistances were found on the same plasmid, a study in Denmark suggested the use of a combination of macrolide and tylosin as the solution for VRE (44). In another study, bacteria are forced to maintain resistance if plasmid addiction systems were present on the same plasmid as the vanA gene (45). The three most prevalent types are vanA, vanB, and vanC, and among these the vanA genotype is considered the most abundant (46, 47). A second variety (vanF) has also been discovered even though it has only been found in *Paenibacillus popilliae* thus far. *P. popilliae* has been proposed as a potential source of vancomycin resistance in enterococci due to a considerable degree of similarity between amino acid sequences of the vanF variant and vanA variant (48). Other potential origins include a variety of organisms that produce glycopeptides even if there is a more likely development of genetic variances as an earlier common source. The initial D-Ala-D-Ala pentapeptide found within the three-dimensional network of peptidoglycans that makes up the bacterial cell wall may be changed to either D-Ala-D-Lactate (D-Ala-D-Lac) or D-Ala-D-Serine (D-Ala-D-Ser) by all forms of vancomycin resistance in enterococci (49). Modifications to the cell wall's structure, brought on by several genes, are the root cause of all forms of vancomycin resistance no matter how many types, the number and arrangement of these genes remain remarkably stable. The sensor gene vanS activates the regulator gene vanR in the presence of a glycopeptide, vanH facilitates the conversion of pyruvate to lactate when the gene complex is active while vanA utilizes lactate to create the alternative D-Ala-D-Lac end of the pentapeptide. Resistance can only exist if the pentapeptide's typical D-Ala-D-Ala terminus is no longer produced. The vanX and vanY genes, respectively, hydrolyze and stop the formation of the pentapeptides and cleave any pentapeptides that might still grow and provide a solution to this problem (49). VanS starts the process of dephosphorylating vanR when a glycopeptide is absent making the gene inactive. Reportedly vanA-type resistance was the first kind among others that induced highly resistant transposon Tn1546 and the other closely linked factors. The vanA ligase catalyzes the ester bond synthesis between D-Ala and D-Lac while vanH dehydrogenase converts pyruvate to D-Lac (30). As a result, the D-Ala-D-Ala dipeptide is changed into the D-Ala-D-Lac dipeptide which significantly reduces the molecule's affinity for glycopeptides (26).

Lipopolysaccharide (LPS) helps Gram-negative bacteria maintain a strong permeability barrier in their outer membrane, blocking the introduction of toxic substances like antibiotics. The important gene *cdsA*, which is involved in the transformation of phosphatidic acid into CDP-diacylglycerol during phospholipid biosynthesis, was the target of all seven suppressors that were examined. These *cdsA* mutations lead to a partial functional impairment as phosphatidic acid accumulation. The fact that these *cdsA* mutations provide a general rise in vancomycin resistance, even in a wild-type cell, means that this suppression is not limited to mutations that lead to abnormalities in outer membrane biogenesis. Researchers demonstrated that the accumulation of phosphatidic acid by methods other than *cdsA* mutations also enhances resistance to vancomycin using genetics and quadrupole time of flight (Q-TOF) liquid chromatography-mass spectrometry (LC-MS) (50). The authors hypothesize that elevated amounts of phosphatidic acid alter the outer membrane's physical characteristics to prevent vancomycin from entering the periplasm and reaching its target, an intermediate needed for the production of the peptidoglycan cell wall (50). Like zoonotic bacteria, resistant bacteria from food animals' intestinal flora contaminate animal corpses after being killed and then pass down the food chain to infect humans' intestines (51). It is possible to determine the prevalence of resistance in various populations and to identify

a potential transmission of resistant bacteria from animals to individuals, and vice versa, by examining the prevalence of resistance of specific indicator organisms like *E. coli* and enterococci in the intestinal tract of various populations of animals and humans.

The stance of the centers for disease control and prevention on the detection of VRSA

The samples processed for isolation and identification of *S. aureus* may grow on a mannitol salt agar or, alternatively, an MRSA chromogenic agar. Whatever method of identification is applied, the obtained *S. aureus* is required to evaluate against vancomycin antibiotic, and, if minimum inhibitory concentration (MIC) is found to be $\geq 4\,\mu g/mL$, it ought to be reported to health departments as reportable isolates. The MIC of vancomycin against *S. aureus* of $\geq 8\,\mu g/mL$ must be sent to CDC for confirmation of VRSA. All the *S. aureus* isolates showing MIC $\geq 16\,\mu g/mL$ against vancomycin are requested by the CDC for further characterization of VRSA precursor organisms. Upon confirmation by the CDC, this is shared with public health partners (52).

The guidelines for declaring different strains of *S. aureus* with respect to vancomycin are as follows:

1. VSSA (*S. aureus* sensitive to vancomycin) presenting MIC $\leq 2\,\mu g/mL$.
2. VRSA (*S. aureus* resistant to vancomycin) presenting MIC $\geq 16\mu g/mL$.
3. VISA (*S. aureus* intermediate susceptible to vancomycin) presenting MIC 4-8 $\mu g/mL$.

VRSA was identified in 2002 in a patient hospitalized for a catheter-related infection. The MIC was noted $>32\,\mu g/mL$ and was related to VRE while molecular analysis showed a vanA positive gene of the Van cluster (53). Vancomycin resistance mechanisms are discussed in the following section, including intrinsic resistance seen in Gram-negative bacteria. Although recently some antibiotic alternatives like bacteriophages are being investigated against potential pathogens (54).

Guidelines for VRSA

According to Hospital Infection Control Practices Advisory Committee (HICPAC), hospitals should develop their institution-specific protocols while the focus should remain as follows (55).

- The clinicians ought to prescribe vancomycin keeping in view the circumstances and consequences.
- Hospital staff should be aware of the vancomycin resistance.
- Detection and prompt reporting of vancomycin resistance in microorganisms from laboratories and hospitals should be early and prompt.
- An appropriate implementation plan for the control of horizontal infection (person to person) of VRE ought to launch.

The CDC suggests decolonizing patients from VRSA to avoid the development of clinical signs and the further spread of resistance. The colonization of VRSA in persons is defined as the presence of VRSA in or on a person but has no development of any clinical signs. The persons may simultaneously colonize with VRSA infection e.g., wound infection, and colonization of VRSA through the colonization of nares. Here it is important to decolonize (reducing the burden of pathogen/organism by eradication strategy). There are approaches suggested for this and among such is decreasing the reservoir of vancomycin-resistant *S. aureus* (VRSA). Certain factors like the disease situation and status of the immune response, capacity of the person to tolerate dose regimen, and risk of transmission to others make the baseline for decolonization. Such a decision further relies on the consultation of the patient's physician and public authorities (52).

Public health concerns

Methicillin-resistant *S. aureus* is not only a recognized source of infection in hospitals (56) but also outside healthcare setups. The excessive use of vancomycin to treat MRSA increases the selective pressure of vancomycin in the population, leading to higher VISAs and VRSA strains (57). The introduction of food animals into Vancomycin-resistant Enterococci production systems was mostly linked to the widespread use of sub-treatment avoparcin to promote the growth of animals in Europe and other countries since the mid-1990s. Avoparcin was prohibited as a growth promoter in the EU in 1997 as described in Commission Directive 97/6/EC. VRE-related human illnesses and outbreaks, as well as the frequency of VRE in asymptomatic human carriers, have grown throughout the EU since 1999. The prohibition of disinfectant growth promoters may have played an important role in reducing VRE carriers in animals and glycopeptide-resistant Enterococci species. Despite the greater frequency of VRE carriers in animals, fewer nosocomial VRE infections were noted in Europe than in the United States even though avoparcin was never prescribed to North American animals. A decade or two ago, people found it difficult to explain food-borne infections except in a few cases of similar VREs identified from humans and

animals which were believed to be spread from animal contact rather than meat intake. Human infections with animal VRE stress resulted in temporary colonization. However, evidence of various molecular typing techniques brought forward a strong correlation between VREs or genetic determinants in animals and humans. These links between animals and human VRE carriers have not been proven to be causal but show that there are several reverse transmissions from wild animals and plants (57).

Concerns about food safety

Antibiotics were initially licensed to be used as growth promoters, while the abrupt ban on their use has compromised health due to critical food animal diseases. On the other hand, this ban has resulted in a reduction of resistance to pathogens of animals and human origin thus enhancing animal health and safety. According to a recent report by Denmark's National Department of Poultry Production, the broiler industry has been "plagued by leg and skin problems" since the late 1990s which could have jeopardized the broiler industry's wellbeing (58).

Food safety concerns about antimicrobial resistance were increased after the ban was implemented. There was a rise in infections which were earlier controlled by the use of medicine in the feed. The utilization of therapeutics in food-producing animals was increased in various parts of Europe. Sales of therapeutic antimicrobials grew from 383 tons in 1999 to 437 tons in 2000 after the EU imposed a ban on growth promoters in 1999. This was due to an increase in tetracycline sales of 36 tons, trimethoprim/sulphonamides sales of 12 tons, and macrolides sales of 12 tons. The use of medicines in pigs increased by 7 tons and use in the poultry sector increased by 13 tons. More than one species of animals showed an increase in the use of medicine by 37 tons. The EU restriction in 1999, as well as the incidents of ailments such as porcine dermatitis, nephritis syndrome, and post-weaning multisystemic wasting syndrome, were factors in the pig business's expansion. Therapeutic antibiotic use in Denmark increased from 48 tons in 1996 to 94 tons in 2001. Tetracycline, which is primarily used in pigs, increased from 12.9 to 27.9 tons (a 116 percent increase) followed by macrolides and lincosamides (7.6 to 14.3 tons, an 88 percent increase), and aminoglycosides (7.1 to 11.9 tons, 68 percent). Experience in Sweden suggests this might be somewhat beneficial in the end, albeit at a greater financial cost. However, it is unknown whether this will apply to the rest of Europe where farming conditions differ from those in Scandinavia (59).

Economic losses due to resistance

In Europe, 9.6 percent of nosocomial infections were caused by Enterococcus species while VRE nosocomial infections were very frequent in Intensive Care Units (ICU). Serious basic health issues including liver transplants, diabetes mellitus, or kidney failure are risk factors associated with VRE colonization and the subsequent development of nosocomial infections. Several investigations have shown that VRE bloodstream infections were common (BSI) and far more deadly than vancomycin-susceptible enterococci BSI (VSE) (60). VRE infections necessitate higher invasive procedures, increased use of antibiotics, and longer hospitalization that led to both increased hospital expenditure and disease risk. In the United States, hospital expenditure for BSI-like VRE infections ranged from $9,949 to $77,558, as per data gathered from a university-based teaching hospital. Another study conducted in the United States discovered that the expenditure associated with VRE-related surgical site infections (SSI) was approximately $12,766. Despite the expense associated with these preventive measures, VRE screening in elevated areas and VRE client isolation also are well-established in many institutions. A study reported the hospitalization expense of VRE-infected patients was more than VSE-infected individuals. This could be because of factors associated with VRE patients' expenses than VSE patients' expenses. The economic impact of VRE infections in hospitalized patients is now being investigated in the United States and other countries.

Conclusion

Vancomycin resistance has been found as an emerging issue that carries a wider range of resistant genes collectively called the "Van cluster". These genes are found among a wider range of microbes that finally share these among each other. The transfer of resistance is extensive among animals, humans, and the environment with the full potential of zoonosis and reverse zoonosis. Significant economic damage is possible and food safety, food security, farm animal health, and public health are at risk. The adoption of a set of guidelines from the Centers for Diseases Controls and Prevention (CDC) ought to be implemented with an active reporting system. However, further protocols are required to control this resistance by developing coordinated but comprehensive therapeutic and preventive strategic plans.

Author contributions

AIA wrote the manuscript. AFA revised the manuscript. 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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Enhancing activity of β-lactam and fluoroquinolones antibiotics by artemisinin and its derivatives against MDR *Escherichia coli*

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Artemisinin and its derivatives had played a biocidal role in biomedical remedies, while they were expected to enhance the activity of antibiotics against multiple drug-resistant (MDR) bacteria. The current study evaluated the interaction of artemisinin (ART), dihydroartemisinin (DHA), artesunate (AS), and artemisinic acid (AA) with β-lactam and fluoroquinolones antibiotics against *Escherichia coli*. Antibiotic strip test (*E*-test), Kirby Bauer's disc test (KB method), and broth microdilution method were adopted for susceptibility analysis, while the checkerboard method was applied to assess synergisms. ART, DHA, AS, and AA showed significantly enhanced antibacterial effects of β-lactam antibiotics against different strains of *E. coli*. The study showed ciprofloxacin to be most effective by presenting the least MIC (0.017125 ± 0.0022 μg/ml), while oxacillin was least effective (MIC 256 μg/ml) against *E. coli*. Synergism between AA and penicillin G (75%), ampicillin (25%), and oxacillin (50%) was observed in all isolates tested. AA and AS significantly decreased the MIC of ampicillin (−0.912 ± 0.908 μg/ml) and ciprofloxacin (−0.901 ± 0.893 g/ml), respectively. Artemisinin and its derivatives increased antibiotic accumulation within *E. coli* in a dose-dependent manner. The time-kill assay significantly reduced the bacterial number within 24 h of incubation. The study thus concludes greater room for improvement in enhancing the efficacy of antibiotics if used with artemisinin and its derivatives.

KEYWORDS

multidrug resistance *E. coli*, beta lactam, fluoroquinolones, artemisinin, fractional inhibitory concentration index

Introduction

Escherichia coli, Salmonella species, and *Staphylococcus aureus* have affected poultry, dairy animals, and pets because they are resistant to a broader range of antibiotics (1–4). Among gram-positive bacteria, a rise in antimicrobial resistance has resulted in novel strains of *S. aureus* like methicillin-resistant *S. aureus*, vancomycin-resistant *S. aureus* (5, 6), and vancomycin-intermediate *S. aureus*. On the other hand, among gram-negative bacteria, *E. coli* is the top prevalent bacteria with extended resistance patterns. These bacteria possess β-lactamases of four classes, i.e., serine penicillinases, Metallo-β-lactamases, cephalosporinases, and oxacillinases (7). China has been found to possess a higher prevalence of extended β-lactamases producing *E. coli* (8).

Despite recent advances in developing antibacterial drugs, every infection is still a widespread, serious, and worldwide problem (9). This problem is accompanied by rapidly increasing multi-drug resistance (MDR) of microorganisms to antibacterial treatments (10). MDR can be mediated by complex mechanisms, such as overexpression of antibiotic-inactivating enzymes, lack of or an alteration in a target site, increased efflux, or lowered penetrability of the drug due to decreased permeability (11). The alarming increase in antibiotic-resistant bacteria highlights the urgent need for more effective drugs to combat bacterial infection (12). Although they do not possess significant antibacterial activity, they bind to β-lactamases and inactivate the β-lactamases, thereby protecting the antibiotics that are typical substrates for these enzymes (13). *Artemisia annua* L., an annual medicinal herb, can grow wild in China and Vietnam's temperate and high-altitude regions (11, 13). Artemisinin, one of the bioactive compounds with anti-malarial activity, has been successfully isolated from *A. annua* (14). Other than anti-malarial activity, it is used to alleviate high fever and treat jaundice (15). Artemisinin was an excellent antibacterial, antifungal, antileishmanial, and antitumor agent (16). Initially, artemisinin was isolated from the traditional Chinese herb *A. annua* L. (sweet wormwood), an active ingredient containing a sesquiterpene lactone (17). Artemisinin and its derivatives, such as artemisinin, dihydroartemisinin, and artesunate, have been widely used against malaria. Artemisinin and its derivatives have recently been found effective in treating viral infections and tumors (18, 19). Previously, we found that artemisinin and its derivatives could protect the sepsis model of mice from challenges with a heat-killed *E. coli* by reducing pro-inflammatory cytokine release and endotoxin level *via* suppressing the activation of toll-like receptor (TLR) 4/TLR9/nuclear factor-kB pathways (20, 21). The antibacterial properties of artemisinin have been tested on a wide range of bacteria, such as *E. coli*, *S. aureus*, *Pseudomonas aeruginosa,* and *Mycobacterium intracellulare* (22).

In brief, artemisinin and its derivatives (Dihydroartemisinin, Artesunate, and Artemisinic Acid) may become potential antibacterial candidates. Therefore, the enhancement of antibacterial activity of various β-lactam and fluoroquinolones antibiotics by Artemisinin and its Dihydroartemisinin against multiple drug resistant *E. coli* were objectives of this study.

Materials and methods

Chemical and antibiotics

Artemisinin, Dihydroartemisinin, Artesunate, and Artemisinic acid were obtained from the China Institute of food and drug control and dissolved following the instruction before use. All antibiotics (ciprofloxacin, imipenem, penicillin G, ampicillin, and oxacillin) were purchased from the North China Pharmaceutical Group Corp (Shijiazhuang, China) and Southwest Synthetic Pharmaceutical Co. Ltd (Chongqing, China). All antibiotics were dissolved and diluted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (23). Antibiotics disks (10 μg) of all five antibiotics and strips of ciprofloxacin, imipenem (0.002–32 mg), penicillin G, ampicillin, and oxacillin (0.016–256 mg) were purchased from Liofilchem srl (Zona Industriale Italy). All standard strains of *E. coli* BNCC 186347, 125787, 125988, and 195617 were purchased from BeNe Culture Collection, Kunshan city, Jiangsu province, China.

Bacterial inoculation

The bacterial suspension was prepared from overnight cultures by the direct colony method. Colonies were taken directly from the plate and suspended in 5 ml of sterile 0.85% saline. The turbidity of the initial suspension was adjusted compared with the 0.5 Mc Farland standard contains about 1×10^8 colony forming units (CFUs)/ml. Ten-fold dilutions of the initial suspension were additionally prepared into sterile 0.85% saline to achieve 1×10^6 CFU/ml (24).

Preparation of antibiotic stock solution

Standard powder forms of penicillin, ampicillin, oxacillin, ciprofloxacin, and imipenem were stored at 4°C till usage. The stock solution of each antibiotic was prepared by weighing and consequently dissolving suitable amounts of the antibiotics, reaching a concentration of 1,024 μg/ml in Mueller-Hinton broth.

Disk diffusion method

The Kirby-Bauer disk diffusion test performed the antimicrobial susceptibility test of the isolates (25). In brief, each test isolate was swabbed uniformly onto the surface of Mueller-Hinton agar plates (26). A drug-sensitive paper sheet containing (10 μg) penicillin, ampicillin, oxacillin, ciprofloxacin, and imipenem was pasted on the agar plate inoculated with the bacteria to be tested. Following incubation, the inhibition zones, in millimeters, were measured in duplicate and scored as sensitive, intermediate, and resistant categories by the critical breakpoints recommended by the Clinical and Laboratory Standards Institute (23).

Determination of the minimum inhibitory concentration

Minimum inhibitory concentration (MIC) values of antibiotics were determined by the microdilution method following the recommendations of Papich (27). Stock solutions of antibiotics were prepared and added to the bottom of a 96-well microtiter plate (Nunc Inc., Roskilde, Denmark). The first well of the 96-well plate was filled with 100 μl of the stock solution, which was then serially diluted. Each well received 100 μl of an overnight *E. coli* culture at a final concentration of 5×10^5 CFU/ml. The microtiter plates were incubated at 35°C for 24 h, and the MIC was determined as the lowest concentration of antibiotics showing no visible bacterial growth (28).

Synergy testing

The synergistic effect of the antibiotic combinations was detected using a checkerboard dilution assay (29). The initial concentration of each drug was twofold greater than the MIC concentration. In a screw cap test tube, 0.25 ml of broth of each two drugs to be tested was added to 0.5 ml of broth containing a suspension of the organism to be tested to reach the final volume of 1 ml. The inoculum of the bacterial suspension (in 0.5 ml of broth) was 2×10^5 CFU to produce a final inoculum of 1×10^5 CFU per ml after adding an equal volume of the antimicrobial solutions. Each test was composed of 36 tubes set horizontally and vertically. Six rows in one direction contained twofold serial dilutions of antibiotic, and six rows in the other direction contained twofold serial dilutions of the drug; two additional rows had twofold serial dilutions of antibiotic and drug alone. The tubes were incubated at 37°C for 24 and 48 h, the tubes were read as those showing turbidity (+) and those showing no turbidity (–). Also, swabs from each tube were streaked on blood agar plates. These plates were seen as those showing growth and those showing no growth. A fractional inhibitory concentration index was used to interpret the results. The FIC of each agent was calculated by dividing the MIC of the drug in combination with the MIC of the drug alone as mentioned in the equations below.

$$FIC\ of\ Drug\ A = \frac{MIC\ of\ drug\ A\ in\ presence\ of\ drug\ B}{MICof\ drug\ A\ \left(Alone\right)}$$

$$FIC\ of\ Drug\ B = \frac{MIC\ of\ drug\ B\ in\ presence\ of\ drug\ A}{MICof\ drug\ B\ \left(Alone\right)}$$

$$FIC\ Index = FIC\ of\ drug\ A + FIC\ of\ drug\ B$$

The sum of both FICs ($\sum$FIC = FIC of antibiotic + FIC of Drug) in each well was used to categorize the combined activity of antimicrobial agents at the given concentrations as synergistic ($0 < \text{FICI} \leq 0.5$), additive ($0.5 < \text{FICI} \leq 1$), indifferent ($1 < \text{FICI} \leq 4$), and antagonistic ($\sum\text{FICI} \geq 4$) [33].

Unit change/percent change in the efficacy of antibiotics

Unit increase or decrease in MIC of antibiotics used in combination with the drug compared to the antibiotic used alone was calculated (formula given below) to find differences among different interactions at the level of 1 μg/ml of antibiotic used alone. To develop a more general understanding, the percentage change in MIC of antibiotics used in combination compared to that used alone was calculated (formula given below).

$$\text{A unit change in MIC} = (MIC\ in\ combination - MIC\ alone)/MIC\ alone$$

$$\text{Percentage change in MIC of antibiotic} = \left((\text{MIC in combination} - \text{MIC alone})/\text{MIC alone}\right) \times 100$$

Killing kinetics

Time–kill assays on *E. coli* were performed using artemisinin, dihydroartemisinin, artesunate, and artemisinic acid at 0.5×, 1×, and 2× the MIC. After 24 h and 48 h of incubation, the number of CFU was assessed by serial dilution. The rate and extent of killing were expressed as viable count ($\log_{10}$ CFU/ml) against time (27).

First significant reduction in bacterial count (μg/ml)

All drug-antibiotic interactions were further analyzed for their quick response. The bacterial count was assessed by their first significant reduction in MIC among different time intervals.

Comparison of between drugs used in combination with antibiotics

To highlight which drug showed a higher effect in combination with antibiotics, the comparison between ART and DHA when used with each antibiotic and each time interval was analyzed. Similarly, AS and AA was compared by comparing MIC of drug-antibiotic combinations.

Statistical analysis

SPSS version 22 for Windows was used to analyze the data. A *t*-test was used to find statistical differences between the experiments, such as the difference between using a combination of antibiotics and just one antibiotic. One-way ANOVA was also used to compare how these treatments affected the fold change of MIC values. It was suspected that a $p \leq 0.05$ value was statistically significant.

TABLE 1 Confirmation of antibiotic resistance profile of *E. coli* against different antibiotics using different tests.

Antibiotic names	E-test (μg/ml)	ZOIs (mm)	Broth microdilution method MICs (μg/ml)
Penicillin	172 ± 88.63	1.245 ± 1.376	215 ± 251.147
Ampicillin	71.125 ± 106.882	14.545 ± 8.97	170.94 ± 271.26
Oxacillin	256 ± 0	0 ± 0	360 ± 174.36
Ciprofloxacin	0.017125 ± 0.0022	37.12 ± 1.596	0.0196 ± 0.012
Imipenem	0.239 ± 0.09262	29.035 ± 1.317	0.625 ± 0.383

Results

Antibacterial response

The study found a significant difference in minimum inhibitory concentrations (MIC) among tested drugs in that DHA and AA differed significantly ($p < 0.05$) between each other and with ART and AS. However, ART and AS showed a non-significant difference ($p > 0.05$) in MIC. ART showed the highest MIC, while DHA expressed a minimum of all four drugs against *E. coli*, exhibiting that the latter showed the highest antibacterial potential among the slots (Supplementary Figure S4).

Antibiotic resistance was confirmed by all three phenotypic methods commonly used against bacteria (Table 1, Supplementary Figure S1). In E-test, ciprofloxacin stood highly productive because it showed a minor MIC (0.017125 ± 0.0022 μg/ml), followed by imipenem, ampicillin, penicillin, and oxacillin. The latter showed a very high MIC (256 μg/ml). Similar findings were noted when the disc diffusion test and broth microdilution methods were applied. MIC values were, however, higher in the case of the broth microdilution method compared to the E-strip test.

Synergism of ART and DHA with antibiotics

The study showed none of the antagonistic responses of ART with all the antibiotics. A similar response was noted in the case of DHA with antibiotics (Table 2, Supplementary Figure S2). The latter showed no interaction as indifferent. In contrast, the former showed 25% of isolates falling in the indifferent category when ART was combined with ciprofloxacin, ampicillin, and

TABLE 2 Percentage of isolates with different synergy interactions of ART and DHA with antibiotics.

Drug name	Antibiotic name/type of drug interactions	Type of synergy interactions (%)			
		Synergistic	Additive	Indifferent	Antagonistic
ART.	Ciprofloxacin	25	50	25	0
	Imipenem	0	50	50	0
	Ampicillin	0	75	25	0
	Oxacillin	0	75	25	0
	Penicillin G	50	50	0	0
DHA.	Ciprofloxacin	50	50	0	0
	Imipenem	0	100	0	0
	Ampicillin	0	100	0	0
	Oxacillin	25	75	0	0
	Penicillin G	50	50	0	0

TABLE 3 Percentage of isolates with different synergy interactions of AA and AS with antibiotics.

Drug name	Antibiotic name	Type of synergy interactions (%)			
		Synergistic	Additive	Indifferent	Antagonistic
AA	Ciprofloxacin	50	50	0	0
	Imipenem	25	75	0	0
	Ampicillin	75	25	0	0
	Oxacillin	50	50	0	0
	Penicillin G	100	0	0	0
AS	Ciprofloxacin	0	50	50	0
	Imipenem	0	75	25	0
	Ampicillin	25	50	25	0
	Oxacillin	0	75	25	0
	Penicillin G	50	25	25	0

oxacillin, while with imipenem, 50% fell in the stated category. Partial synergism was noted highest response compared to all other interactions when ART and DHA were combined with antibiotics.

In combination with antibiotics, AS and AA showed higher percentages of isolates falling into the sensitive category than ART and DHA (Tables 2, 3). All the *E. coli* isolates showed partial and complete synergism when AA was used in combination with antibiotics against (Table 3). Penicillin G, ampicillin, and oxacillin in combination with AA showed 100, 75, and 50% of isolates falling in the complete sensitive category, while imipenem and ciprofloxacin showed 75 and 50% of isolates falling into partial sensitive drug interaction. On the other hand, artesunate, in combination with imipenem, oxacillin, and ciprofloxacin, showed 75 75, and 50% of isolates showed partial synergism.

Change in MIC of antibiotics in combination with ART and DHA

The analysis of unit changes in MIC of antibiotics in combination with drugs (ART & DHA) compared to the antibiotics used alone showed the highest decrease in the case of oxacillin with ART and ampicillin with DHA (Table 4, Supplementary Figure S3). The comparison of the unit decreases for ART and antibiotics was found uniform as there was a non-significant difference among antibiotics in combination with ART. The same was noted among antibiotics combined with DHA.

Change in MIC of antibiotics in combination with AS and AA

A comparison of a unit change in MIC of antibiotics in combination with AA and AS showed a non-significant difference ($p > 0.05$) with each other indicating a smooth response by drug interaction assay (Table 5). A more remarkable change in MIC was noted in the case of ampicillin (-0.912 ± 0.908 μg/ml) in combination with AA, followed by ciprofloxacin (-0.901 ± 0.893 μg/ml) combination while in the case of AS; there was a more significant change in penicillin G (-0.9 ± 0.0306 μg/ml). The fact indicates that 0.912 ± 0.908 μg/ml of ampicillin antibiotic was reduced in combination with AA compared to 1 μg/ml of it used alone. The same goes for others in this trial, where a significant reduction in MIC was observed. Expressing percentage decrease in MIC was found more in penicillin G and oxacillin when combined with ART than DHA (Supplementary Figure S5). Ampicillin, imipenem, and ciprofloxacin showed a more significant reduction in MIC when combined with DHA. However, penicillin G showed a more substantial decrease in MIC when combined with AS, while all other antibiotics showed more MIC reduction when combined with AA (Supplementary Figure S6).

TABLE 4 Comparative unit (means ± SD) change in minimum inhibitory concentration (μg/ml) of antibiotics used in combination with ART and DHA.

Antibiotics	ART	DHA
Penicillin G	-0.888 ± 0.0729^{a}	-0.875 ± 0.068^{a}
Oxacillin	-0.925 ± 0.9225^{a}	-0.762 ± 0.728^{a}
Ampicillin	-0.631 ± 0.758^{a}	-0.844 ± 0.825^{a}
Imipenem	-0.7624 ± 0.727^{a}	-0.812 ± 0.787^{a}
Ciprofloxacin	-0.755 ± 0.718^{a}	-0.870 ± 0.892^{a}

Different superscripts within a column indicate significant differences ($p < 0.05$). The formula "Unit change in MIC = (MIC in combination-MIC alone)/MIC alone."

TABLE 5 Comparative unit (means ± SD) change in minimum inhibitory concentration (μg/ml) of antibiotics used in combination with AA and AS.

Antibiotics	AA	AS
Penicillin G	-0.8375 ± 0.075^{a}	-0.9 ± 0.0306^{a}
Oxacillin	-0.8375 ± 0.816^{a}	-0.8 ± 0.81^{a}
Ampicillin	-0.912 ± 0.908^{a}	-0.894 ± 0.885^{a}
Imipenem	-0.800 ± 0.810^{a}	-0.850 ± 0.833^{a}
Ciprofloxacin	-0.901 ± 0.893^{a}	-0.851 ± 0.834^{a}

Different superscripts within a column indicate significant differences ($p < 0.05$). Formula "Unit decrease = (MIC in combination-MIC alone)/MIC alone."

Time kill assay and first reduction in bacterial count (CFU/ml)

The first reduction in MIC was described to find earlier efficacy of antibiotics with drug combinations against *E. coli* to provide a baseline for adequate time intervals for dose

TABLE 6 Comparison of time kills assay (bacterial count, CFU/ml) under the effect of AS and AA.

Name of antibiotic	Drug name	0 h	2 h	4 h	6 h	8 h	24 h
Ciprofloxacin	AS	297.0 ± 156.42^{a}	166.0 ± 174.02^{a}	72.00 ± 82.126^{b}	40.25 ± 42.161^{c}	5.75 ± 11.50^{d}	0.00 ± 0.00^{e}
	AA	447.00 ± 254.649^{a}	264.25 ± 303.983^{a}	185.25 ± 218.007^{a}	66.75 ± 82.062^{a}	24.50 ± 32.265^{b}	3.25 ± 6.500^{c}
Imipenem	AS	292.75 ± 256.860^{a}	151.25 ± 251.900^{a}	123.75 ± 208.823^{a}	102.25 ± 196.581^{a}	69.50 ± 139.000^{a}	39.00 ± 78.00^{a}
	AA	596.50 ± 141.6^{a}	496.25 ± 144.7^{a}	400.00 ± 163.1^{a}	248.25 ± 168.8^{b}	54.00 ± 68.13^{c}	1.00 ± 2.000^{d}
Ampicillin	AS	335.25 ± 216.5^{a}	280.00 ± 189.2^{a}	206.50 ± 152.5^{a}	112.50 ± 88.98^{a}	28.50 ± 37.89^{b}	10.25 ± 20.5^{c}
	AA	441.00 ± 186.790^{a}	300.00 ± 213.815^{a}	235.00 ± 222.577^{a}	90.251 ± 08.014^{b}	21.50 ± 36.638^{c}	1.00 ± 2.000^{d}
Oxacillin	AS	385.25 ± 226.553^{a}	271.25 ± 196.315^{a}	218.25 ± 155.127^{a}	136.75 ± 140.555^{a}	22.50 ± 26.032^{b}	0.50 ± 1.000^{c}
	AA	374.25 ± 187.416^{a}	269.75 ± 199.796^{a}	169.00 ± 184.808^{a}	123.25 ± 163.700^{a}	68.50 ± 99.915^{a}	24.25 ± 48.500^{b}
Penicillin G	AS	290.75 ± 198.028^{a}	143.75 ± 131.677^{a}	91.00 ± 140.490^{a}	62.50 ± 100.125^{a}	6.00 ± 12.000^{b}	$.00 \pm .00^{c}$
	AA	357.00 ± 199.371^{a}	177.25 ± 107.608^{a}	128.75 ± 85.745^{b}	38.25 ± 46.843^{c}	17.00 ± 24.042^{d}	0.00 ± 0.00^{e}

Different superscripts within a row indicate a significant difference ($p < 0.05$).

TABLE 7 Comparison of the time-kill assay (bacterial count) under the effect of ART and DHA.

Name of antibiotic		0 h	2 h	4 h	6 h	8 h	24 h
Ciprofloxacin	ART	190.25 ± 125.128^{a}	13.00 ± 16.269^{b}	4.50 ± 8.347^{c}	2.50 ± 5.00^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
	DHA	326.75 ± 303.418^{a}	239.00 ± 278.713^{a}	195.00 ± 252.521^{a}	113.00 ± 192.897^{a}	8.25 ± 16.500^{a}	0.00 ± 0.00^{b}
Imipenem	ART	287.50 ± 239.511^{a}	174.75 ± 246.384^{a}	126.25 ± 210.270^{a}	107.75 ± 193.498^{a}	4.50 ± 9.000^{a}	2.00 ± 4.00^{a}
	DHA	503.75 ± 187.41^{a}	381.00 ± 177.40^{a}	253.25 ± 212.734^{a}	193.25 ± 182.872^{a}	44.00 ± 48.833^{b}	22.50 ± 35.707^{c}
Ampicillin	ART	308.50 ± 168.761^{a}	193.75 ± 189.804^{a}	111.00 ± 121.403^{a}	47.50 ± 75.372^{b}	11.25 ± 16.029^{c}	0.00 ± 0.00^{d}
	DHA	357.00 ± 211.781^{a}	286.25 ± 232.213^{a}	169.00 ± 177.281^{a}	108.50 ± 119.551^{a}	2.75 ± 4.272^{b}	0.00 ± 0.00^{c}
Oxacillin	ART	286.75 ± 144.657^{a}	183.50 ± 173.221^{a}	144.00 ± 159.689^{a}	69.50 ± 111.683^{a}	14.25 ± 20.271^{b}	0.00 ± 0.00^{c}
	DHA	386.75 ± 238.315^{a}	288.75 ± 262.500^{a}	183.50 ± 208.058^{a}	116.25 ± 127.369^{a}	34.50 ± 54.751^{b}	0.00 ± 0.00^{c}
Penicillin G	ART	300.00 ± 161.107^{a}	123.75 ± 83.731^{a}	60.50 ± 103.989^{b}	56.25 ± 96.472^{c}	8.50 ± 10.116^{d}	0.00 ± 0.00^{e}
	DHA	374.50 ± 262.062^{a}	265.25 ± 258.965^{a}	125.50 ± 204.386^{a}	91.75 ± 156.585^{a}	16.25 ± 18.768^{a}	5.50 ± 11.00^{a}

regimens. Time kill assay showed zero bacterial counts after 24 h of incubation of *E. coli* against ciprofloxacin in combination with AS and penicillin G in combination with AS and AA. In contrast, all other combinations of antibiotics with drugs showed some bacterial count (CFU/ml) (Table 6). The study showed a significant bacterial count reduction started at the 4th hour of incubation in case ciprofloxacin combined with AS and penicillin G in combination with AA. At the 6th hour of incubation, the first significant reduction of MIC was noted in the case of ampicillin and imipenem were used in combination with Similarly, ciprofloxacin with AA, ampicillin with AS, oxacillin with AS, and penicillin G with AS showed the first reduction in their MICs at 8th hour of incubation. It was noteworthy that oxacillin in combination with AA could show the first reduction in MIC after 24 h of incubation.

ART and DHA showed better antibiotic interaction than AS and AA against *E. coli*. There was no bacterial count (CFU/ml) after the 8th and 24th hour of incubation when ciprofloxacin was used with ART, while other zero CFU/ml were noted after the 24th hour of all different drug combinations except for imipenem with ART and DHA; and penicillin G with DHA (Table 7). The first reduction in bacterial count (CFU/ml) was started at 2 h of incubation in the case of ciprofloxacin with ART. In contrast, at the 4th-hour incubation, this response was noted in the case of penicillin in combination with ART. Only ampicillin with ART showed a first significant reduction in MIC at the 6th hour of incubation.

In contrast, at the 8th hour of incubation, imipenem with DHA, ampicillin with DHA, oxacillin with ART, and DHA oxacillin with DHA did not significantly reduce bacterial count (CFU/ml) throughout the time-kill assay. It was also noted that there were higher standard deviations and some of the combinations, which reflected significant variations of response existing with strains of *E. coli*.

Comparison between drugs in a combination of each antibiotic at different time intervals

This section described the difference between ART and DHA (*t*-test) when combined with antibiotics at 2, 4, 6, 8, and 24th

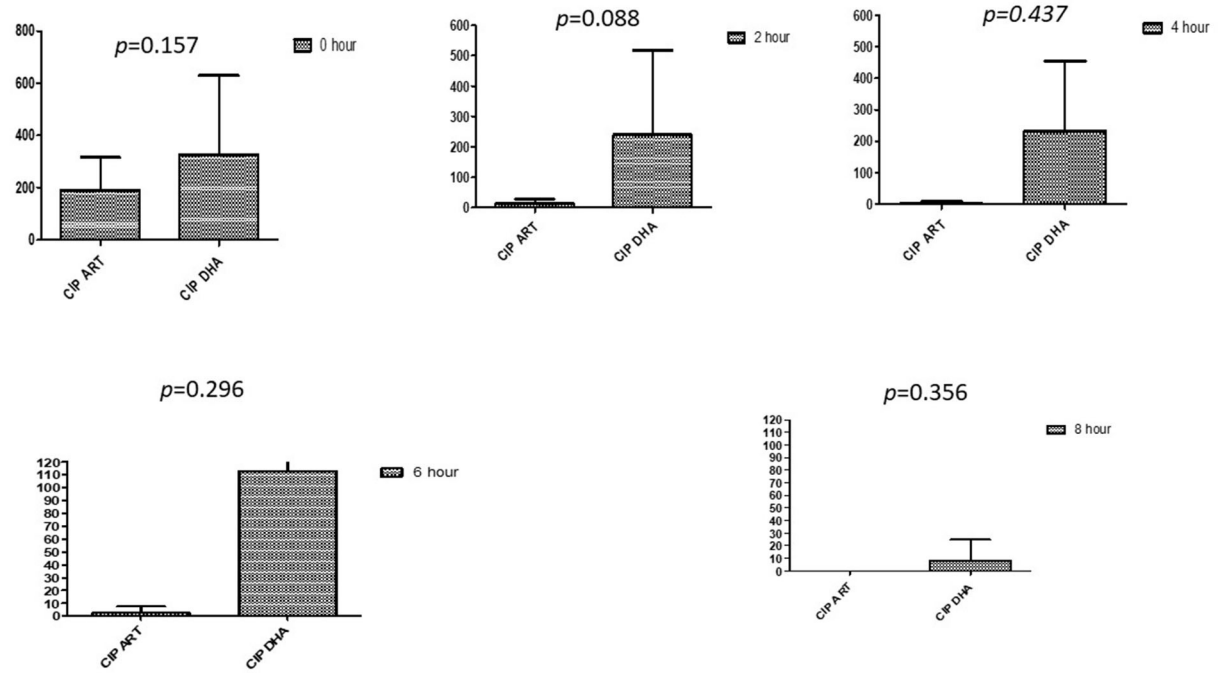

FIGURE 1
Compare ciprofloxacin's minimum inhibitory concentrations (μg/mL) when combined with ART and DHA on E. coli. CIP ART, Ciprofloxacin combined with ART; CIP DHA, Ciprofloxacin combined with DHA; $p < 0.05$ indicate a significant difference among MICs of antibiotic combined ART and DHA.

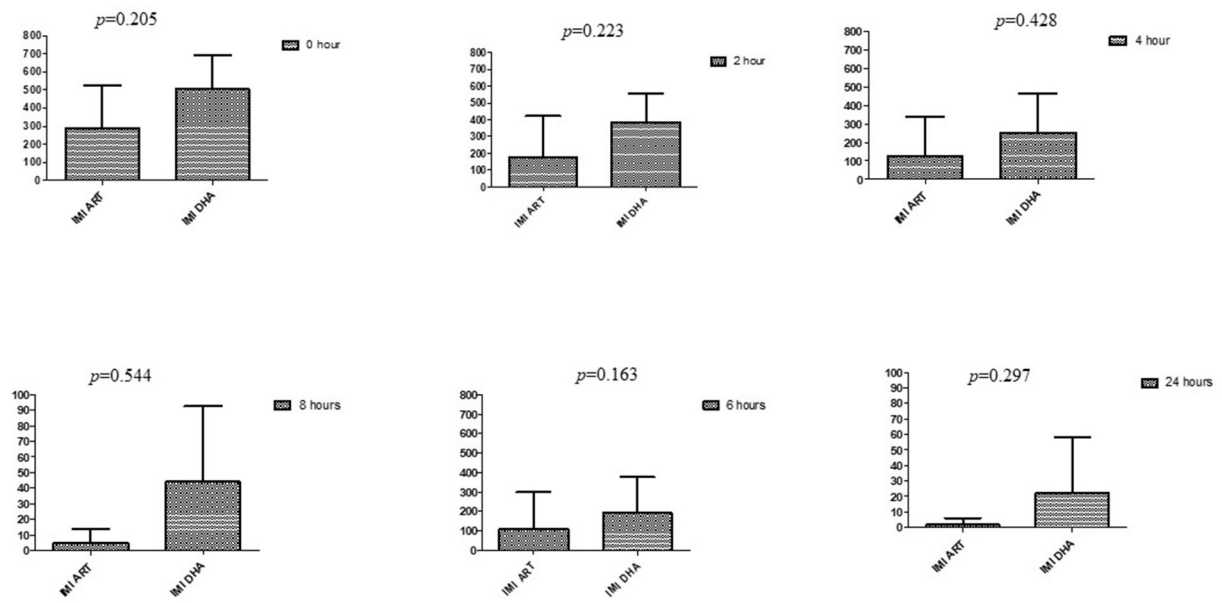

FIGURE 2
Comparison of imipenem's minimum inhibitory concentrations (μg/mL) when combined with ART and DHA on E. coli. IMI ART, Imipenem combined with ART; IMI DHA, Imipenem combined with DHA; $p < 0.05$ indicate a significant difference among MICs of antibiotic combined with ART and DHA.

hour of incubation. Similarly, AA and AS were compared with each antibiotic to find a better efficacious drug (Figures 1–9). The comparison between ART and DHA in combination with ciprofloxacin showed a non-significant ($p > 0.05$) difference at 2, 4, 6, and 8th hour of incubation (Figure 1). A similar response of comparison of ART and DHA with each imipenem,

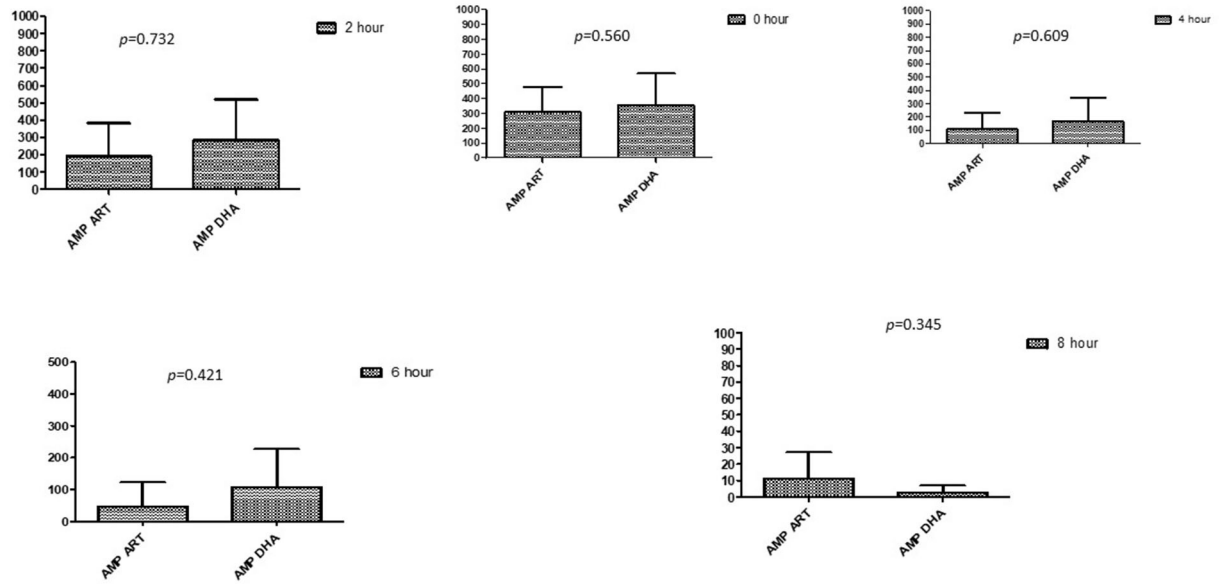

FIGURE 3
Comparison of ampicillin's minimum inhibitory concentrations (μg/mL) when combined with ART and DHA on E. coli. AMP ART, Ampicillin combined with ART; AMP DHA, Ampicillin combined with DHA; $p < 0.05$ indicate a significant difference among MICs of antibiotic combined ART and DHA.

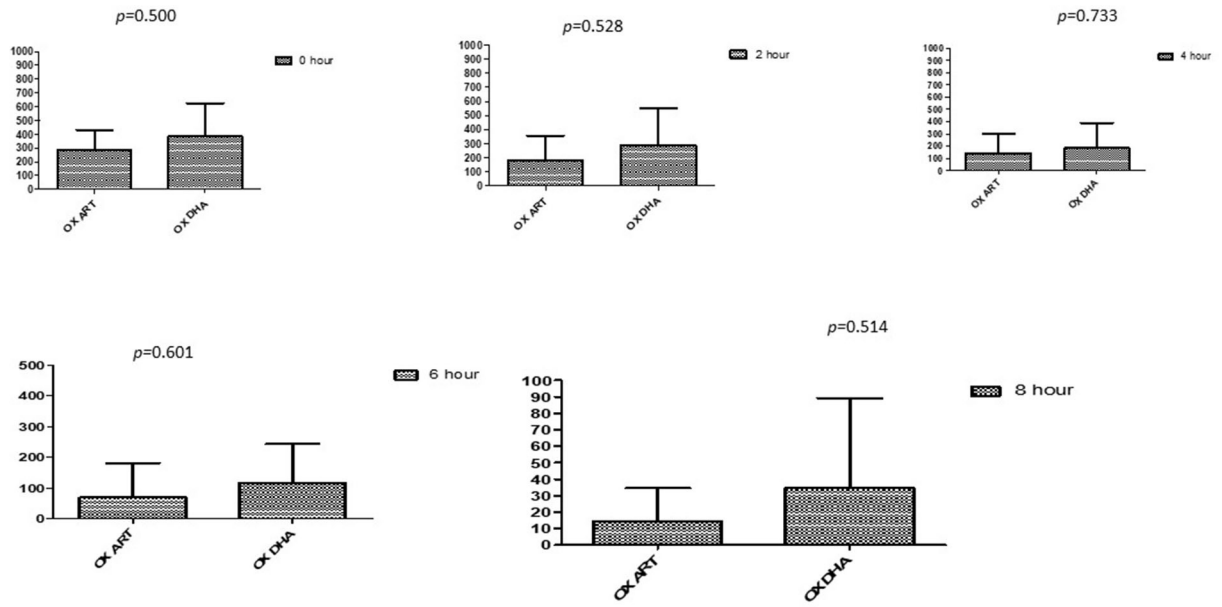

FIGURE 4
Comparison of Oxacillin's minimum inhibitory concentrations (μg/mL) when combined with ART and DHA on E. coli. OX ART, Oxacillin combined with ART; OX DHA, Oxacillin combined with DHA; $p < 0.05$ indicate a significant difference among MICs of antibiotic combined ART and DHA.

ampicillin, oxacillin, and penicillin G was noted at each time interval (Figures 6–9). A similar response was noticed when AA and AS were compared with each antibiotic at every incubation period (Figures 6–9). However, a comparison of AS and AA in combination with ciprofloxacin showed $p = 0.055$ and $p = 0.088$ at 2 and 4th hour, respectively, of incubation. Similar p-values ($p = 0.084$, $p = 0.055$, and $p = 0.082$) were found at 0, 2, and 4 h of incubation when AA and AS in combination with imipenem were compared. Overall, all drugs presented continued support in reducing the antibiotic quantity to combat *E. coli.*

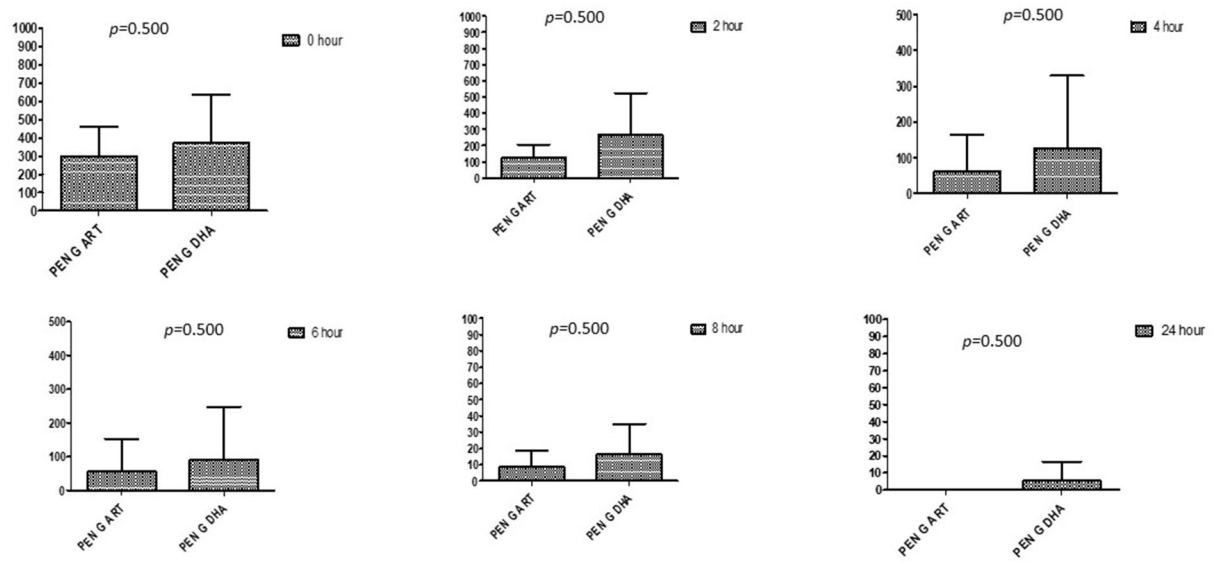

FIGURE 5
Comparison of minimum inhibitory concentrations (μg/mL) of Penicillin G when combined with ART and DHA on E. coli. PENG ART, Penicillin G combined with ART; PEN G-DHA, Penicillin G combined with DHA; $p < 0.05$ indicate a significant difference among MICs of antibiotic combined with ART and DHA.

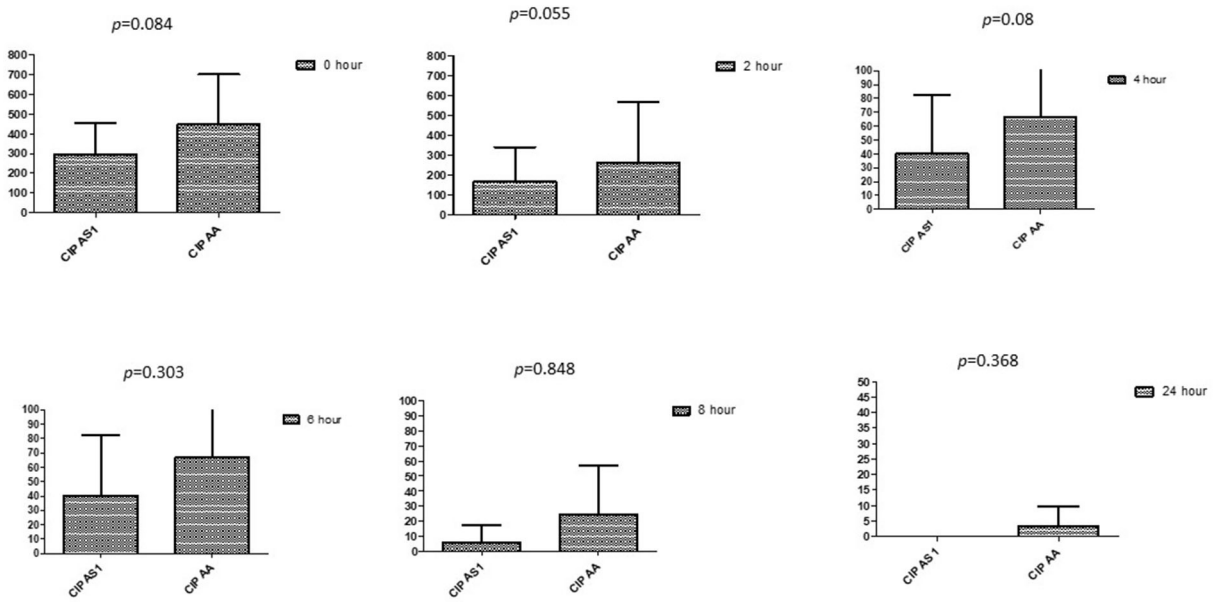

FIGURE 6
Comparison of ciprofloxacin's minimum inhibitory concentrations (μg/mL) when combined with AA and AS on E. coli. CIP AS, Ciprofloxacin combined with AS; CIP AA, Ciprofloxacin combined with AA; $p < 0.05$ indicate a significant difference among MICs of antibiotic combined AS1 and AA.

Discussion

The anti-malarial drugs artemisinin and its derivatives (artesunate, dihydroartemisinin, artemether, and arteether) have been clinically used to treat malaria (18, 19, 30). Artesunate was also revealed to increase the susceptibility of various β-lactam antibiotics against *E. coli* by increasing antibiotics accumulation *via* inhibiting the efflux pump system, AcrAB-TolC, an essential and significant multi-drug efflux pump system within *E. coli* (31, 32). Results showed that oxacillin had the greatest MIC, while ciprofloxacin had the lowest MIC, supported by those reported by CLSI (23). The antibacterial activity of drugs at

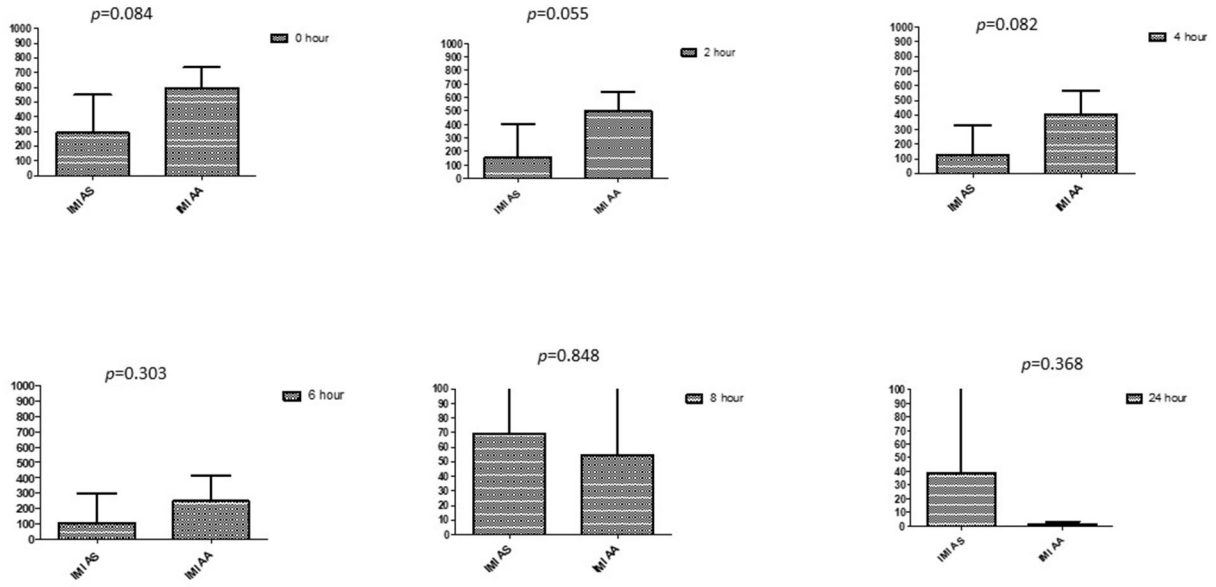

FIGURE 7
Comparison of imipenem's minimum inhibitory concentrations (μg/mL) when combined with AA and AS on E. coli. IMI-AS, Imipenem combined with AS; IMI-AA, Imipenem combined with AA; $p < 0.05$ indicate a significant difference among MICs of antibiotic combined with AS and AA.

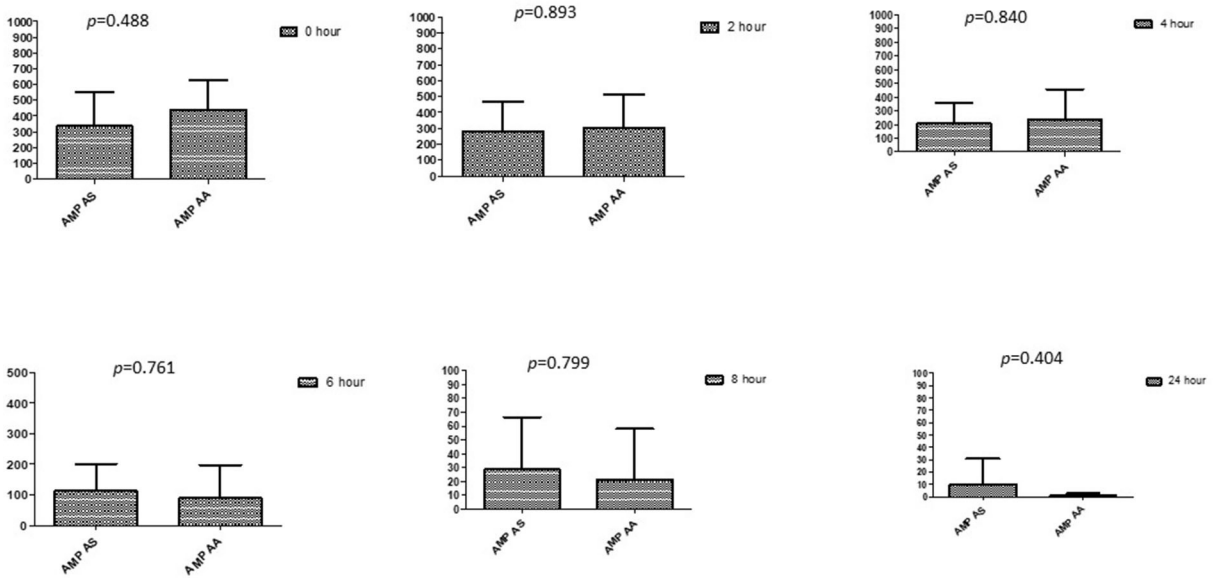

FIGURE 8
Comparison of ampicillin's minimum inhibitory concentrations (μg/mL) when combined with AA and AS on E. coli. AMP-AS, Ampicillin combined with AS; AMP-AA, Ampicillin combined with AA; $p < 0.05$ indicate a significant difference among MICs of antibiotic combined with AS and AA.

higher concentrations in the current study was in line with the previous research (33). However, in contradiction to the current study, Wu et al. (34) did not find the antibacterial activity of DHA and ART. Variation in the activity of DHA and AA from each other was in line with the findings of Wang et al. (35). Resistance to antibiotics using all three phenotypic tests was in line with Berge et al. (36). However, the broth microdilution method identified a higher MIC than the *E*-strip test (23).

No antagonistic effect of ART and DHA with all antibiotics was also noted (37). DHA has not shown indifferent interaction but synergistic and additive effects with antibiotics, previously described as an antitumor anti-malarial agent by Wu et al. (37). The additive effect of AS with imipenem, oxacillin, and

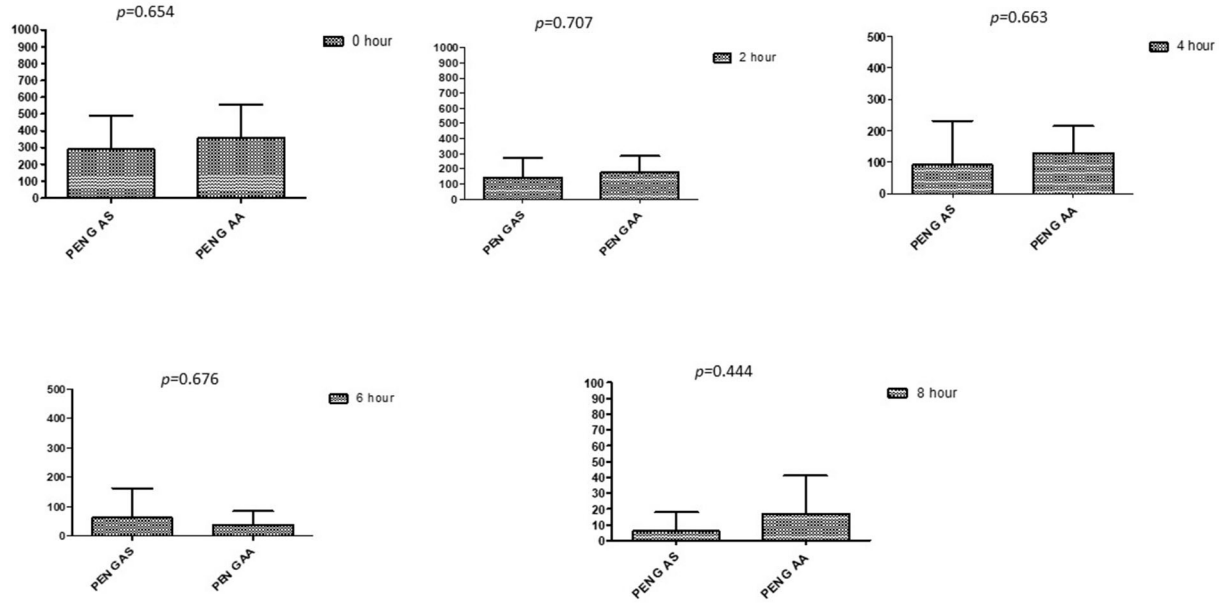

FIGURE 9
Comparison of minimum inhibitory concentrations (μg/mL) of Penicillin G when combined with AA and AS on E. coli. PEN G-AS, Penicillin G combined with AS; PEN G-AA, Penicillin G combined with AA; $p < 0.05$ indicate a significant difference among MICs of antibiotic combined with AS and AA.

ciprofloxacin was once also identified (38). Huang et al. (39) has also observed the property of artemisinin and its derivatives, as found in the current study. A comparison of antibiotic MIC changes with AA and AS revealed no significant difference ($p > 0.05$), confirming a smooth response by drug interaction test. To the best of our knowledge, these responses were seen by Li et al. (20) while using AS, but for AA, research was not found so far. The enhancement in antibiotic property of AA in the current study contradicted the previous studies that noted no or negligible antibiotic activity. Wu et al. (34) observed a reduction in penicillin G's MIC by AS than by AA while using ART and DHA with different antibiotics. Jingsu Meng also observed these values using ART, AS, and DHA (40). However, no AA higher standard deviations study for some combinations reflected substantial variability in *E. coli* reactivity. All drugs tested in the (41) study showed lower *E. coli* burdens. In our study, all four drugs, including ART, AS, DHA, and AA, have shown similar effects in combination with different antibiotics. It might be due to the effect described by Baucheron S (42). In addition to drug accumulation within the bacterial cell, it might also be possible to inhibit some drug transportation channels, Odds saw this effect when using Artemisinin (9). ART and AS were previously studied, but minimal information is available in combination with fluoroquinolones. No drug has shown an antagonistic effect when used with antibiotics, indicating that all drugs play an equally important role in lowering antibiotic resistance.

Conclusion

The current study presented the enhanced activity of antibiotics in combination with artemisinin and its derivatives. Time-kill assay showed significant efficacy of synergistic combinations at earlier hours of incubation, indicating their potential to use in outbreaks. The study thus proposes that apart from developing new antibiotics, existing ones with enhancing monomers like artemisinin and its derivative be employed. Further studies are required to document efficacy and safety parameters in lab animals and at the farm level.

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

Conceptualization and methodology: SU, LW, JL, and WG. Validation: JL, LW, and WG. Formal analysis: SU, LW, AA, AM, and MS. Investigation: SU and LW. Data curation: SU, AA, and LW. Writing—original draft preparation and visualization: SU. Writing—review and editing: AA, SU, AM, MG, AI, SA, and KC. Supervision and funding acquisition: JL. Project administration:

JL and LW. 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.2022.1048531/full#supplementary-material

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Cost-benefit ratio of anthelmintic treatment and its comparative efficacy in commercial dairy farms

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Intestinal parasitic infection is one of the major challenges in obtaining optimal production and maintaining the health and welfare of all animals including cattle and buffaloes. Anti-parasitic treatments appear to be a reliable countermeasure. However, the effectiveness and selection of suitable anthelmintics require situational assessments in a given locality. In the current study, the efficacy and impact of benzimidazole (albendazole) were assessed in a total of 400 (100 each) on the performance of buffaloes, buffalo-heifer, cattle, and cattle-heifers at two commercial dairy farms in the Province of Punjab, Pakistan. Additionally, the cost-benefit ratio was calculated by assessing the inputs (medication, feed, and labor cost) and outputs (milk and weight gain). The qualitative and quantitative examination of helminth eggs in each type of animal indicated a prevalence of 73.3, 78.3, 76.6, and 85.0% in cattle, cattle-heifers, buffaloes, and buffaloes-heifers, respectively. Specifically, a highest rate (10.0–13.3%) of *Haemonchus* sp. infection was only observed in cattle and heifers, while *Fasciola* sp. infections (10.0–11.6%) were the most often found species in buffaloes and heifers. The highest anthelmintic impacts (egg per gram of feces, $p < 0.001$) were observed on day 14 post-medication. Until 60 days of post-anthelmintic treatment, an average increase of 0.8 and 0.7 L in milk production per day in cattle and buffaloes, respectively while a total of 11.45 and 9.45 kg body weight were noticed in cattle-heifer and buffaloes-heifer, respectively. Cumulative cost-benefit analysis indicated a positive correlation between treated and non-treated animals. These

findings reiterate the importance of anthelmintic drugs in reducing the impacts of parasites on the productivity, health, and well-being of an animal under high infection challenges.

KEYWORDS

anthelmintic, efficacy, cattle, buffaloes, heifers, cost-benefit ratio

Introduction

Livestock plays a major role in the economy of Pakistan; it contributes to 61.9% of the total agriculture revenue and shares 14.0% of Pakistan's gross domestic product (1). To supply cheap, high-quality and surplus milk, meat, and meat products for human consumption, farmers are favoring exotic highly productive breeds over local animal breeds (2). As low as 30–35 million people are engaged in the livestock sector in Pakistan, highlighting the potential and dependency of the economy on livestock (3). The major constraint to sustainable animal production comes from several diseases (4–13). Helminth infections not only reduce productivity but also compromise the quality of food. Parasitized animals reduce live weight gain, increased age of puberty, low productivity, and higher susceptibility to other pathogens; collectively contributing to severe economic losses to stakeholders (14, 15). Infection with various species of parasites in cattle and buffaloes is a growing global problem (16–29). The most prevalent cattle helminths include *Strongylus* sp., *Paramphistome* sp., *Strongyloides* sp., *Moniezia* sp., *Toxocara vitulorum*, *Trichuris* sp., *Fasciola* sp., and *Bunostomum* sp. (30). Depending upon animal rearing standards, resistance against parasites, and biosecurity measures, the prevalence of helminth infections is variable and ranges in the world from 0.78 to 84.1% (31). This prevalence ranges in Pakistan from 33.68 to 51.0% (32). As a countermeasure, broad-spectrum anthelmintic drugs such as albendazole and Ivermectin were used to significantly improve the health and well-being of animals which then favorably impact the productivity and standards of animal-origin food and food products (33). The recorded increment of milk production upon post-anthelmintic medication was estimated to be 0.42 L per day (34). The efficacy of albendazole against *Ostertagia ostertagi* ranges from 84.9 to 99% depending upon the developmental life stages of the parasite (35). Basically, anthelmintic drugs are effective in controlling parasitic infections, but excessive use of anthelmintics (under doses and narrow spectrum drugs) induced an increase in the resistant parasite population (36).

The present study was conducted to evaluate the effect of anthelmintic treatment on milk production and live weight gain in cattle, buffaloes, and their heifers. Moreover, the efficacy of the drug was monitored to ascertain existing anthelmintic resistance and a cost-benefit ratio was performed.

Materials and methods

Study area and sampling

The study was carried out during the spring season on two private dairy farms, consisting of buffaloes from the Sheikhupura district and cattle from the Rajanpur district of Punjab Province, Pakistan. A total of 400 samples (100 each) from cattle, cattle-heifer, buffaloes, and buffalo-heifer were collected for this study.

Classification of animals

Animals were selected based on the following criteria: (i) Adult cattle and buffaloes were in the first trimester of lactation. (ii) Age of cattle-heifers and buffalo-heifers ranged from 5 to 8 months. (iii) Animals were not dewormed during the last 3 months prior to the study. (iv) Animals' eggs per gram (EPG) $\geq$ 200 in feces were included in the treated and control group (37). A total of 60 animals for each group of cattle and cattle-heifer (Holstein Friesian); and buffaloes and buffalo-heifers (Nili Ravi breed) were included for anthelmintic treatment to proceed with the present study. Live body weight was estimated by taking the girth circumference in centimeters (cm) with measuring tape and compared it with tables (http://bairnsley.com/Weight%20by%20Girth.htm) (37). The average adult buffalo and cattle weights were 500 and 550 kg, and buffalo-heifer and cattle-heifers were 90 and 150 kg, respectively. All animals at each farm were kept approximately under the same managemental conditions.

Samples collection and examination

Fecal samples were collected from the rectum of each animal and placed in labeled Ziplock® plastic bags. The samples were shipped in iceboxes to the Laboratory for microscopic examination. These samples were processed by both qualitative (floatation and sedimentation) and quantitative (modified McMaster egg counting) techniques as previously described (38–40).

Feeding management of experimental animals

All experimental animals were kept under the same environmental conditions with a slight difference in the types of concentrate and fodder. Briefly, cattle and cattle-heifers were fed with a concentrate of Dairy Pellet (crude protein: 18–21%) of Maxim International Pvt. Ltd. and silage, while buffaloes and buffalo-heifers were fed with manually prepared concentrate (No recorded crude protein) containing a mixture of different food ingredients (cotton seed cake, rice bran, wheat bran, bread pieces, and mustard oil) and seasonal fodders.

Anthelmintic and its efficacy

The average EPG of all animal groups before medication (day 0) is shown in Tables 2, 3. Following these findings, animals were classified into control and treatment groups. The animals of treatment groups were medicated with albendazole (Valbazen 113.6 mg/ml; Pfizer Pharma, New York) at the rate of 10 mg/kg body weight. The efficacy was calculated upon average variation in egg reduction compared with day 0. Fecal egg count reduction testing (FECRT) was used to identify the helminths' resistance against albendazole. This test was applied till 60 days post-medication with 2 weeks intervals compared with the EPG count of infected animals before/after treatment (41). The weight gain of treated groups was subtracted from control groups to estimate the actual weight gain upon the anthelmintic appliance. The anthelmintic efficacy was estimated using the following formula:

$$\text{Effectiveness} = 100 \times (\text{Pre} - \text{treated}\ EPG - Post - treated\ EPG)/\text{Pre} - \text{treated EPG}.$$

Economic evaluation of the farms

For the cost-benefit ratio, the considered input variables were the cost for treatment, feed, and labor while output variables were milk production and estimated live weight gain (42). Local market prices of these variables in each studied area were considered according to their region for this study. Briefly, the treatment cost of anthelmintic for adult cattle and buffalo was estimated to be US$ 1.2/animal, while for cattle-heifer and buffalo-heifer it was US$ 0.4 and 0.2/animal, respectively. The price and details of feed costs were estimated as previously described by Rashid et al. (2). Cost of input parameters including feed [concentrate (US$ 0.4/kg for cattle and 0.27 US$ 0.4/kg for buffalo) and fodder (silage of US$ 0.048/kg for cattle and seasonal fodder of 0.029 US$/kg for buffalo)], treatment and labor were encountered. On dairy farms, one labor was engaged for 10 adults or 20 heifers with a pay package of US$ 114.59/month (https://www.reference.com/geography/average-salary-pakistan-d487909030150b6f). Income consists of milk production (cattle for US$0.54/L and buffalo for 0.76/L) and lives weight (US$ 3.82/kg) gain was taken for the *cost-benefit ratio* (CBR). Live weight gain/loss of heifers was measured at days 0, 7, 15, 30, 45, and 60 according to the formula.

$$\text{Income from variation in relative body weight}: [\text{Live weight at last visit} - \text{Live weight at day 0}] \times Market\ price\ (kg)$$

Statistical analysis

Descriptive statistical and two-way ANOVA analysis was performed on EPG and body weight gain in control and treated groups using GraphPad Prism 7 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com).

TABLE 1 Prevalence of helminth in cattle, cattle-heifers, buffaloes, and buffalo-heifers.

Helminthes type	No. of cattle (%)	No. of cattle heifer (%)	No. of Buffalo (%)	No. of Buffalo heifer (%)
Fasciola	3(5.0)	4(6.7)	6(10.0)	7(11.7)
Ostertagia	5(8.3)	3(5.0)	5(8.3)	5(8.3)
Trichostrongylus	3(5.0)	2(3.3)	4(6.7)	4(6.7)
Oesophagostomum	2(3.3)	3(5.0)	5(8.3)	3(5.0)
Strongyloides	5(8.3)	6(10.0)	5(8.3)	5(8.3)
Toxocara vitolurum	2(3.3)	1(1.7)	2(3.3)	3(5.0)
Haemonchus	6(10.0)	8(13.3)	–	–
Moniezia	4(6.7)	4(6.7)	4(6.7)	4(6.7)
Mixed infection	14(23.3	16(26.6)	15(25.0)	20(33.33)
Total infected	44	47	46	51
Overall prevalence	73.3	78.3	76.7	85.0

A total of 60 animals were taken for each group.

TABLE 2 Descriptive analysis [mean EPG and standard error (SE)] with 95% confidence interval (CI) in control and medicated groups of cattle and cattle-heifers.

Days	Cattle						Cattle-heifers						*P-value*
	Control ($n = 20$			Treatment ($n = 20$)			Control ($n = 20$			Treatment ($n = 20$)			
	Mean ± SE	Increment %	95% CI	Mean ± SE	Efficacy %	95% CI	Mean ± SE	Increment %	95% CI	Mean ± SE	Efficacy %	95% CI	
0	690.0 ± 12.4		664.1–715.9	652.5 ± 40.3	-	568.1–736.9	742.5 ± 52.0		718.2–766.8	680.0 ± 41.4		593.3–766.7	<0.0001
7	737.5 ± 13.0	6.9	710.3–764.7	77.5 ± 14.7	88.1	46.7–108.3	810.0 ± 70.0	9.1	777.3–842.7	55.0 ± 12.5	91.9	28.8–81.2	
14	785.0 ± 13.6	13.8	756.5–813.5	40.0 ± 10.6	93.9	17.7–62.3	867.5 ± 59.1	16.8	839.8–895.2	37.5 ± 8.0	94.5	20.7–54.3	
30	860.0 ± 12.4	24.6	834.1–885.9	55.0 ± 9.5	91.6	35.1–75.0	942.5 ± 63.4	26.9	912.8–972.2	70.0 ± 11.7	89.7	54.5–94.5	
45	902.0 ± 14.3	30.8	872.6–932.4	112.5 ± 8.8	82.8	94.1–130.9	1002.5 ± 61.7	35.0	973.6–1031.0	125.0 ± 10.6	81.6	102.9–147.1	
60	927.5 ± 10.6	34.4	905.4–949.6	170.0 ± 8.4	74.0	152.4–187.6	1042.5 ± 76.6	40.4	1007.0–1078.0	197.5 ± 12.8	71.0	170.7–224.3	
P-value (between group)	<0.0001												

Anthelmintic efficacy was evaluated by fecal egg count reduction testing (FECRT) with reference to pre-medication in each group. Here n is the total number of animals, SE is the standard error, CI is confidence interval, and FECR is fecal egg count reduction rating. Fecal egg count of control and medicated animals cattle and cattle-calf were taken to find out the anthelmintic efficacy by fecal egg count reduction.

TABLE 3 Descriptive analysis [mean EPG and standard error (SE)] with 95% confidence interval (CI) in control and medicated groups of buffaloes and buffalo-heifers.

Days	Buffaloes						Buffaloes –heifers						*P*-value
	Control ($n = 20$)			Treatment ($n = 20$)			Control ($n = 20$			Treatment ($n = 20$)			
	Mean ± SE	Increment %	95% CI	Mean ± SE	Efficacy %	95% CI	Mean ± SE	Increment %	95% CI	Mean ± SE	Efficacy %	95% CI	
0	737.5 ± 11.4	-	713.6–761.4	785.0 ± 50.8	-	678.7–891.3	755.0 ± 12.0	-	729.9–780.1	815.0 ± 50.3	-	709.8–920.2	<0.0001
7	787.5 ± 14.0	6.8	758.2–816.8	62.5 ± 12.0	92.0	37.5–87.5	820.0 ± 12.3	8.6	794.4–845.6	50.0 ± 10.9	93.9	27.2–72.8	
14	805.0 ± 14.5	9.2	774.7–835.3	37.5 ± 8.0	95.2	20.7–54.3	847.5 ± 11.2	12.3	824.1–870.9	17.5 ± 6.6	97.9	3.8–31.2	
30	892.5 ± 12.7	21.0	865.9–919.1	130.0 ± 9.2	83.4	110.8–149.2	947.5 ± 10.6	25.5	927.7–967.3	45.0 ± 10.2	94.5	23.7–66.3	
45	942.5 ± 19.6	27.8	901.4–938.6	177.5 ± 9.2	77.4	158.2–196.8	1040.0 ± 11.2	37.8	1016.0–1064.0	147.5 ± 7.7	81.9	131.4–163.6	
60	1005.0 ± 15.8	36.3	972.0–1038.0	222.5 ± 9.9	71.7	201.7–243.3	1145.0 ± 12.0	51.7	1120.0–1170.0	200.0 ± 10.3	75.5	178.5–221.5	
P-value (between group)	<0.0001												

Anthelmintic efficacy was evaluated by fecal egg count reduction testing (FECRT) with reference to pre-medication in each group.

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The percentage of EPG reduction within 2 weeks interval was calculated with reference to pre-medication (days 0) while the increment in milk production was calculated from the control group and analyzed by Student *t*-test. Moreover, the value of CBR was calculated from the total income and cost. The threshold value of 5% was considered for all the statistical tests.

Results

Approved experimental protocol

Samples were collected according to instructions and guidelines approved by animal Ethics committee No. DR 1112.

Infestation prevalence

A total number of eight types of helminth species were present in the studied animals. The most prevalent helminths were *Ostertagia* sp. (8.3, 5.0, 8.3, and 8.3%), *Strongyloides* sp. (8.3, 10.0, 8.3, and 8.3%) followed by *Oesophagostomum* sp., *Haemonchus* sp., *Trichostongylus* sp., *Moniezia* sp., *Toxocara vitulorum*, and *Fasciola* in cattle, cattle-heifer, buffaloes, and buffaloes-heifers, respectively. The overall prevalence of helminth infection in cattle and cattle-heifers was recorded to be 73.3 and 78.3%, respectively, whereas in buffaloes and buffalo-heifers it was 76.6 and 85.0%, respectively ($p > 0.05$). Specifically, a higher prevalence of *Haemonchus* sp. (10.0–13.3%) was observed in cattle and cattle-heifers, followed by *Fasciola* (10.0–11.6%) in buffaloes and buffalo-heifers, respectively while a lower prevalence of *Toxocara vitulorum* was observed in both animal species (cattle and buffaloes) (Table 1).

Anthelmintic efficacy

The reduction of EPG on day 7 in treated cattle and cattle-heifers was 88.1 and 91.9% whereas in buffaloes and buffalo-heifers it was 92.0 and 93.8%, respectively. The highest EPG reduction rate observed on day 14 in treated cattle and cattle-heifers was 93.8 and 94.4% respectively. In buffaloes and buffalo heifers, the maximum reduction in EPG count was 95.2 and 97.8%, respectively on day 14. The reduction in EPG reached 73.9 and 70.9% in cattle and cattle-heifers, respectively while in buffaloes and buffalo-heifer reduction was 71.6 and 75.5% on 60 days of post-anthelmintic treatment. The anthelmintic was highly significant ($p < 0.001$) in helminth reduction with respect to the control group (Tables 2, 3).

Anthelmintic effect on production

The average milk yield of control and treated cattle was 18.5 ± 0.36 and 19.3 ± 0.32 L/day, respectively ($p > 0.05$) with differences of 0.8L/day whereas in buffaloes it was 7.5 ± 0.22 and 8.2 ± 0.23 L/day ($p > 0.05$) with differences of 0.7 L/day (buffalo). The mean weight of cattle-heifers ranged from 151.4 to 153.0 kg/heifer while buffalo-heifers ranged from 89.9 to 92.8 kg/heifer at pre-medication (day 0). The weight gain in treated heifers after 60 days was significant ($p < 0.05$) compared with the control group. After 60 days of post-anthelmintic treatment, the mean weight gains in treated cattle-heifer and buffalo-heifers were recorded on days 0, 7, 15, 30, 45, and 60 with a total gain of 11.5 and 9.5 kg, respectively (Figure 1).

Cost-benefit ratio

The CBR values indicate the ratio of income/loss per unit cost. The highest CBR value was recorded for the animal groups of treated adult cattle (2.515) followed by control adult cattle (2.423), treated adult buffalo (1.759), control adult buffalo (1.618), treated buffalo-heifer (1.351), treated cattle-heifer (1.056), control buffalo-heifers (0.935), and control cattle-heifers (0.748). Higher CBR values indicate farm profitability while lesser value shows a loss in the rearing of those animals (Table 4).

Discussion

The current study provides the data on the most prevalent helminth species (*Haemonchus* sp. in cattle and *Fasciola* sp. in buffaloes), mixed parasitic infection, AE, and CBR. In the field condition, a higher prevalence of fasciolosis (20%) in cattle and buffaloes in Toba Tek Singh, Pakistan was observed (43). A higher prevalence of 23.3, 26.67, 25 and 33.33% of mix infection was observed in cattle, cattle-heifer, buffaloes, and buffalo-heifers in the studied animals. Similarly, the prevalence of mixed infection was found at 7, 18, 9, and 23% in cattle, cattle-heifer, buffaloes, and buffalo-heifers, respectively in a study conducted in the same province similar to the current study. This previous study's finding (0.00–3.27) for the prevalence of *Oesophagostomum* was slightly different (3.3–8.3) from the current study (15). The similarity (mixed infections) and difference (*Oesophagostomum*) were due to the same study area and different feeding systems. The distribution of these parasites at dairy farms are mainly attributed to the contaminated fodder (44) and intermediate host. Moreover, since feed and water are available *ad libitum* to all free-moving animals at dairy farms (45), the incidence is likely to occur frequently. Generally, the mean EPG in heifers was higher than in adult animals pertaining to the higher susceptibility of heifers

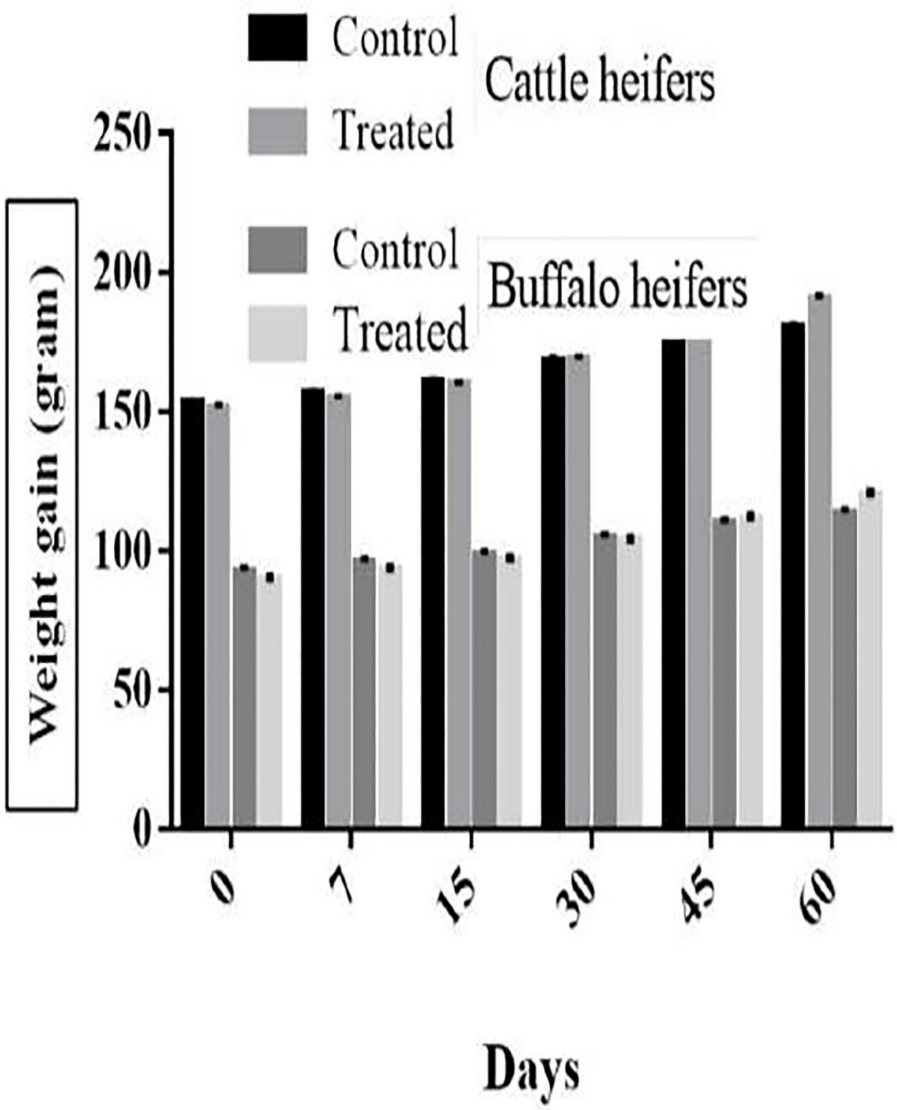

FIGURE 1
Anthelmintic effect on the weight gain of cattle and buffaloes heifers.

than immune-compromised adult animals (46). Moreover, the mean EPG in the buffaloes breed was higher than cattle breed representing their susceptibility or no previous anthelmintic treatment. The EPG in control groups increased with the passage of time due to the persistent and continuous expansion of intestinal parasites as already described by Saqib et al. (37). The maximum reduction in EPG was on the 14th day post-medication. However, a higher reduction in percentage was observed in buffaloes and buffalo-heifers indicating a reduced anthelmintic resistance as compared to cattle breeds (47) that are treated regularly favoring the establishment of anthelmintic resistance. The efficacy of anthelmintic was not 100% in any animal species/breed which might be the problem of drug resistance. It might be due to the regular or under-dose usage of the same group of medicine. Therefore, it is recommended to apply alternative treatment, and a combination of two groups (37). Generally, investors consider exotic cattle breeds over the buffaloes at dairy farms mainly due to a higher milk yield (2). In this study, the average increase in milk production in cattle and buffalo was 0.8 and 0.7 L/ day, respectively, which confirms previous studies undertaken in the Netherlands (48). This increase in milk production in cattle was lower than in buffaloes and this can be associated with less reduction of EPG in cattle in the local production system. The average weight of cattle-heifers was higher than buffalo-heifers at the same age which might be due to the improved genetic potential in the cattle breed. Owing

TABLE 4 Cost-benefit ratio (CBR) of treated and control groups.

Species	Animal group	Animal type	Treatment cost		Per animal per day feed cost						Total cost and income for 60 days			
			Dose (ml)	Cost	Conc. Qty. (kg)	Cost	Fodder Qty. (kg)	Cost	Total feed cost	Labor cost	Total cost (Feed+Labor)	Qty. in kg/unit price (milk and weight)	income	CBR
Cattle	Treated	Adult	50	1.2	6	2.4	28	1.34	224.4	23.0	248.6	1158.0/0.54	625.3	2.525
		Heifer	15	0.4	3	1.2	20	0.95	129.0	11.5	140.9	39.0/3.82	148.8	1.056
	Control	Adult	-	-	6	2.4	28	1.34	224.4	23.0	247.4	1110.0/0.54	599.4	2.423
		Heifer	-	-	3	1.2	20	0.95	129.0	11.5	140.5	27.5/3.82	105.1	0.748
Buffalo	Treated	Adult	50	1.2	8	2.14	35	1.0	188.4	23.0	212.6	492.0/0.76	373.9	1.758
		Heifer	10	0.2	3	0.8	15	0.435	74.1	11.5	85.8	30.4/3.82	115.9	1.351
	Control	Adult	-	-	8	2.14	35	1.0	188.4	23.0	211.4	450.0/0.76	342.0	1.618
		Heifer	-	-	3	0.8	15	0.435	74.1	11.5	85.6	21.0/3.82	80.0	0.95

Income and cost are mentioned in United State Dollars (USD). Here Conc. Qty is the concentrate quantity, ml is milliliter and CBR is the cost-benefit ratio.

to the increasing genetic profile, the first conception rate of cattle heifers is earlier at 14–16 months of age than buffalo heifers which are at 22 months of age (49, 50). The weight gain of treated buffalo and cattle-heifers was 500 and 650 g/day while in the control group it was 350 and 460 g/day, respectively, which is in accordance with the results of a previous study on a dairy farm in Pakistan and the Manawatu region of New Zealand (49, 51). Additionally, the CBR of treated animals was higher than that of the non-treated ones (37) in adult cattle followed by adult buffaloes, buffalo-heifers, and cattle-heifers. It is noteworthy, that infrastructure cost to control environmental conditions and auto-machine, which is necessary for exotic animal dairies was not considered. On the other hand, native buffaloes do not require extra management as they are fully acclimatized and adapted to the native environment. Considering this difference in managemental cost, the CBR of cattle will be considerably lower than buffaloes. It was also observed in this study that non-treated cattle-heifers and buffalo-heifers were not profitable, mainly due to their susceptibility to parasitic infections and extra requirements of feed at this stage of development, which can be interfered with by the existing parasites.

Conclusions

Taken together, these studies indicate that anthelmintic treatment may positively impact EPG reduction and can cause an increase in milk production and gain in body weight in both cattle and buffaloes at studied dairies. Collectively, this improved efficiency of production favors the direct and positive cost-benefit ratio for dairy farmers.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by DR 1112. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MR and AC performed experiments. MR, AC, and NZ drafted MS. TR and MTA did proofreading and correction. MIR did data analysis. AB provided sources for sampling and

data history of animals. AA, AM, and MMA provide the funding and proofreading of MS. ME, MM, and GH did data curation. 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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Effect of *Allium sativum* and *Nigella sativa* on alleviating aluminum toxicity state in the albino rats

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The study objective was to evaluate *Allium sativum's* potential and *Nigella Sativa's* combination's potential to reduce aluminum toxicity and return to the normal state. In the present study, a hundred albino rats were randomly divided into five equal groups. The first group was used as a control group; the other four groups were exposed to aluminum 1,600 ppm. The second exposed to aluminum only; the third and fourth groups were treated with *Allium sativum* 5% and *Nigella sativa* 5%, respectively, while the fifth group was treated with a mix of *Allium sativum* 2.5% and *Nigella sativa* 2.5% for 8th weeks. After 8 weeks, the aluminum administration was stopped, and the second group was divided into three groups. The groups were treated with *Allium sativum* 5% and *Nigella sativa* 5%, and a mix of *Allium sativum* 2.5% and *Nigella sativa* 2.5%, respectively. The first group was the control group (continued from the first experiment). Garlic and *Nigella sativa* were crushed and added to feed while receiving aluminum chloride daily at a dose of 1.6 ml/l was added to the drinking water. Histopathological changes in the liver, kidney, and testes were investigated after 8 and 16 weeks, and blood samples were collected after 4, 8, and 16 weeks for biochemical blood parameters. The results showed that the histopathological examination of the liver, kidney, and testes showed signs of congestion in blood vessels after aluminum exposure. Meanwhile, the treatment with *Allium sativum* or *Nigella sativum* or the mixture between them had positive effects on evading the harmful effects of aluminum in the liver, Kidney, and testes tissues. In addition, there were protective effects for *Allium sativum* and *Nigella sativa* against

aluminum on serum creatinine, urea, ALT, and AST concentrations. The present study concluded that supplementation *with Allium sativum* or *Nigella sativa* or their combination could reduce aluminum toxicity and return the liver, kidney, and testes to normal.

KEYWORDS

aluminum toxicity, *Allium sativum*, *Nigella sativa*, histopathological abnormalities, liver, kidney, testes

Introduction

Aluminum is a trivalent action found in its ionic form in most animal and plant tissues and natural water everywhere (1). It is the third most prevalent element and the most abundant metal in the earth's crust, representing ∼8% of total mineral components (2). The sources of Al are especially corn, yellow cheese, salt, herbs, spices, tea, cosmetics, aluminum ware, and containers. Also, Al is widely used in antacid drugs, as well as in food additives and toothpaste (3).

Aluminium's toxic action is to cause oxidative stress by producing reactive free radicals that can overwhelm the cell's antioxidant defenses and cause cellular injuries where the ion interacts with plasma membrane moieties, cytoplasmic biomolecules, mitochondria, and nuclear structures. In contrast, oxidative injuries are caused by the oxidation of proteins, lipids, and nucleotides, which results in the generation of altered functional biomolecules with impaired operational capabilities for cellular homeostasis (4–6).

Aluminum is a neurotoxic agent that causes oxidative stress (7). It is known to cause toxic effects on various systems, including the brain, bone, Kidney, and blood (8). It affects several enzymes and other biomolecules relevant to Alzheimer's disease (9, 10). Plants are the green factors on our planet; they convert carbon dioxide and water to carbohydrates and nitrogen to amino acids. Besides food, plants are considered the natural green pharmacy, providing drugs to maintain good health and restore humans' failed health. The medical arts originated when humankind first began to use remedial measures to eliminate their pains, suffering, and other illnesses (11).

Furthermore, *Nigella sativa*, also known as black seed, black cumin, or habatul Barakah, has long been used in the Middle East as a traditional medicine for various complaints, headache, cough, flatulence, as a choleretic, antispasmodic, uricosuric and have a positive effect of the immune system (12). *Nigella sativa* includes a variety of active ingredients such as nigellicine, nigellimine, nigellidine, and alphahederin, as well as thymoquinone, thymohydroquinone, dithymoquinone, thymol, and carvacrol (13). Also, *Nigella sativa* protects against renal ischemia-reperfusion-induced oxidative injury, nephrotoxicity, and hepatotoxicity induced by disease or chemicals (14).

Allium sativum belongs to the family Liliaceae and the genus Allium (15). *Allium sativum* is commonly used in food, and its medical properties have been well-recognized throughout history and ancient civilizations. Traditional medical practitioners have considered *Allium sativum* an excellent medicinal plant with many therapeutic potentials. *Allium sativum* includes secondary metabolites such as flavonoids, polyphenols and tannins, as well as bioactivity such as antibacterial, antifungal, anti-inflammatory, pancreatic-amylase, glucoamylase enzyme inhibitors, antiplatelets and has excellent anti-oxidant properties, which scavenged reactive oxygen species (ROS), enhanced cellular antioxidant enzymes, and increased glutathione in the cells (16, 17).

The present study was designed to study the aluminium's toxic effects on the liver, kidney, testis, and some blood measurements and to study the ability of medicinal plants to treat this effect and to return the organs to their normal states after the exposure period.

Materials and methods

Study location

This study was conducted at the animal house laboratory, animal production department, faculty of agriculture, Al-Azaher University, Cairo, Egypt, located on the Nile River, 160 km (100 miles) inland from the Mediterranean Sea and 135 km (80 miles) west of the Red Sea, latitude 27.18°N, and longitude 31.19°E.

Animals and experimental design

One hundred male albino rats, apparently healthy and clinically free of diseases, with a body weight of 80–100 g and aged 1.5 months, were included in this study. Rats were obtained from El Osman Farm, Cairo, Egypt, housed in stainless steel cages, and provided with food, water, and *libitum*. Aluminum

chloride was received daily at a dose of 1.6 ml/l added to the drinking water (18, 19).

Exposure to aluminum toxicity

Rats were randomly divided into five equal groups (20 rats/each); the Control group (G1), Aluminum chloride 1,600 ppm/rat (G2), Aluminum chloride 1,600 ppm + *Allium sativum* 5% (G3), Aluminum chloride 1,600 ppm + *Nigella sativa* 5% (G4), and Aluminum 1,600 ppm + *Allium sativum* 2.5%+ *Nigella sativa* 2.5% (G5) treated groups, for 60 days (Figure 1).

Return to normal status after exposure to aluminum toxicity

After exposure to aluminum toxicity, for 8 weeks, returning to normal status was examined by subdividing 15 rats into three groups (5 rats/each); the ferst group was saved as the control group (which continued from the first experiment); the second and therd subgroups treated with *Allium sativum* 5% and *Nigella sativa* 5%, respectively; the fourth subgroup treated with a mix of *Allium sativum* 2.5% and *Nigella sativa* 2.5% (Figure 1).

Histopathological examination of the groups during exposure to aluminum toxicity and return

Histological samples were taken after 8 and 16 weeks. Immediately after collection, the livers, kidneys and testes were immersed in a formalin concentration of 10% for 2 days, washed in water, dehydrated in ascending grade of ethyl alcohol, cleared by xylene, and embedded in melted paraffin wax. The samples were examined by a laboratory microscope (H & E., Stain, X 100). The liver and kidney blocks were sectioned at six-micron cut and stained with eosin and hematoxylin, according to (20).

Blood sampling

Blood samples were obtained from rats by withdrawing blood from the orbital venous plexuses using a capillary tube at the fourth, eighth, and 16th weeks. Blood samples were centrifuged at 3,000 rpm for 10 min, and then sera and plasma samples were harvested and stored at−20°C until further assays. Serum total protein and albumin were determined according to Burtis (21) and Gindler and Gindler and Westgard (22) using kits supplied by SPINREACT, S Chemical Company, SPAIN. Aspartate transaminase (AST), alanine transaminase (ALT), Creatinine, and urea were determined according to Mathieu et al. (23) and Tabacco et al. (24) using kits supplied by LINEAR CHEMICALS Chemical Company, SPAIN.

Statistical analysis

Statistical analysis of the presented data was performed using the (25), followed by Duncan's multiple range test (26) to test the effect of Aluminum, *Allium sativum,* and *Nigella sativa* after 4, 8, and 16 weeks of the experiment. The normal distribution of the data was confirmed using the Kolmogorov–Smirnov test. The p values ≤ 0.05 of significance were considered.

Results

Exposure to aluminum toxicity

Histopathological liver

The effect of *Allium sativum* or *Nigella sativa* on the histopathology of albino rats' liver in the 8th week after treatment during aluminum exposure was examined compared to that of the control rats and presented in Figure 2.

Histopathological sections of *Allium sativum* 5%, *Nigella sativa* 5%, the combination, and the control group showed well-maintained lobular structure, portal tracts consisting of the hepatic artery, portal vein, bile duct, the central veins; some were normal and some congested (Figures 2A,C–E). The hepatocytes were normal in arrangement, cytoplasm, and nuclei. Meanwhile, in the aluminum 1,600 ppm treated group (Figure 2B), hepatic sinusoids revealed mild congestion compared with the control group, which showed typical structures for the liver. This indicates that the treatment with *Allium sativum* or *Nigella sativa* or the mixture between them had positive effects on evading the harmful effects of aluminum in liver tissues.

Histopathological kidney

The effect of treating with *Allium sativum* and *Nigella sativa* during exposure to aluminum on the kidneys' histopathology after the 8 weeks of treatment was examined in comparison with that of the control rats (Figure 3).

The study showed the effect of aluminum and treatment with *Allium sativum* and *Nigella sativa* on the histopathology of rats' Kidneys compared with the control group. Sections in the kidney tissue showed preserved architecture. The cortex revealed normal glomeruli and tubules. The medulla revealed normal tubules and mildly congested blood vessels of interstitial tissue.

Meanwhile, at 1,600 ppm, aluminum-treated group sections in the kidney tissue showed preserved architecture, and the cortex revealed normal glomeruli and tubules with congestion of the cortical blood vessels (Figure 3B). In comparison, treatment

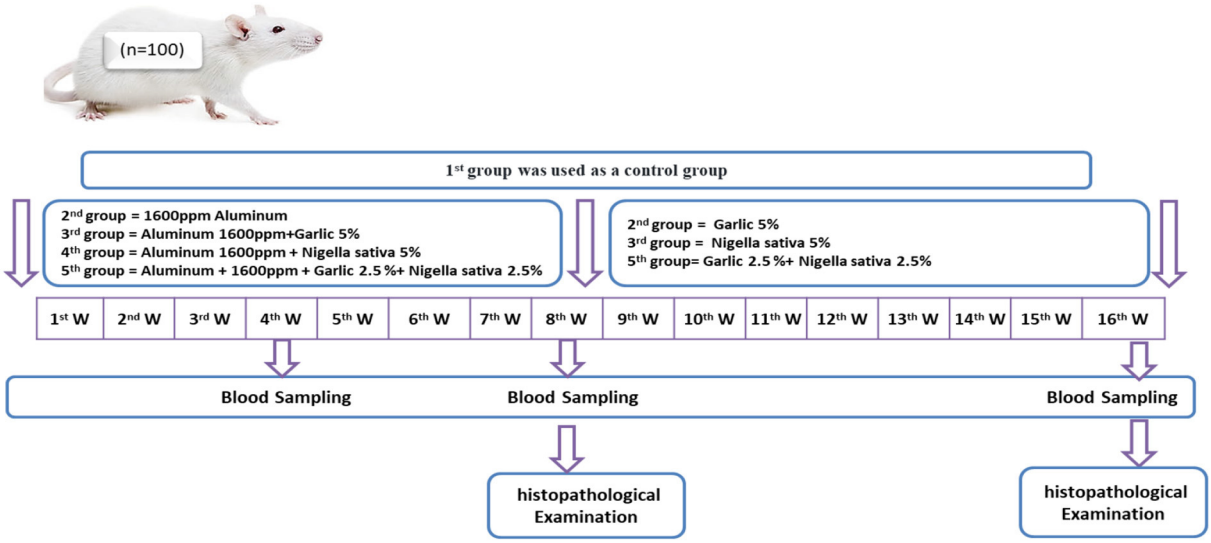

FIGURE 1
Schematic diagram showing the experimental protocol for the effect of *Allium sativum* and *Nigella sativa* on reducing the negative consequences of Aluminum and returning liver, Kidney, and test to' natural state in the Albino rats.

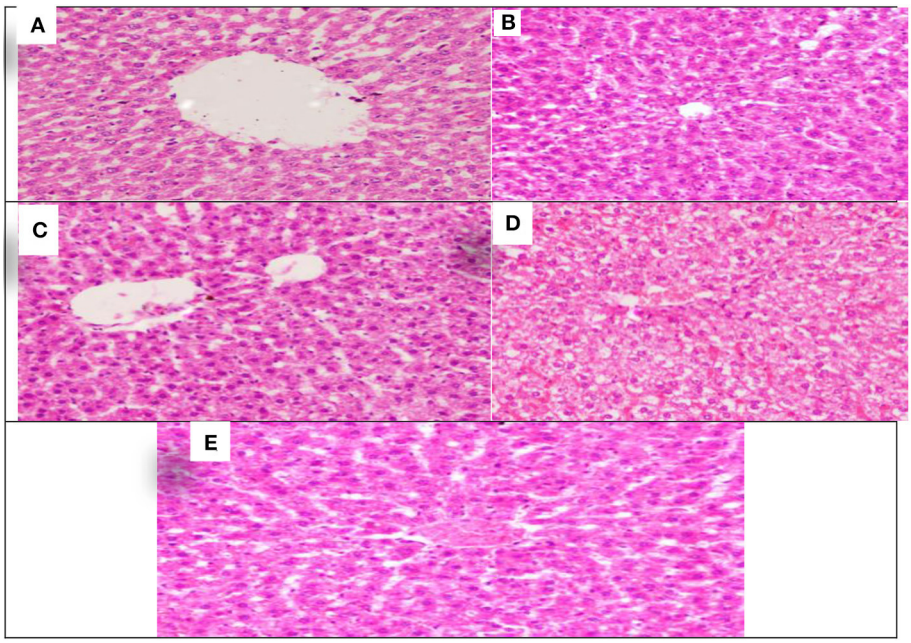

FIGURE 2
Schematic diagram showing the effect of exposure to aluminum and medicinal plants and their mix on the histopathology of rats' liver at 60 days after treatment. **(A)** Control group. **(B)** 1,600 ppm Aluminum. **(C)** 1,600 ppm Aluminum + *Allium sativum* 5%. **(D)** 1,600 ppm Aluminum + *Nigella sativa* 5%, and **(E)** 1,600 ppm Aluminum + *Allium sativum* 2.5% + *Nigella sativa* 2.5%.

with medicinal plants *Allium sativum* 5%, *Nigella* 5%, and the mixture between them (*Allium sativum* 2.5% + *Nigella* 2.5%), showed average results in Kidney samples. Sections in the kidney tissue showed preserved architecture; the cortex revealed normal glomeruli and tubules, and the medulla revealed normal tubules and mildly congested blood vessels of interstitial tissue. This indicates that the treatment with *Allium sativum* or Nigella or the mixture between them had positive effects on evading the harmful effects of aluminum in Kidney tissues (Figures 3C–E).

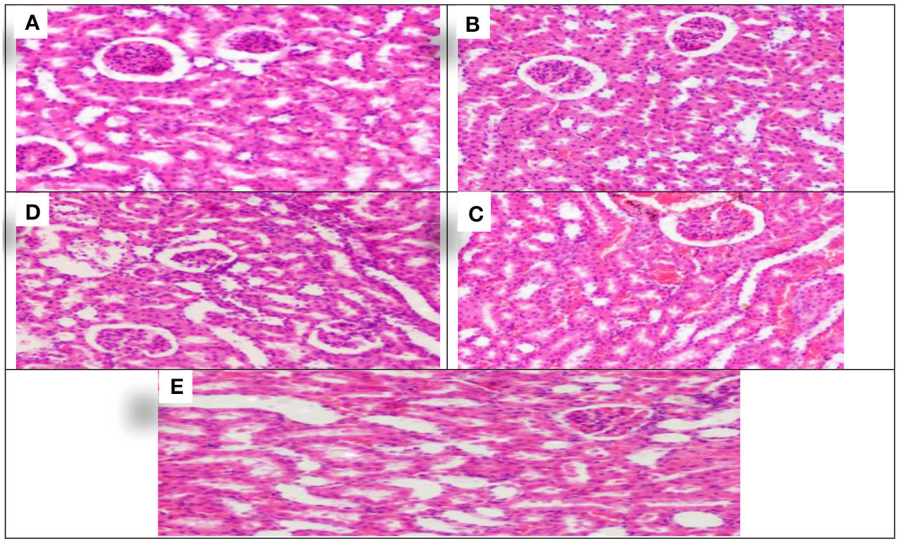

FIGURE 3
Schematic diagram showing the effect of each aluminum, medicinal plant, and mix with aluminum and medicinal plants on histopathology of rats' Kidney 60 days after treatment. **(A)** Control group. **(B)** 1,600 ppm Aluminum. **(C)** 1,600 ppm Aluminum + *Allium sativum* 5%. **(D)** 1,600 ppm Aluminum + *Nigella sativa* 5%, and **(E)** 1,600 ppm Aluminum + *Allium sativum* 2.5% + *Nigella sativa* 2.5.

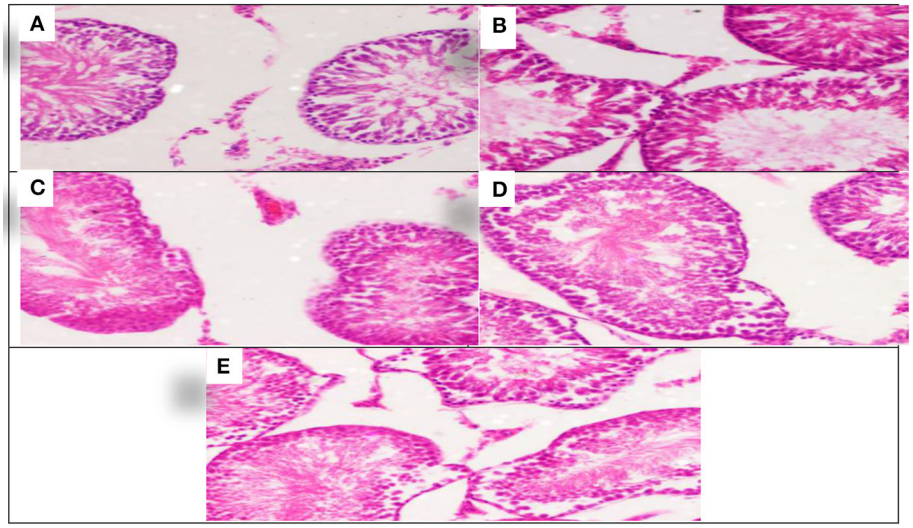

FIGURE 4
Schematic diagram showing the effect of each aluminum, medicinal plant, and mix with aluminum and medicinal plants on histopathology of rats' Tests 60 days after treatment. **(A)** Control group. **(B)** 1,600 ppm Aluminum. **(C)** 1,600 ppm Aluminum + *Allium sativum* 5%. **(D)** 1,600 ppm Aluminum + *Nigella sativa* 5%, and **(E)** 1,600 ppm Aluminum + *Allium sativum* 2.5% + *Nigella sativa* 2.5%.

Histopathological testes

The effect of each *Allium sativum* and *Nigella sativa* during exposure to aluminum on the testes histopathology after 8 weeks of treatment were compared to the control rats (Figure 4).

Aluminum, *Allium sativum,* and *Nigella sativa* affect the histopathology of rats' testicular tissue in the 8th week compared with the control group. Sections in the testicular tissue showed preserved histological architecture. The testicular seminiferous tubules consisted of normal basement membrane spermatogonia arranged in a stratified manner, spermatocytes, and spermatozoa in the lumen. However, Sertoli cells were normal in number and arrangement. The testicular tissue structure in all groups, including lobular architecture: normal

TABLE 1 Serum Biochemical Parameters (mg//dl) and in control, *Allium sativum* and *Nigella sativa* at fourth and 8th week during exposure to aluminum 1,600 ppm toxicity ($n = 20$/group, mean ± SEM).

Parameter (mg//dl)	Time	Control	Aluminum$^{1,600\ ppm}$	Aluminum$^{1,600\ ppm}$			Sig
				Allium sativum$^{5\%}$	N. *Sativa*$^{5\%}$	*Allium sativum*$^{2.5\%}$ + N. *Sativa*$^{2.5\%}$	
Total Protein	4th	8.80 ± 0.10^{a}	6.36 ± 0.08^{d}	7.65 ± 0.09^{c}	7.77 ± 0.07^{c}	8.14 ± 0.09^{b}	*
	8th	8.74 ± 0.04^{a}	6.14 ± 0.04^{d}	7.20 ± 0.10^{c}	7.34 ± 0.12^{c}	8.25 ± 0.08^{b}	**
Albumin	4th	5.77 ± 0.03^{a}	$4.73 \pm 0.09c$	5.17 ± 0.03^{bc}	4.87 ± 0.32^{c}	5.63 ± 0.09^{b}	***
	8th	5.76 ± 0.12^{a}	4.17 ± 0.03^{b}	5.54 ± 0.24^{a}	4.50 ± 0.21^{b}	5.40 ± 0.23^{a}	***
Creatinine	4th	0.72 ± 0.01^{c}	1.22 ± 0.01^{a}	0.94 ± 0.01^{b}	0.95 ± 0.02^{b}	0.75 ± 0.02^{c}	***
	8th	0.78 ± 0.03^{b}	1.65 ± 0.16^{a}	0.88 ± 0.01^{b}	0.96 ± 0.02^{b}	0.75 ± 0.02^{b}	***
Urea	4th	34.33 ± 1.86^{c}	56.00 ± 2.08^{a}	43.67 ± 1.76^{b}	48.33 ± 2.03^{b}	35.00 ± 0.58^{c}	***
	8th	33.00 ± 1.73^{b}	53.73 ± 3.39^{a}	49.33 ± 3.76^{a}	52.60 ± 2.33^{a}	40.67 ± 1.76^{b}	***
ALT	4th	45.33 ± 2.03^{b}	57.00 ± 1.53^{a}	48.50 ± 0.29^{b}	44.00 ± 2.65^{b}	44.67 ± 1.20^{b}	***
	8th	41.00 ± 0.58^{b}	66.00 ± 2.08^{a}	43.33 ± 2.33^{b}	44.00 ± 2.08^{b}	45.67 ± 1.76^{b}	***
AST	4th	30.46 ± 1.86^{c}	34.33 ± 8.11^{a}	35.50 ± 14.43^{ab}	32.50 ± 4.04^{bc}	31.63 ± 2.96^{c}	***
	8th	31.53 ± 1.45^{cd}	39.03 ± 2.40^{a}	36.00 ± 2.65^{b}	32.10 ± 1.53^{b}	31.40 ± 1.15^{d}	***

[a,b,c,d]Means with different superscripts in the same row are *Means in the same row differ significantly ($p \leq 0.05$); **Means in the same row differ significantly ($p \leq 0.01$); ***Means in the same row differ significantly ($p \leq 0.001$).

seminiferous tubules and Sertoli cells, was normal without histological changes (Figures 4A–E).

Serum blood biochemical parameters

The results of biochemical blood parameters in control, *Allium sativum,* and *Nigella sativa* at the fourth and 8th week during exposure to aluminum 1,600 ppm toxicity are presented in Table 1.

The total protein and albumin concentrations (mg/dl; mean = SEM) at D30 and D60 was significantly decreased ($P < 0.01$) in the aluminum 1,600 ppm treated group, while there were no significant differences between *Allium sativum* 5% and *Nigella Sativa* 5% in the total protein concentration. Additionally, the change in total protein concentration in the animal treated with the *Allium sativum* 2.5% plus *Nigella Sativa* 2.5% was non-significant and approached the control group (Table 1).

The study revealed that treating with *Allium sativum* 5% and *Nigella Sativa* 5% maintained the creatinine concentration (mg/dl; mean = SEM) at the normal level in the fourth and 8th week, and there were no significant ($P > 0.01$) differences between control group at the 8th week. In addition, the animals treated with a mix of medicinal plants (*Allium sativum* 2.5% + *Nigella sativa* 2.5%) had the same creatinine concentration (mg/dl; mean= SEM) as the control group in the fourth and 8th week.

The urea concentration (mg/dl; mean = SEM) had no significant differences between the mix of medicinal plants (*Allium sativum* 2.5% + *Nigella Sativa* 2.5%) treatment and the control group in the fourth and 8th week. In contrast, the urea concentration was significantly higher ($P < 0.01$) in comparison to those of the Control group after the fourth and 8th week.

Regarding liver enzyme function, both AST and ALT levels (mg/dl; mean = SEM) showed significant ($P < 0.01$) higher levels between the aluminum 1,600 ppm treated group and other groups in the fourth and 8th week. However, the treatment with *Allium sativum* and *Nigella sativa* decreased ALT and AST percentages (Table 1).

Return to normal status after exposure to aluminum toxicity

Histopathological liver

Using *Allium sativum* 5% and *Nigella sativa* 5%, and a mix of them (*Allium sativum* 2.5% + *Nigella sativa* 2.5%) to return the normal state after exposure to aluminum toxicity, as well as histopathology of rats' liver at the 16th week is shown in Figure 5.

The study obtained a positive effect due to *Allium sativum* and *Nigella sativa* in improving the harmful effects that the liver was exposed to during the treatment with aluminum. The optimum effect was the mixture between *Allium sativum* and *Nigella*, where liver tissue returned to its normal state (Figure 5D).

Sections in the liver tissue of all treated groups showed preserved lobular architecture. Also, the portal tracts consisted of the hepatic artery, portal vein, bile duct, and central veins; some were normal or congested. The hepatocytes were normal in arrangement, cytoplasm, and nuclei, and Congestion in the hepatic sinuses was not detected (Figures 5A–D).

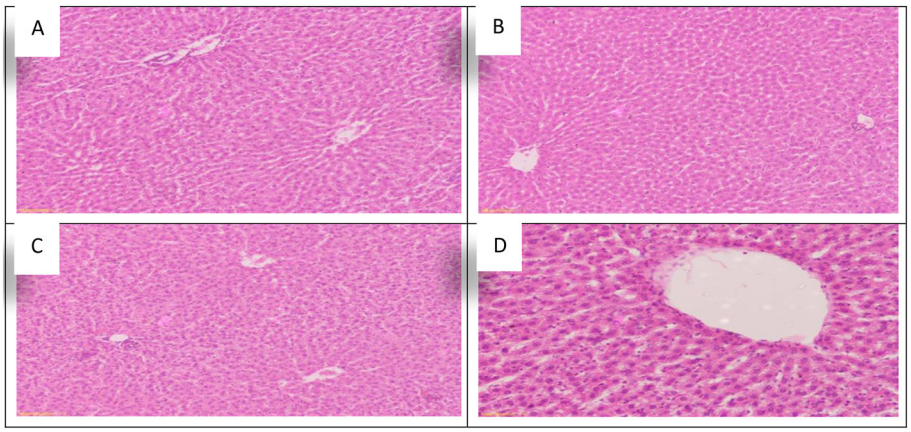

FIGURE 5
Schematic diagram showing the effect of each aluminum, medicinal plant, and mix with aluminum and medicinal plants on histopathology of rats' liver during return to normal after exposure to aluminum toxicity at 60 days after treatment. **(A)** Control group. **(B)** *Allium sativum* 5%. **(C)** *Nigella sativa* 5%, and **(D)** *Allium sativum* 2.5% + *Nigella sativa* 2.5%.

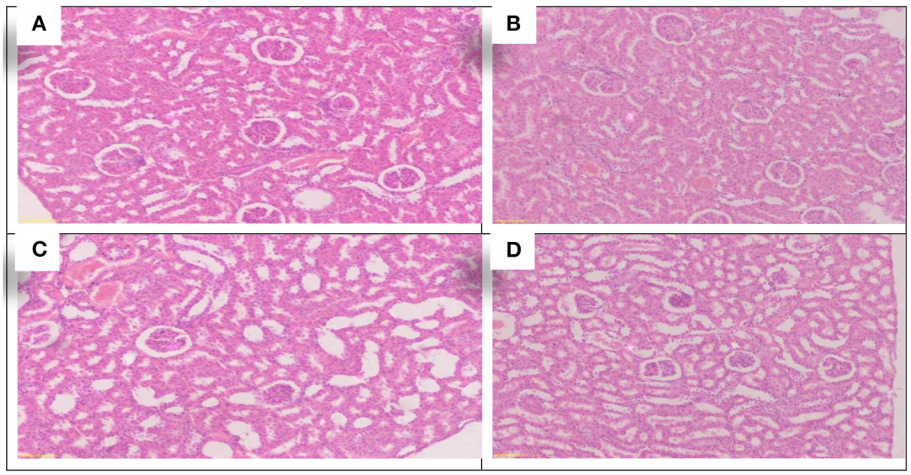

FIGURE 6
Schematic diagram showing the effect of each aluminum, medicinal plant and mix with aluminum and medicinal plants on histopathology of rats' Kidney during return to normal after exposure to aluminum toxicity at 60 day after treatment. **(A)** Control group. **(B)** *Allium sativum* 5%. **(C)** *Nigella sativa* 5%, and **(D)** *Allium sativum* 2.5% + *Nigella sativa* 2.5%.

Histopathological kidney

Using *Allium sativum* 5% and *Nigella sativa* 5%, and a mix of them (*Allium sativum* 2.5% + *Nigella sativa* 2.5%) for return to normal state after exposure to aluminum toxicity, as well as histopathology of rats' kidneys at the 16th week, is shown in Figure 6.

The study found that the rats treated with *Allium sativum* 5% and *Nigella sativa* 5% had well-maintained kidney tissue structure, the cortex revealed normal glomeruli, tubules with minimal congestion in the *Allium sativum* 5% treated groups, and mild congestion in the *Nigella sativa* 5% treated groups of the cortical blood vessels. In addition, the medulla revealed normal tubules and minimal congested blood vessels of interstitial tissue in the *Allium sativum* 5% and average tubules and interstitial tissue in the *Nigella sativa* 5% treated groups (Figures 6B,C).

For the treatment with *Allium sativum* 2.5% plus *Nigella sativa* 2.5%, the results showed preserved architecture in kidney tissue; the cortex revealed normal glomeruli and tubules, while the medulla revealed normal tubules and minimal congestion of blood vessels of interstitial tissue (Figure 6D).

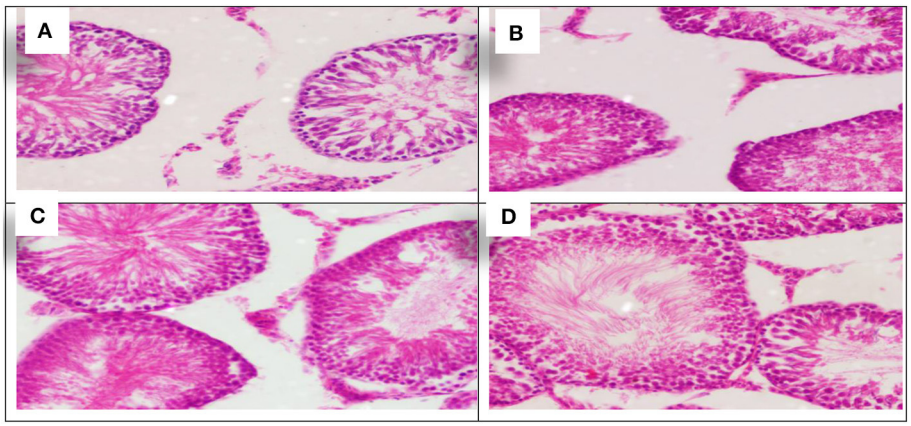

FIGURE 7
Schematic diagram showing the effect of each aluminum, medicinal plant, and mix with aluminum and medicinal plants on histopathology of rats' testicular tissues during return to normal after exposure to aluminum toxicity at 60 days after treatment. **(A)** Control group. **(B)** *Allium sativum* 5%. **(C)** *Nigella sativa* 5%, and **(D)** *Allium sativum* 2.5% + *Nigella sativa* 2.5%.

Histopathological testes

Using *Allium sativum* 5% and *Nigella sativa* 5%, and a mix of them (*Allium sativum* 2.5% + *Nigella sativa* 2.5%) for return to normal state after exposure to aluminum toxicity, as well as histopathology of rats' testes at the 16th week, are shown in Figure 7.

The sections showed similarity in all groups of testicular tissue, which showed preserved lobular architecture with normal seminiferous tubules and Sertoli cells. The testicular tissue showed preserved histological architecture, and the testicular seminiferous tubules consisted of normal basement membrane spermatogonia arranged in a stratified manner, spermatocytes and spermatozoa in the lumen. Sertoli cells were normal in number and arrangement (The sections showed similarity in all groups of testicular tissue, which showed preserved lobular architecture with normal seminiferous tubules and Sertoli cells. The testicular tissue showed preserved histological architecture, and the testicular seminiferous tubules consisted of normal basement membrane spermatogonia arranged in a stratified manner, spermatocytes and spermatozoa in the lumen. Sertoli cells were normal in number and arrangement (Figures 7A–D).

Serum blood biochemical parameters

The results of biochemical blood parameters in control, aluminum, *Allium sativum,* and *Nigella sativa* at the 16th week during return to normal after exposure to aluminum 1,600 ppm toxicity are presented in Table 2.

There was a significant increase in the total protein concentration (mg/dl; mean = SEM) of the rats treated with *Allium sativum* 5%, *Nigella Sativa* 5%, and a mix of the *Allium sativum* 2.5% plus *Nigella Sativa* 2.5% than the control group at 16th week. However, the Creatinine concentration (mg/dl; mean = SEM) in the 16th week had no significant changes among *Allium sativum* 5% and mix of the *Allium sativum* 2.5% plus *Nigella Sativa* 2.5% treated and control group, while the rats treated with *Nigella sativa,* 5% had a higher Creatinine concentration than other groups (Table 2).

For urea concentration (mg/dl; mean = SEM), no significant differences were found in the urea levels (mg/dl) among *Allium sativum* 5%, *Nigella sativa* 5%, and mix of the *Allium sativum* 2.5% plus *Nigella Sativa* 2.5% treated groups than Control group at 16th week. Also, the liver function enzymes showed no significant differences in the ALT and AST (mg/dl; mean = SEM) levels between the *Allium sativum* 2.5% mix plus *Nigella Sativa* 2.5% treatment and control group at the 16th week. While the *Nigella sativa* 5% treated group was significantly higher ($p < 0.01$) in the ALT and AST level (mg/dl; mean ± SEM) in comparison with the Control group (Table 2).

Discussion

This experiment successfully evaluated the effect of *Nigella sativa* and *Allium sativum* supplementation on reducing the adverse effects of aluminum and returning the Albino Rats' liver, kidney, and testes to their natural state. Furthermore, in this study, *Allium sativum* and *Nigella sativa* were used during exposure to aluminum toxicity and after the aluminum treatment was discontinued.

The current study revealed adverse effects of exposure to aluminum where hepatic sinusoid congestion and the liver's central veins were normal in some places but congested in others. Following the treatments with *Nigella sativa* and *Allium sativum*, it found beneficial effects in avoiding the harmful effects of aluminum on liver tissues. Moreover, the rats treated with *Nigella sativa* showed normal liver tissue, the preserved lobular

TABLE 2 Serum Biochemical Parameters (mg//dl) in control, aluminum, *Allium sativum*, and *Nigella sativa* at 16th week during exposure to aluminum 1,600 ppm toxicity (n = 5/group, mean ± SEM).

Parameter	Time	Control	*Allium sativum*$^{5\%}$	N. *Sativa*$^{5\%}$	*Allium sativum* $^{2.5\%}$ + N. *Sativa*$^{2.5\%}$	Sig
Total	16th	8.80 ± 0.10^{b}	8.14 ± 0.04^{c}	9.14 ± 0.04^{a}	8.14 ± 0.04^{c}	***
Albumin	16th	5.77 ± 0.03^{b}	5.27 ± 0.07^{c}	5.87 ± 0.03^{b}	6.17 ± 0.03^{a}	***
Creatinine	16th	0.72 ± 0.01^{a}	0.94 ± 0.02^{a}	0.85 ± 0.03^{b}	0.70 ± 0.01^{a}	***
Urea	16th	34.33 ± 1.86^{ab}	37.67 ± 1.33^{a}	35.67 ± 1.20^{ab}	31.33 ± 0.67^{b}	*
ALT	16th	45.33 ± 2.03^{b}	50.00 ± 0.58^{ab}	51.33 ± 1.76^{a}	45.67 ± 0.88^{b}	**
AST	16th	30.46 ± 1.86^{b}	31.76 ± 1.45^{a}	31.36 ± 1.45^{a}	30.23 ± 0.88^{b}	***

[a,b,c,d]Means with different superscripts in the same row are *Means in the same row differ significantly ($p \leq 0.05$); **Means in the same row differ significantly ($p \leq 0.01$); ***Means in the same row differ significantly ($p \leq 0.001$).

architecture; the portal tracts consisted of the normal hepatic artery, portal vein, and bile duct, and the hepatocytes were normal in an arrangement of cytoplasm and nuclei. *N. Sativa* administration protects hepatic tissue from the deleterious effects of toxic metals (27). In addition, *N.sativa* provided cytoprotectant by improving the histoarchitecture of the liver and decreasing the number of apoptotic cells (28).

In rats with hepatic ischemia, biochemical parameters such as total oxidative status and oxidative stress index were measured in hepatic tissue. The results indicated that *N. sativa* treatment protects the rat liver from hepatic ischemia-reperfusion injury by increasing antioxidant enzymatic activities significantly (29). Furthermore, Through its antioxidant and anti-apoptotic properties, *N. Sativa* was effective in protecting against hepatotoxicity (28).

Several studies showed that *Allium sativum* could protect the liver cells from some toxic agents. Dietary inclusion of *Allium sativum* powder protects against rat hepatotoxicity, improves antioxidant status, and modulates oxidative stress (30). In comparison, the co-administration of leaves' wild garlic has reduced histological injuries in the hepatic and renal rats (31). In addition, *Allium sativum* attenuated the hepatotoxicity effect of nitrate in rats. *Allium sativum* extract may reduce lipid peroxidation and enhance the antioxidant defense system (32). Another study suggested that *Allium sativum* with a high dose has the potential ability to induce liver damage (33).

The above results on the Kidney section revealed that rats treated with *Nigella sativa* produced normal Kidney samples, with sections of kidney tissue displaying preserved architecture. Normal glomeruli and tubules were found in the cortex, and normal tubules were found in the medulla. This indicates that *Nigella sativa* had positive effects on evading the harmful effects of aluminum in Kidney tissues. The protective effect of N. Sativa against tubular necrosis in rat kidneys was practical; it also found a significant reduction in total oxidative status and oxidative stress index levels in kidney tissue (34, 35). Furthermore, N. Sativa acts in the kidney as a powerful free radical scavenger, preventing the toxicon both biochemical and histopathological parameters (36).

Albrakati et al. (37) reported that *Allium sativum* extract supplementation significantly increased renal structure and function changes, indicating its renoprotective effect by protecting cellular membrane integrity. However, it did not show any change in the kidney histology compared to the control group in the rats treated with *Allium sativum* extract, according to Shiju et al. (38).

The testicular tissue showed preserved histological architecture, and the testicular seminiferous tubules consisted of normal basement membrane spermatogonia arranged in a stratified manner, spermatocytes and spermatozoa in the lumen. Sertoli cells were normal in number and arrangement. Most of the therapeutic properties of the *Nigella sativa* are due to the presence of thymoquinone (TQ) and its protective role on testicular toxicity in male rats; as they monitor decreased total antioxidant capacity and finally, administration of TQ may decrease the destructive effects of testicular tissue (39).

Testicular tissue is susceptible to free radicals and oxidative stress because the tissue has a high cell division rate. It was postulated that increases in oxidative stress could reduce testosterone secretion by testicular Leydig cells (40). Also, Lotfi et al. (41) concluded that the *Allium sativum* could help to reduce the severity of damage in the testicular tissues of diabetic rats through its hypoglycemic, antioxidant, and anti-inflammatory properties; *Allium sativum* extract was associated with reduced glucose levels, oxidative stress, interleukin-1 β levels, and gene expression of inducible nitric oxide synthase and increased testosterone levels and sperm quality.

In addition, the biochemical measurements ensure that the *Nigella sativa* and *Allium sativum* supplementation could reduce the adverse effects of aluminum and return the Albino Rats' liver, kidney, and testes to their natural state. There were no significant differences between *Allium sativum* 5% and *Nigella sativa* 5% in the total protein concentration. The animals treated with a mix of aluminum and medicinal plants showed that total protein concentration approached the control group. These results follow those of Al-Logmani and Zari (42). They reported that treatment of diabetic and non-diabetic rats with 5% *Nigella sativa* oil for seven weeks did not significantly affect the serum

concentration of total protein as compared with untreated diabetic and control rats. Ghalehkandi et al. (43) showed that treatment of rats with *Allium sativum* aqueous extract had no significant changes in the serum value of total protein. Also, they found that combinative use of *Allium sativum* and CrCl3 does not affect the serum value of total protein.

In the present study, the serum creatinine and urea concentration were measured as indicators of nephrotoxicity for all the groups of rats. The serum creatinine and urea levels were very close between animals treated with *Nigella sativa* and the control group. Abul-Nasr et al. (44) revealed that *N. Sativa* had a nephroprotective effect as they lowered the values of serum creatinine and urea; also, N. *Sativa* had protective effects on albino rats. Estimating urea and creatinine reflects kidney function status, and their elevation is associated with renal impairment (45).

Treatment with *Allium sativum* and Nigella for liver enzyme function decreased ALT and AST percentage after the 8th week of aluminum exposure. No significant differences were reported in the AST and ALT concentrations among *Allium sativum*, *Nigella sativa*, and control groups. Hassanin and Hassan (46) found no significant effect on serum transaminases (AST and ALT) in male guinea pigs treated with *Nigella sativa*. At the same time, Al-Jishi and Haifa (47) showed that *Nigella sativa* seed powder (180 mg/kg rat/day) increased ALT activity, but no changes were noticed in AST activity in rats. In addition, Samson et al. (48) showed a significant decrease in serum ALT and AST compared with the control group in the rats treated with *Allium sativum* (200, 400, and 600 mg/kg/day).

It was noted that the feed intake in the group treated with *Allium sativum* was lower than the rest of the groups due to the lack of palatability for the rat to eat *Allium sativum*, and this made the mixture better in results, in addition to an improvement in the proportion of feed intake. This may be explained by garlic and Nigella's potential as a natural antioxidant, in addition to the ability of garlic to activate liver enzymes that help the body to get rid of toxins. Concerning kidney functions, as the percentage of fibrosis decreased and there was an improvement in the structures of the liver and kidneys, the black bean works to speed up the mechanism of protecting the liver and kidneys. The black seed also inhibits hepatic and renal toxicity.

Conclusion

In conclusion, aluminum has adverse effects on animal and human health. Treatment with *Nigella sativa* and *Allium sativum* reduces aluminium's negative consequences and helps the Liver, Kidney, and Testes return to their natural state. Moreover, the blood biochemical findings in the present study ensured the efficacy of *Nigella sativa* and *Allium sativum* on aluminum toxicity. Furthermore, diets rich in medicinal plants (*Allium sativum* and Nigella) could be beneficial in alleviating aluminum toxicity.

Data availability statement

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

Ethics statement

This study was reviewed and approved by Animal Production Department, Faculty of Agriculture, Al-Azaher University, Cairo, Egypt (no: ASS; 2022/05/1161).

Author contributions

Conceptualization, project administration, funding acquisition, and supervision: MEA, SA, and MAM. Methodology and writing—review and editing: MEA, MA, YSM, and AA-D. Formal analysis: FA-S, AA-D, MA, and AEA. Investigation: MEA, SA, MAM, and MA. Writing—original draft preparation: MEA, AA-D, and MA. All authors have read and agreed to the published 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.

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Corrigendum: Effect of *Allium sativum* and *Nigella sativa* on alleviating aluminum toxicity state in the albino rats

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The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Investigation of respiratory disease outbreaks of poultry in Bangladesh using two real-time PCR-based simultaneous detection assays

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For rapid and sensitive pathogen screening from field outbreaks, molecular techniques such as qPCR-based simultaneous detections are efficient. Respiratory diseases are the most detrimental diseases to the poultry industry and need to be addressed because of their major economic losses. In the current study, we have applied two different detection assays: one for simultaneous detection of avian influenza virus (AIV; M gene) and subtyping (H5, N1, H9, N2) using TaqMan probe chemistry (TaqMan multitarget) and another for simultaneous detection of Newcastle disease virus (NDV), infectious bronchitis virus (IBV), and infectious laryngotracheitis virus (ILTV) using SYBR Green chemistry (SYBR Green multitarget). Two individual qPCRs were conducted for the detection of four pathogens. Surveillance of tissue (n = 158) and oropharyngeal swab (206) samples from multiple poultry flocks during the years April 2020–July 2022 applying the TaqMan and SYBR Green multitarget qPCRs revealed that 48.9% of samples were positive for respiratory infections, of which 17.2% were positive for NDV, 25.5% were positive for AIV, 9.9% were positive for IBV, and only a single positive (0.3%) for ILTV. Among the AIV, 35% were highly pathogenic subtype H5N1 and 65% were low pathogenic subtype H9N2. Co-infections of 2–3 respiratory viruses were also accurately detected. Respiratory viral pathogens are quite common in Bangladeshi poultry and can be successfully detected using multitarget simultaneous real-time quantitative polymerase chain reaction (RT-qPCR) assays like those adopted in the current study. Increased mass surveillance, along with the molecular characterization of the circulating respiratory viruses, is crucial to control the epidemic and subsequently save the Bangladeshi poultry industry.

KEYWORDS

outbreak investigation, RT-qPCR assays, simultaneous detection, respiratory viral pathogens, mixed infection

Introduction

The worldwide poultry industry is expanding rapidly in response to the rising demand for animal-derived protein. In Bangladesh, poultry is reared both in the backyard and commercial settings where more than 50% of the households keep chickens and almost 30% of the households rear ducks (http://www.bbs.gov.bd/). The Bangladeshi commercial poultry industry comprises broilers, layers, and slow-growing colored meat-type chickens (Sonali) that produce ∼0.2 million metric tons of poultry meat and 5,210 million table eggs per year (1). However, 60–70% of commercial hens are kept in small-scale facilities with limited biosecurity and management methods, which enhances disease susceptibility (2).

Intensification of commercial farming leads to the accumulation of multiple pathogens at a single site, in particular, a wide range of respiratory viral pathogens cause huge economic losses (3). The presence of more than one pathogen in hosts at the same time causes the complexity of diseases (3–8). Globally, poultry production encounters more viral infections compared to infections with other pathogens (9). Among different viral pathogens of poultry, avian influenza virus (AIV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), and infectious laryngotracheitis virus (ILTV) are the most prevalent and can produce disease independently, concurrently, or in association with bacterial agents and mycoplasmas (8, 10–13). Furthermore, the biosecurity of poultry farms in Bangladesh is quite unsatisfactory, particularly in small- to medium-scale poultry farms (14, 15), allowing pathogens to enter the farms. Early diagnosis of respiratory pathogens can minimize mortality in the infected flocks as well as could suggest effective supportive treatment options. The success of vaccination depends on the genetic background of the circulating strains factor and the selection of broadly protective vaccines against avian viral diseases (16, 17).

Respiratory infections are very common in commercial poultry and duck populations of Bangladesh (18–21). Therefore, rapid detection is required, followed by prevention and control. The current study emphasizes the prevalence of respiratory viruses in poultry and their early, accurate detection. Several laboratory methods such as virus isolation in embryonated eggs and organ cultures and serological tests are being used for decades for detecting and differentiating avian respiratory viral infections (3, 22–24). These methods are time-consuming and laborious, particularly when used separately for a single pathogen (25–27). Nowadays, rapid diagnostic tests have been developed for the detection of either viral nucleic acids or viral antigens, including rapid chromatographic and, molecular techniques (28–33). However, those techniques detect only one specific pathogen at a time. The duplex or multiplex PCR has the ability to amplify and differentiate multiple specific nucleic acids. Many countries have implemented respiratory disease surveillance, and the use of multiplex real-time PCR assays has aided in detecting circulating viruses and outbreaks, as well as estimating disease burden (29–32). Recently, a PCR-based panel assay for the detection of multiple poultry respiratory pathogens has been established using a real-time PCR system in a single reaction with SYBR Green reagent (3). A mini version of the Riems Influenza a Typing Array (RITA) assay (34, 35) has also been used in the subtype detection of AIVs and identification of other avian viral pathogens in Bangladesh (20, 36). The RITA assay is very useful for detecting the AIV subtype in a single run, but it is not cost-effective for a country with limited resources. As a result, selecting particular primers and probes for a specific target run may reduce PCR costs while simultaneously enabling rapid diagnosis. The aim of the study was to detect respiratory viral pathogens (AIV, NDV, IBV, and ILT) and selective subtyping of AIVs using two multitarget real-time quantitative polymerase chain reaction (RT-qPCR) simultaneous detection assays from outbreak samples. We adopt previously established assays (34, 35) in a format of multitarget simultaneous runs for the detection of respiratory viral pathogens that are common and endemic in Bangladesh.

Materials and methods

Virus strains from repositories

Ten (10) of each positive AIV– subtype H5N1 ($n = 5$) and H9N2 ($n = 5$) along with NDV, IBV, five (5) ILTV, and 10 mycoplasma-positive (referred to as negative here) samples were collected from virus repositories at the Department of Pathology and of which three vaccine strains were purchased from commercial sources (Nobilis ND LaSota, NOBILIS® MA5 + CLONE 30, and Nobilis® ILT, MSD animal health) to adopt and validate the simultaneous RT-qPCR assays.

Outbreak samples

A total of 364 poultry flocks, including layer ($n = 97$), broiler and broiler breeder ($n = 115$), Sonali ($n = 63$), duck ($n = 79$), pigeon ($n = 8$), and turkey ($n = 2$) with a history of respiratory signs from different commercial poultry flocks in the Mymensingh division of Bangladesh were investigated from April 2020 to June 2022. Dead birds from those natural outbreaks were submitted to the Department of Pathology, necropsy was performed, and gross pathological changes were recorded. A total of 158 tissue samples (lung and trachea from each individual animal were pooled) were collected in sterile tubes. Tissues were homogenized to prepare a 20% w/v suspension in minimum essential medium (MEM, Thermo

Fisher Scientific, USA) containing a mixture of streptomycin and penicillin (50 μg/ml), and the supernatant was collected following centrifugation at 3,000 rpm for 10 min. In addition, 206 oropharyngeal swabs were collected from different poultry flocks with a similar history. Five individual birds were randomly chosen, and swabs were pooled in the tube containing MEM and antibiotics and considered single-swab samples. Samples were kept at −70°C until further analysis.

Nucleic acid extraction and real-time PCRs

Viral DNA or RNA was extracted using a Monarch nucleic acid purification kit (New England Biolabs Inc., USA). One-step RT-qPCR was performed using TaqMan probe-based and SYBR Green chemistry. The current study used target primers developed for specific diseases in previous investigations (28, 35, 37–39), which are listed in Supplementary Table 1.

TaqMan multitarget RT-qPCR for screening and subtyping of avian influenza virus

The TaqMan probe-based single-step RT-qPCR assay (referred to as "TaqMan multitarget" here) was optimized using a Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs Inc., USA) and AIV-specific oligonucleotides as used previously (Supplementary Table 1). The final reaction volume was 12.5 μl, containing 2.5 μl of DNA or RNA template, 6 μl of 2× RT-PCR reaction mix, 1 μl of RT- PCR Enzyme Mix, 1 μl of nuclease-free water, and 2 μl of primer probe mix (10 pmol each). The RT-qPCR thermocycling conditions were 55°C for 10 min (reverse transcription) and 95°C for 1 min (initial denaturation), followed by 45 cycles at 95°C for 10 s (denaturation) and 60°C for 30 s (annealing and elongation) with the reading of fluorescence in this step. The Optimized TaqMan Multiplex is used for the simultaneous detection of generic AIV (M gene) and subtype of AIV (H5, H9, N1, and N2 genes).

SYBR green multitarget RT-qPCR for screening of other respiratory pathogens

The detection of NDV, IBV, and infectious laryngotracheitis (ILT) using single-step RT-qPCR SYBR Green assay (referred to as "SYBR Green multitarget" here) was carried out by specific target primers against avian paramyxovirus 1 NP gene, IBV UTR region, and ILT glycoprotein gene-specific oligonucleotides, respectively (Supplementary Table 1). The assay was carried out using a Luna Universal One-Step RT-qPCR Kit (New England Biolabs Inc., USA) containing SYBR Green reagents following a previously established protocol (23). The final reaction volume was 12.5 μl, including 2.5 μl of the RNA template, 5 μl of 2× RT-PCR SYBR reaction mix, 0.5 μl of Luna WarmStart RT Enzyme Mix, 2.5 μl of nuclease-free water, and 2 μl of primer mix (10 pmol each). The RT-qPCR thermocycling conditions were 55°C for 10 min (reverse transcription) and 95°C for 2 min (initial denaturation), followed by 45 cycles at 95°C for 10 s (denaturation) and 60°C for 30 s (annealing and elongation) with a reading of fluorescence in this step. Immediately after PCR, a melting curve analysis was performed with a continuous temperature increment of 0.5°C/s between 65 and 95°C. Samples yielding cycle threshold (Ct) values ≤32 with an appropriate melting temperature (Tm) were considered positive.

Laboratory validation

In total, 45 reference samples, AIV ($n = 10$), NDV ($n = 10$), IBV ($n = 10$), ILTV ($n = 5$)-positive samples (virus from the repository and vaccine strains), and avian mycoplasma-positive here considered as negative sample ($n = 10$), were tested using single-target single-run and multitarget (TaqMan multitarget and SYBR Green multitarget) RT- qPCR in parallel. The threshold cycle (Ct) values of each individual run were compared with the Ct obtained at multitarget runs. The reproducibility of the multitarget RT-qPCRs was recorded by three independent runs. The sensitivity and specificity of the applied multitarget RT-qPCRs tested in the reference samples were verified, and true-positive and negative results were calculated using the web-based tool (https://www.medcalc.org/calc/diagnostic_test.php). The false-negative and false-positive results were verified using the positive and negative control within each sample panel.

Results

Outbreak investigations

A total of 364 poultry flocks, including layer, broiler and broiler breeder, Sonali, duck, pigeon, and turkey flocks, were investigated. Each type of bird has varied flock sizes (Table 1), and the vaccination schedule for respiratory pathogens was also varied, however, in most cases vaccination was not done. In general, the affected flocks reported clinical signs of off-feed, gasping, sneezing, coughing, drowsiness, decreasing output, and varying degree of mortality. Table 1 displays the outbreak overview for various bird species. The outbreak mostly occurred from pre- to post-winter seasons (October–May) in Bangladesh when the average temperature

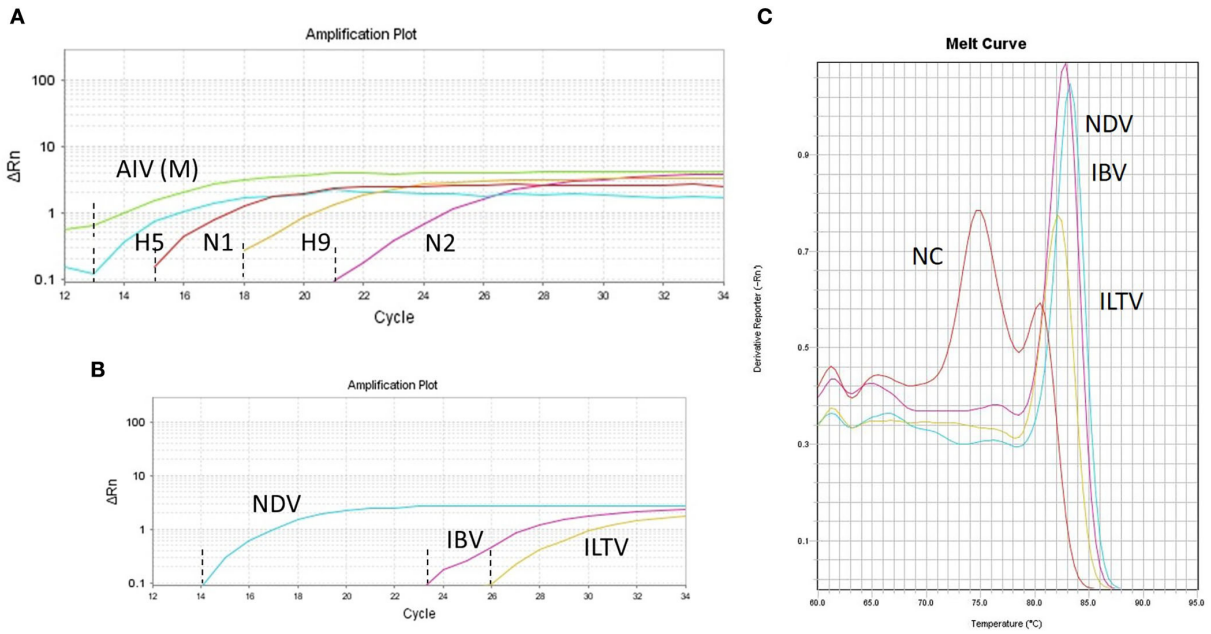

FIGURE 1
RT-qPCRs amplification plots for specific target viral pathogens. **(A)** Simultaneous detection of generic avian influenza virus (AIV-M) and subtyping of AIV (H5, H9, N1, and N2) in a single run. **(B)** Simultaneous detection of Newcastle disease virus (ND), infectious bronchitis virus (IBV), and infectious laryngotracheitis virus (ILT) along with the melt curve **(C)** in a single run. NC denotes a negative control that shows a melt curve at a different temperature (∼75°C). The Ct values of individual gene is indicated by dash lines **(A,B)** generated from the number of cycles.

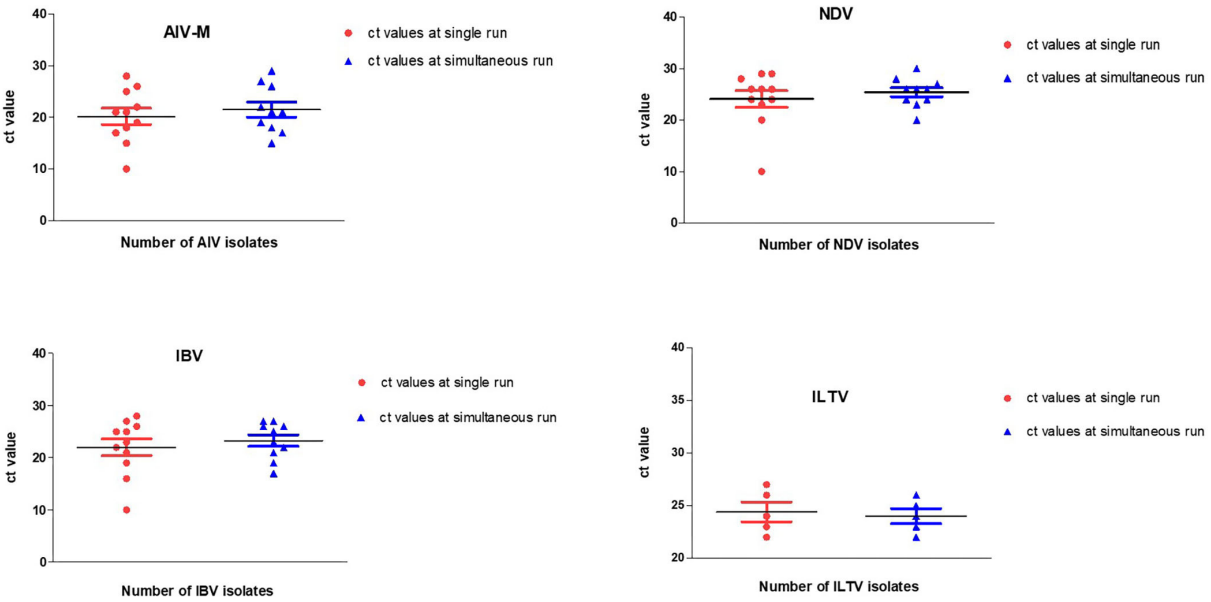

FIGURE 2
Scatter plots representing Ct values at two independent RT-qPCR runs. There were no significant variations in Ct values generated using single-target single run and multitarget parallel RT-qPCR simultaneous run.

TABLE 1 Summary of respiratory outbreaks in poultry in Bangladesh in the current study.

Bird type	Number of flocks	Flock size range	Vaccination	Clinical signs	Mortality average	Major necropsy findings
Layer	97	50–3,5000	Yes	gasping, sneezing, coughing, drowsiness, diarrhea, off feed, certain drop in egg production	3.6%	Congestion in trachea and lung; cloudy air sac, highly fragile liver, hemorrhages in cecal tonsil, intestine, pancreas, muscle, liver, brain, proventricular gland and gizzard
Broiler and Broiler breeder	115	100–12,500	Yes/No[a]	gasping, sneezing, coughing, ruffled feather, depression, off feed, certain drop in egg production (Breeder only)	7.3%	Hemorrhages in muscle, congestion in the trachea and lung, cloudy air sac, ascites; hydropericardium, fragile liver
Sonali	63	1,800–6,800	No	Respiratory distress, depression and off feed	5.8%	Congestion in the trachea, lung, cloudy air sac, liver, intestine and mesentery; mottled spleen
Duck	79	200–7,500	No	Most of them apparently healthy sometimes develop nervous disorder	2.5%	Congestion in the trachea and lung hemorrhages in the intestine and brain
Pigeon	8	20–50	No	respiratory sound, drowsiness, nervous signs, sudden death	35%	Congestion in the trachea and lungs, mottled spleen, hemorrhages in the intestine and brain
Turkey	2	15–30	No	respiratory distress, depression and off feed	2%	Congestion in the trachea and lungs; hemorrhages in the intestine

[a]Small-sized broiler flocks were mostly unvaccinated but bigger flock sizes and breeders were vaccinated properly.

ranges from 15 to 30°C. At necropsy, the dead or affected birds showed congested and hemorrhagic trachea, cloudy air sac, hemorrhages in the intestine, brain, blackish, fibrinous, edematous, hemorrhagic, and congested and consolidated lung as shown in Supplementary Figure 1. Following the acquisition of respiratory clinical characteristics, samples from several commercial poultry flocks were examined using simultaneous RT-qPCRs.

Application of RT-qPCR-based simultaneous assay

Two multitarget parallel RT-qPCR assays (TaqMan multitarget and SYBR Green multitarget) were applied, which were originally based on the "Riems Influenza a Typing Array" (34, 35). Each multitarget parallel assay's PCR plate or strip (depending on the number of samples to be analyzed in each RT-qPCR) was set so that each column can include six unknown samples, one positive and one negative control (Supplementary Table 2).

As all target viruses have RNA genome except ILTV (DNA genome), we applied RT-qPCR methods for SYBR Green multitarget parallel assay to facilitate simultaneous run. TaqMan multitarget RT-qPCR exclusively amplified the M, H5, H9, N1, and N2 genes of AIV in a single run (Figure 1A). Other respiratory viruses, such as NDV, IBV, and ILTV, were detected as well using SYBR Green multitarget RT-qPCR but in a separate run (Figure 1B). The construction of a dissociation melt curve (Tm) in SYBR Green multitarget RT-qPCR at temperatures between 83 and 84°C further aided the accurate identification of specific target viruses as the negative control produced an unspecific Tm at 75°C (Figure 1C). The final interpretation for multitarget RT-qPCRs was based on the Ct (cycle threshold) values on the sigmoid curve and the Tm values on the melt curve (SYBR Green multitarget only), and it was fairly

good for detecting pathogen target genes (Figure 1). Ct values above 35 were considered for negative interpretation of the target pathogens.

Comparison of viral load in single and simultaneous RT-qPCR

All reference (positive and negative) viruses ($n = 45$) were tested blindly against all target genes using multitarget parallel RT-qPCR assays and compared with single-target RT-qPCRs. The resulting Ct and Tm values in specific targets at positive controls were then used as a baseline for the evaluation of other samples. Triplicate runs of the selected reference samples were employed in both single-target and multitarget parallel RT-qPCR assays. Each test performed using single- or multiple-target assays in two different thermal cyclers on two different days yielded almost similar Ct values (Figure 2). The mean Ct value for each target in two independent assays did not differ considerably, indicating that the applied simultaneous TaqMan and SYBR Green multitarget detection approaches are as specific as single-target RT-qPCR runs.

The semi-quantitation and detection limits for the multitarget simultaneous assays are given in Table 2. The applied TaqMan multitarget and SYBR Green multitarget simultaneous RT-qPCR assays maintained linearity of magnitude and, using the slope from the linear equation, the overall efficiency was estimated to be 99% as of single-target single RT-qPCR run. The three replicates also do not differ with the generation of Ct values and thereafter have shown the less standard error of the average Ct values (Table 2).

Detection of target respiratory pathogens

Among the 158 tissue samples, there were 26 positives (16.5%) for NDV, 54 positives (34.2%) for AIV, 19 positives (12%) for IBV, and only a single positive (0.8%) for ILTV. On the other hand, 206 oropharyngeal swabs were detected 18 NDV (8.7%), 39 AIV (18.9%), and 17 IBV (8.3%) with no ILTV detection. Figure 3 shows the percentage of flocks afflicted by viral infections by bird type from tissue (Figure 3A) and swab (Figure 3B) samples.

Altogether, 48.9% of samples carried respiratory infections of which the highest detected (16.5%) pathogen was low pathogenic avian influenza (LPAI) subtype H9N2, followed by NDV (13.2%), IBV (9.9%), highly pathogenic avian influenza (HPAI) subtype H5N1 (9.1%) with only 0.3% ILTV (Figure 4A). Thus, within the AIV, 35% belonged to the HPAI subtype H5N1 and 65% were LPAI subtype H9N2. In addition, mixed viral infection was detected using the multiplex simultaneous RT-qPCRs, and the positive virus co-infection was found within the AIV, NDV, and IBV as depicted in Figure 4B. Briefly, 13 samples carried co-infections of which 7 were positives for both AIV and NDV, 2 positives for NDV and IBV, 3 positives for AIV and IBV while a single flock was positive for triple infection with AIV H9N2, NDV, and IBV. The 13 positive co-infected samples were further reconfirmed using single RT-qPCRs for each target independently, yielding almost identical amplification results.

Discussion

The ability to detect and differentiate causative viruses from surveillance or outbreak samples quickly helps in controlling the spread of viruses. The current study focuses on the detection of respiratory viral infections in poultry and the circulation of either high or low pathogenic subtypes of AIV in Bangladesh. Another important aim is the application of rapid and sensitive real-time PCR-based multitarget simultaneous assays (TaqMan multitarget and SYBR Green multitarget) for the successful detection of respiratory viral pathogens. In this study, we used tissue (pooled lung and trachea) and oropharyngeal swab samples from dead or sick animals when flock morbidity and mortality were noticed. The detection and identification of avian respiratory pathogens, particularly AIV, IBV, and NDV, have been the subject of numerous research studies due to the significance of these viruses in commercial poultry.

Riems influenza a typing array (RITA) is an RT-qPCR-based low-density array diagnostic approach for the detection of 14 HA and 9 NA subtypes of AIVs (34), and an improved version of this method has been developed recently (35). The assay uses a hydrolysis probe technique (TaqMan), one of the most powerful and widespread methodologies in diagnostic microbiology (40). The RITA represents a competitive, fast, and sensitive subtyping tool that requires neither new machinery nor additional training of staff in a lab where RT-qPCR is already established (34). Researchers have used PCR extensively as an indispensable diagnostic technology to detect viruses since its introduction because of its high sensitivity and specificity. Based on this assay, we applied two multitarget simultaneous assays, one is TaqMan probe-based RT-qPCR for AIV detection and subtyping in a single run. The sample gives positive results against the target generic gene of AIV (M) and simultaneously detects the AIV subtypes (H5, H9, N1, and N2) within a short period of time. Multiplex PCR is the simultaneous detection of more than one virus in a single-tube reaction, whereas our approach is the uniplex PCR in multiwell layout. Due to the endemic nature of H5N1 and H9N2 in Bangladesh (19), the current study concentrated on subtyping these viruses. Recently circulated global havoc of the HPAI H5N8 subtype is not yet detected in Bangladesh; therefore, it is opted out of current multitarget assays. However, it is quite possible to incorporate N8 detection into the same TaqMan multitarget assays to detect the N8 subtype simultaneously in a future study. Another assay is SYBR Green-based RT-qPCR that can detect target genes against three

TABLE 2 Detection limit (Ct) and reproducibility of reference samples in applied multitarget simultaneous RT-qPCR assays.

Reference samples	Single target RT-qPCRs		Multitarget simultaneous RT-qPCRs	
	Average Ct[a]	S.E.M[b]	Average Ct[a]	S.E.M[b]
AIV (1–10)	21.2	±0.13	21.5	±0.12
NDV (1–10)	25.5	±0.24	25.4	±0.27
IBV (1–10)	23.2	±0.18	23.3	±0.15
ILTV (1–5)	24.4	±0.19	24.0	±0.18
Negative[c] (1–10)	Undetermined	N/A	Undetermined	N/A

[a]Average Ct value from triplicate independent single-target and multitarget simultaneous assay runs.
[b]Standard error of the average Ct values.
[c]Known negative for AIV, NDV, IBV, and ILTV.

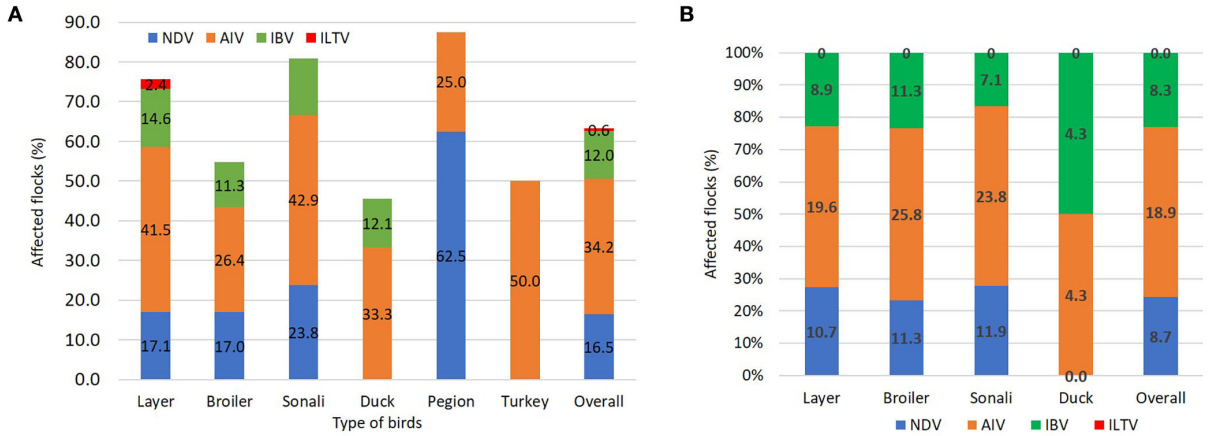

FIGURE 3
Detection of respiratory pathogens by applying the multitarget simultaneous RT-qPCRs. **(A)** The stacked column showing the percentage of respiratory pathogens in tissue samples among different bird types. **(B)** The stacked column showing the percentage of respiratory pathogens in oropharyngeal swab samples among different bird types.

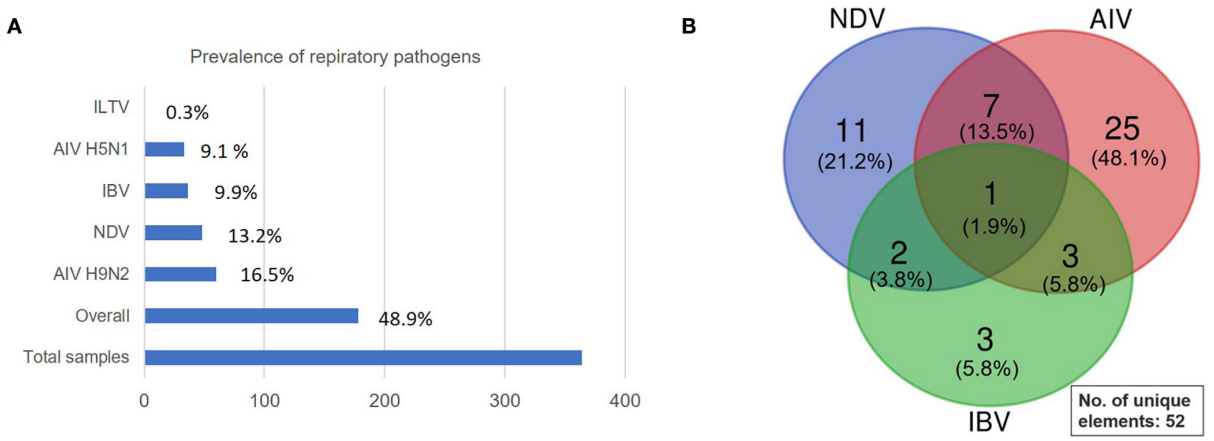

FIGURE 4
Overall prevalence of respiratory pathogens. **(A)** A clustered bar showing the overall prevalence of respiratory pathogens identified in the current study. **(B)** Venn diagram showing the presence of co-infections of 2–3 different respiratory viruses detected by multitarget simultaneous RT-qPCR assays.

other respiratory pathogens (NDV, IBV, and ILT) simultaneously in a single run. Such molecular approaches have been employed in the past with various target pathogen combinations and have proven to be highly useful (34, 39, 41).

Before applying the assays in field samples, we evaluated the assays with reference ($n = 45$) samples from the laboratory repositories. As those target primers have already been established and sensitivity and specificity were evaluated, sensitized (34, 37–39), and are using for many years, we further combined those targets as per the requirement of the current study. Further specificity of the multitarget assays was ensured when negative commercial flocks were turned out negative by applying the simultaneous RT-qPCR assays. The most commonly determined respiratory diseases in different poultry farms during the study period were avian influenza (AI), with a prevalence of 25.5% (HPAI H5N1 and LPAI H9N2), followed by Newcastle disease (ND) and infectious bronchitis (IB), each with a prevalence of 13.2 and 9.9%, respectively. ILT, on the other hand, was identified only in a layer flock, indicating an occasional infection. In the current study, AI and ND offered considerable challenges in the layer, broiler, Sonali, and pigeon flocks, whereas AI and IB had a significant impact on duck flocks. The death of the two turkeys examined here may not have been caused by any of the viral diseases tested here; however, one turkey was found to be positive for the LPAI subtype H9N2. These similar fundamental respiratory viral diseases have been identified previously in many other countries in Asia and Africa, including Bangladesh (4, 24, 31–33, 42, 43). For many years, Bangladesh has reported ∼30–50% prevalence of respiratory infections in commercial poultry (22, 23, 42, 43), also in line with our observation, which has led to slow growth and productivity, generating substantial economic losses in the poultry industry. The overall mortality shown in the investigated flocks, however, may not solely be the result of the following four viral infections. Other co-infections and poor biosecurity might have accelerated the infectivity and death rate (41, 44, 45). In the current investigation, using the multitarget simultaneous RT-qPCR assays a total of 13 farms successfully detected co-infections of either AIV and NDV, IBV and NDV, or AIV and IBV, as well as a triple infection with AIV H9N2, NDV, and IBV.

Despite the fact that all the flocks evaluated in this study were suffering from respiratory diseases, none of them tested for *Mycoplasma gallisepticum* (MG) infections. The gross pathological observation, on the other hand, suggested that there was a good chance of obtaining MG-positive pathogens alone or in combination with other detected viral pathogens. As a result, a new investigation is needed to detect mycoplasma and other bacterial infections as a single or mixed pathogen. According to our current investigation, respiratory disease outbreak is one of the most challenging problems in the poultry sector of Bangladesh, which could be due to minimum biosecurity practices in the farms and inadequate vaccination programs. For the reduction of respiratory diseases in poultry, rapid and sensitive detection is required. Continuous monitoring of circulating viruses and reviewing farm vaccination programs using vaccines similar to the circulating strains in addition to effective vaccination are the strategies that need to be implemented.

Conclusion

Respiratory pathogens are a major threat to the poultry industry in Bangladesh. AIV (HPAI H5N1 and LPAI H9N2), NDV, and IBV are the common respiratory pathogens circulating among poultry flocks. The TaqMan and SYBR Green multitarget simultaneous RT-qPCRs assays were successfully employed for screening the outbreak samples (tissue and swabs), and the interpretation was accurate, rapid, and reproducible. The specific laboratories having RT-PCR facilities could easily adopt the assays for simultaneous detection of respiratory viral pathogens within a shorter period of time as well as be able to do selective subtyping for AIVs that are endemic in the respective countries.

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 current study has been approved by the Ethical Committee of Bangladesh Agricultural University Research System under the approval number BAURES/ESRC /ET_28122. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

RP and CK wrote the first draft of the manuscript. RP, CK, IH, and AH organized samples and data collection. RP, CK, IH, AH, JB, MN, and MI analyzed formally and interpreted the results. RP, CK, IH, AH, JB, MN, MI, and EC edited and critically reviewed the manuscript. RP and EC designed the study and supervised the study. All authors have read and agreed to the published 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.

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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.2022.1036757/full#supplementary-material

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Molecular detection of *Theileria* spp. in native sheep and estimation of hemato-biochemical parameters from Sulaimani province/Iraq

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Theileriosis, the hemoprotozoan infection, is an endemic condition in tropical and subtropical areas. In this study, conventional PCR analysis was applied to detect the natural infection of native sheep with theileriosis and estimate its effect on hemato-biochemical parameters. The study was carried out in five regions of Sulaimani province, northern Iraq. From May to October 2021, a total of 360 blood samples were collected randomly from the jugular vein of sheep belonging to 23 flocks with a history of tick infestations. After PCR for theileriosis, the hematobiochemical parameters were evaluated by an automatic analyzer using commercial kits. The PCR results represented that 71.7% of the examined sheep were infected with *Theileria* parasites. The highest prevalence rate (74.6%) was reported in Said Sadiq, and the lowest prevalence (69.5%) was from Bazian. The infection rates in Mawat, Qaradagh, and Sharazoor were 73.1, 70.3, and 71.8%, respectively. The hemogram data revealed a significant decrease in erythrocyte count, hemoglobin concentration, and hematocrit values. Erythrocyte indices also showed significant increases in MC, MCH, and MCHC levels, but no significant differences were detected between the counting of leukocytes, lymphocytes, and granulocytes. Biochemical analysis revealed a significant decrease in total protein, albumin, and creatinine levels with a significant increase in urea and AST levels in infected sheep with theileriosis. Alteration in hemato-biochemical parameters from infected animals can outline the impact of theileriosis on body metabolism and blood factors in naturally infected sheep.

KEYWORDS

***Theileria*, sheep, blood, PCR, Sulaimani**

Introduction

Theileriosis is a hemoprotozoan disease caused by Apicomplexan parasites of the genus *Theileria*. Infection with *Theileria* spp. is considered an important devastating disease of small ruminants, appearing in subacute, acute, or chronic forms (1). Theileriosis is associated with substantial economic losses in livestock due to high morbidity and mortality, especially in tropical and subtropical areas (2, 3). Several species cause theileriosis in ovines with various pathogenicities. *T. lestoquardi, T. luwenshuni,* and *T. uilenbergi* produce malignant infections, while *T. ovis, T. separata,* and *T. recondita* produce mild-type infections. Also, natural infection with *T. annulata* in sheep has been reported worldwide (4). Other species of sheep have also been reported, including *Theileria* sp. OT1, *Theileria* sp. OT3, and *Theileria* sp. MK (5, 6). The parasite was transmitted through vector ticks belonging to the genera *Rhipicephalus*, *Hyalomma,* and *Haemaphysalis* (7). As a common vector-borne livestock infection, *Theileria* infects leukocytes and erythrocytes (8).

Theileriosis occurs after establishing schizont stages within the cytoplasm of susceptible host lymphocytes, with subsequent development of merozoites invading the erythrocytes (9). Other stages of the parasite's life cycle occur in the tick's gut, leading to the development of infective sporozoites (10). Previous studies represented that various factors play a role in the incidence and dissemination of ovine theileriosis (11, 12). The main dispersing factor associated with the prevalence of theileriosis is the existence of susceptible hosts and ectoparasitic vectors (13, 14). The dormant stage of infection is vital in disease epidemiology because chronic carriers act as reservoirs for tick-borne infection. Molecular techniques enable the sensitive and specific detection of pathogens (6). Furthermore, changes in blood biomarker profiles can provide valuable information on the severity of the infection and help measure the prognosis of the disease and determine the applied therapy (15, 16).

Blood and biochemical profiles, including total proteins, globulins, albumin, glucose, bilirubin, cholesterol, urea, and creatinine, can be helpful in disease diagnosis, and serum levels of ALT and AST enzymes give essential evidence about the health status of muscular and liver tissues. These hematobiochemical data effectively distinguish between healthy and diseased animals in veterinary studies (14). Hematological parameters, such as total erythrocyte count, hematocrit, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin concentration, and total and differential leukocyte count, provide vital information on the health status of the animal (17).

Detection of ovine theileriosis and verification have been performed adequately through different diagnostic methods, including PCR and sequencing of amplified DNA products. In contrast, there was little about its effects on hemato-biochemical parameters. Accordingly, the study was designed to define the impact of natural *Theileria* infection on body metabolism and blood factors in naturally infected sheep.

FIGURE 1
Iraqi map showing the study areas in Sulaimani.

Materials and methods

Study areas and sampling

The study was carried out in the Sulaimani province and its surrounding districts in the northern region of Iraq, namely, Bazian, Mawat, Qaradagh, Said Sadiq, and Sharazoor (Figure 1). The number of sheep in the included districts was estimated at 60,000 sheep. From May to October 2021, blood samples were randomly collected from 360 native sheep within 23 herds with a history of tick infestations. The sample size accounted for about 0.6% of the sheep in the study area, calculated based on a 95% confidence interval and a 5% error margin. The age of the included animals was more than 1 year, and of both sexes. About 7 mL of blood was drawn aseptically from the jugular vein. Two mL of blood were placed in an anticoagulant tube (EDTA) for hematological evaluation and molecular detection, and the rest in free anticoagulant tubes for serum collection. The samples were kept at a low temperature until transported to the laboratory, then processed, and the obtained sera were collected in Eppendorf and stored at −20°C until used for biochemical analysis. After hematological estimation, the remaining blood samples were stored similarly in a deep freezer for DNA extraction.

Laboratory examination

Molecular study

The genomic DNA was extracted from whole blood samples using a DNA extraction kit from GeNet Bio (South Korea). Following the manufacturer's instructions, the extracted DNA concentration (ng/mL) was measured using a NanoDrop spectrophotometer and stored at −20°C until used. By applying a conventional PCR assay, the *Theileria* parasite was detected targeting the 18S rRNA gene, using a previously designed specific oligonucleotide primer set with a 1098 bp portion of the gene (F: 5′-AGTTTCTGACCTATCAG-3′ and R: 5′-TTGCCTTAAACTTCCTTG-3′) described by Allsopp et al. (18).

For the PCR assay, the GeNet Bio green master mix from South Korea was used to screen all extracted DNA samples in a total volume of 20 μL. Amplification was achieved in a programmable thermocycler (Prime, UK) with the reactions starting with predenaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The final extension was carried out at 72°C for 7 min, then ended by cooling at 4°C. After amplification, 7 μL of PCR products were stained with ethidium bromide and run on a 1.5% agarose gel in TBE buffer for 80 min with 200 mA and 90 volts. After that, the specific band was visualized using a UV illuminator. The amplified positive samples were compared to the 1098 bp of the 18S rRNA gene.

Hematological studies

The hematological parameters studied included total erythrocyte count (millions/μL), hemoglobin concentration (Hb, g/dL), and hematocrit value (HCT, %). Several erythrocytic indices were also estimated, including Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Total Leukocytes Counts (TLC), Lymphocytes (LYM), and Granulocytes (GRA) using a hematology analyzer for infected and healthy animals (19).

Biochemical study

Sera from infected and uninfected animals were analyzed for biochemical parameters *via* an auto-analyzer (Alpha Classic, Sweden). The studied biochemical profiles, including total serum protein (TSP), serum albumin (SA), blood urea nitrogen (BUN), serum creatinine (CRE), and Aspartate Transaminase (AST), were estimated through spectrophotometry using commercial test kits (20).

Statistical analysis

Data analysis was performed through the Statistical Package for Social Sciences (SPSS version 24.0, IBM, USA). A comparison of the hematobiochemical parameters between infected and uninfected sheep was made using an independent samples *t*-test. A probability level of <0.05 was considered significantly different.

Results

The data of the present study revealed that of 360 native sheep examined, 71.7% were positive for *Theileria* protozoa. The PCR-based diagnosis of *Theileria* revealed the highest prevalence rate in Said Sadiq (74.6%) and the lowest prevalence in Bazian (69.5%), as shown in Figure 2 and Table 1.

The hematological profiles of infected sheep represented a significant decrease in total erythrocyte count, hemoglobin, and hematocrit. Furthermore, the erythrocytic indices of MCV, MCH, and MCHC recorded significantly higher values.

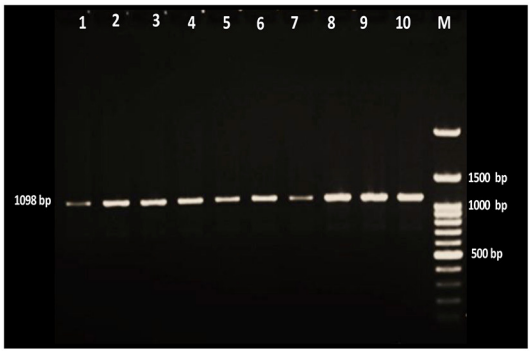

FIGURE 2
Gel electrophoresis of amplified *Theileria* positive products with ethidium bromide stain. Lane M is a 100-bp DNA ladder, and lanes 1–10 were positive samples for the *Theileria* parasite.

TABLE 1 Occurrence of *Theileria* spp. in sheep according to regions of the Sulaymaniyah province.

Location	Examined samples	Number infected	Prevalence (%)
Bazian	82	57	69.5
Mawat	67	49	73.1
Qaradagh	74	52	70.3
Said Sadiq	59	44	74.6
Sharazoor	78	56	71.8
Total	360	258	71.7

TABLE 2 Hematological and biochemical parameters of infected and uninfected sheep with *Theileria* spp.

Parameter	Infected sheep (258)	Uninfected sheep (102)	*P*-value
Erythrocytes	7.85 ± 0.04	8.66 ± 0.07	0.000
Hemoglobin	9.46 ± 0.05	9.90 ± 0.01	0.000
Hematocrit	27.86 ± 0.16	29.65 ± 0.30	0.000
MCV	35.49 ± 0.10	34.23 ± 0.17	0.000
MCH	12.04 ± 0.03	11.43 ± 0.06	0.000
MCHC	33.94 ± 0.03	33.40 ± 0.03	0.000
Leukocytes	10.70 ± 0.24	10.55 ± 0.37	0.747
Lymphocytes	5.52 ± 0.15	5.62 ± 0.25	0.738
Granulocytes	2.60 ± 0.16	2.68 ± 0.28	0.800
Total Proteins	6.22 ± 0.03	7.43 ± 0.06	0.000
Albumin	3.15 ± 0.02	3.76 ± 0.03	0.000
AST	110.91 ± 1.58	79.47 ± 1.38	0.000
Creatinine	0.98 ± 0.01	1.26 ± 0.02	0.000
Urea	34.43 ± 0.58	21.43 ± 0.62	0.000

Values are presented as means ± SEM. MCV, mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concertation; AST, Aspartate transaminase. Statistical analysis = independent samples t-test.

Furthermore, a decrease in total leukocytes, lymphocytes, and granulocytes was nonsignificant ($P > 0.05$).

Serum concentrations of total protein, albumin, and creatinine decreased significantly in infected sheep. On the contrary, significant increases in AST and urea concentrations were recorded in animals infected with *Theileria* (Table 2).

Discussion

Ovine theileriosis represents the endemic condition in the study area with a significant impact on the livestock industry. The present study showed that the prevalence of theileriosis in native sheep was 71.7%, in agreement with the study data reported by Abdullah and Ali (6) 5 years earlier, with a prevalence rate of 76.4% in the Sulaimani province. A lower prevalence than the current study has been reported in other locations. Infection rates of 62.5% in Iran, 54.4% in Oman, and 53.0% in Pakistan were found by Zarei et al. (21), Al-Hamidhi et al. (17), and Shahzad et al. (22), respectively. In other studies, Nangru et al. (23) and Riaz and Tasawar (14) reported 44% in India and 39.5% in Pakistan.

In the current study, samples were collected from small ruminant flukes with a history of tick infestation. Such a high prevalence rate indicated that tick infestation is a chief factor for the persistence of theileriosis in flukes. The significantly higher prevalence of theileriosis in tick-infested animals compared to tick-free sheep was reported by Ullah et al. (24). The highest prevalence of *Theileria* spp. was recognized in Said Sadiq, while the lowest was in Bazian based on PCR. The results indicated different prevalence rates of ovine theileriosis at different sampling sites. Different factors such as husbandry practices, nutritional deficiencies, breed, sex, flock size, climatic conditions (humidity, temperature), and contagious infections directly affect health status. Sheep infections with *Theileria* spp. are associated with the intra-leukocyte schizont and intra-erythrocyte piroplasm stages, which affect the hemato-biochemical changes, and the severity of these changes is related to the virulence of the parasite strain, the infectious dose, the animal breed, and immune status (25).

The present study results show that *Theileria* infection in ovine led to alterations in hemato-biochemical parameters. The hemogram showed a significant decrease in total erythrocyte count, hemoglobin concentration, and hematocrit value. Furthermore, significantly higher values of the MCV, MCH, and MCHC were recorded from PCR-positive samples. These findings agreed with previous studies (24, 26). Infection with *Theileria* spp. results in increased oxidative stress in infected animals, leading to increased fragility of erythrocytes due to membrane lysis and a lower concentration of hemoglobin (27). The reduction of erythrocyte counts might be associated with phagocytosis of the infected cells by an endothelial system of the spleen, lymph nodes, and other organs (28). The total leukocytes, lymphocytes, and granulocyte counts did not differ between the infected and uninfected sheep. Al-Hamidhi, Elshafie, Yaghfoori, Morrison, Johnson and Babiker (17) observed the same finding, while Shahzad (21) reported a significant decrease in WBCs, monocytes, and lymphocytes. A decrease in total cell count is presented by clinical signs like hemolytic anemia, dyspnea, and lethargy (27).

The biochemical analysis showed significantly lower levels of total protein, albumin, and creatinine concentrations with a significant increase in blood urea levels and serum AST enzyme activities of animals infected with *Theileria.* In agreement with the study findings (16, 22), also recognized that *Theileria* parasites cause a significant decrease in protein concentration. The reduction in serum total protein content is due to hypoalbuminemia caused by decreased albumin synthesis, which is the direct or indirect effect of the parasite on hepatocytes, leading to liver failure. Decreased dietary protein intake and diarrhea have also been related factors. Hypoproteinemia and hypoalbuminemia might also result from the accumulation of extravascular proteinaceous fluid after the lymph nodes are affected (29).

A significant decrease in serum creatinine concentration in infected animals was consistent with previous reports by Shahzad et al. (22) in sheep infected with theileriosis.

In contrast (30), reported a nonsignificant decrease in creatinine concentration from infected sheep with theileriosis. Furthermore, current data contradict what was reported by Col and Uslu (16) and (31), with a reverse pattern of a nonsignificant increase in serum creatinine levels in cattle and sheep. The concentration of blood urea in the current study was significantly increased, following the findings of Baghshani et al. (30) and Al-Fetly (31). However, Riaz and Tasawar (14) have reported a nonsignificant increase in urea levels among infected sheep. Increased urea concentrations in animals infected with *Theileria* could be related to kidney damage (16) and strongly associated with the level of parasitemia (14).

The measurement of enzyme concentration is another laboratory trial for disease evaluation. An increase in serum enzyme activity indicates the occurrence of necrosis or destruction in the liver or muscle tissues (32). Detection of AST and ALT serum concentrations provides a significant indication for determining the physiological function of the liver. An increase in the level of AST concentration was significant in *Theileria*-infected sheep compared with uninfected. Other researchers have reported similar findings (19, 30, 32). This rise in AST levels might be associated with hepatic dysfunction or muscular distress (16). Furthermore, the toxic metabolites of *Theileria* spp. have harmful effects on liver cells, which could raise the level of enzymes (33).

Conclusions

The study data denoted a high prevalence of *Theileria* infection among sheep, confirming the endemicity of the disease in the studied area. *Theileria* spp. are dominant hemoparasites in the study area, confirming the high population of transmitter vector ticks for different *Theileria* spp. Under traditional husbandry conditions, subclinically infected animals remain a source of tick infection. The annual costs of animal loss and treatment should be considered. Alteration in some hematological and biochemical profiles of infected sheep reveals the influence of theileriosis on metabolic disturbances due to its harmful effects on the body's organs and systems. Hence, a reduction in animal production might result from chronic infection with theileriosis.

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 Animal Care and Use Committee at the College of Veterinary Medicine, University of Sulaimani. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

SA conducted the design, methodology, and writing of the manuscript. HD and NS participated in the data interpretation and editing of the manuscript. 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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Food-borne zoonotic echinococcosis: A review with special focus on epidemiology

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Echinococcosis is a neglected, WHO-listed cyclozoonotic parasitic disease that is caused by a number of species belonging to the genus *Echinococcus*. This disease is widespread across the globe, resulting in heavy economic losses for farmers and cystic disease in aberrant human hosts. This review paper briefly discussed taxonomy, a brief history, the magnitude of economic losses, host spectrum and life cycle, risk factors, and clinical manifestations. Furthermore, the copro- and sero-ELISA-based prevalence of echinococcosis on different continents was summarized. Finally, the authors analyzed the frequency and use of molecular epidemiology in the taxonomy of *Echinococcus* species based on molecular markers. This review will serve as a quick reference to *Echinococcus*.

KEYWORDS

***Echinococcus*, prevalence, epidemiology, prevention, global scenario**

Introduction

Infectious diseases, including parasitic infestations, are important health problems in both animals and humans (1–4), which cause economic losses and severe illness (5–10). Parasites are capable of causing acute, chronic, and debilitating types of diseases, leading to production losses in animals (11–15), and many pieces of research have shown the positive role of alternative/complementary medicine in treating parasitic diseases. Echinococcosis is a neglected silent cyclozoonotic parasitic disease caused by the metacestode stages of the genus *Echinococcus* belonging to the family Taeniidae, affecting a wide spectrum of animal species, including livestock and wildlife, and it also has zoonotic implications (16–19). The genus *Echinococcus* contains at least nine valid species with different strains and genotypes, namely *E. granulosus*, *E. multilocularis*, *E. vogeli*, *E. oligarthra*, *E. canadensis*, *E. equinus*, *E. felidis*, *E. shiquicus*, and *E. ortleppi*, which are important ones (20, 21).

Echinococcosis is included in the World Health Organization's (WHO) list of neglected tropical diseases. This disease is widespread in its distribution and persists in a variety of environmental conditions in temperate, circumpolar, tropical, and sub-tropical regions (Figure 1). The parasite survives well in arid climatic conditions and subpolar oceanic environmental conditions. Eurasia, Australia, Africa, and South America have a very high disease prevalence, and 50 million people are infected with the disease worldwide (22, 23). Cystic echinococcosis (CE) encompasses a wide geographical area

from the eastern parts of Asia to northern America and from the upper northern hemisphere to the southern countries of the African continent (24, 25).

History

Variable-sized cysts comprise the metacestode stages of *E. granulosus*, which are filled with a transparent liquid. The presence of this clear liquid led to the coining of the term "hydatids." A number of scientists and physicians, including Hippocrates, Galen, Aretaeus, Wolckerus, and Bonet, described the different features of hydatids in their timelines. Some researchers described them as an accumulation of serum and mucus in between the laminar cell layers. Francesco Redi first provided evidence of hydatids' metacestode nature and reported that cysticerci could move like animals (26). Jacob Hartman, a professor of medicine at the University of Königsberg/Germany, confirmed the animal-like nature of cysticerci, describing it as a small, spherical structure with a metacestode bladder. Edward Tyson, a professor at Oxford, also found the motility of the hydatids of *Cysticercus tenuicollis* to be like that of living creatures. Simon Pallas in the Netherlands described hydatids as a distinct group of bladder worms with small bodies on the inner walls of hydatids. Later, Ephraim Goeze discovered tapeworm scoleces in these small bodies called brood capsules. In 1801, Karl Rudolphi introduced the word *Echinococcus* to the world of science (27).

In the mid-nineteenth century, two different forms of echinococcosis were identified: cystic echinococcosis (CE) and alveolar echinococcosis (AE). There remained a heated debate over the aetiological agents responsible for the causation of CE and AE. Two schools of thought emerged, one with "unicasts," believing that both forms occur due to the same species of *Echinococcus,* and the other with "dualists," claiming that two different species are responsible for causing two different forms of the disease. However, the understanding of the dualist school of thought was supported by Professor Adolf Possely. Clear experimental proof supporting the concepts of a dualist school of thought was provided in 1928 by Posselt in his work "Der Alveolarechinokokkus und seine Chirurgie" (alveolar *echinococcus* and its surgery). He provided plausible arguments that two different species cause CE and AE, as their morphology, anatomy, and clinical signs clearly differ from each other (28).

Economic ravages of echinococcosis

Echinococcosis is a neglected tropical disease of public health concern and has serious economic implications. Out of nine known species of *Echinococcus*, *E. granulosus* and *E. multilocularis* pose significant threats to human and animal

FIGURE 1
Geographical distribution of echinococcosis.

health in addition to substantial economic losses (29). Treatment costs, production losses, and mortality in infected animals and aberrant human hosts are economic and social calamities caused by this infection. The cosmopolitan distribution of these ailments has led to losses of USD 3 billion annually (30). It has been reported that echinococcosis has led to annual economic losses of USD 212.35 million in India, USD 232.3 million in Iran, and USD 7.708 million in Turkey (31). Followed by *Fasciola hepatica*, CE is the most important cause of condemnation of livestock viscera. It has been estimated that in Chile, CE leads to economic losses amounting to USD 14.35 million per year (32). Echinococcosis causes substantial economic losses in Pakistan. Losses due to the disease have been estimated at USD 276.20 per 100 infected goats and sheep and USD 165.72 per 100 infected large ruminants and camels. There are losses in terms of quantity and quality of milk, wool, and meat, retarded growth, decreased fertility, and carcass condemnation (33).

Life cycle of the *Echinococcus* species

Echinococcus species enjoy two different host species: the intermediate host and the definitive host. The definitive hosts of this cestode are the carnivores, especially the dogs that carry this parasite in their small intestines. Both wild and domesticated ruminants, camels, and humans serve as intermediate hosts of different *Echinococcus* species (23, 34, 35). The life cycles of different species of *Echinococcus* are depicted in Figure 2. All of the species enjoy a heteroxenic life cycle. The size of adult worms varies from 2 to 11 mm, with 2–7 proglottid segments. Scolex has two rows of rostellar hooks. Each proglottid has a single genital opening, and the mature segment is called the penultimate segment. After fertilization, eggs are fully developed in the uterus and released into the environment along with dog feces. Contaminated water or vegetation ingested by the intermediate host leads to the release of oncospheres from embryonated eggs that penetrate the intestinal wall and spread to various body tissues through circulation. Cyst formation primarily occurs in the lungs and liver, and such infected tissue, when eaten by the canids, releases protoscoleces. They develop into mature worms, completing the life cycle (36–39).

Risk factors for cystic echinococcosis

The dynamics of the completion of the life cycle of the *Echinococcus* species are well supported by the presence of dogs. Home slaughtering practices and feeding the dogs with hydatid cysts facilitate the completion of the parasite's life cycle. Younger children have a great affection for pets, including dogs. Children of such an age are likely at greater risk of suffering from echinococcosis. Regions with widespread grassland are best suited for grazing sheep, goats, and cattle. The pastoral dogs and the nomadic lifestyle play a role in the completion of the synanthropic life cycle of *E. granulosus* (40). Lack of alertness about the life cycle, poor hygiene, open slaughtering practices, close involvement of ruminants and dogs, and improper disposal of condemned carcasses/offal support the spread of echinococcosis (33). In South Asian countries like Pakistan and India, the slaughtering of food animals is carried out either in an open environment or in slaughterhouses that are easy for dogs to access. Such access by the dogs and ingestion of the offal leads to the completion of the *Echinococcus* life cycle (18). A general overview of risk factors for *E. granulosus* infection in dogs and humans is described in Figures 3, 4.

Zoonotic significance in terms of DALYs

The burden of human echinococcosis can be expressed in disability-adjusted life years (DALYs), which are used to express the burden of human cases of CE and AE. The annual global burden of AE is approximately 18,200 cases, which leads to almost 666,000 DALYs (41). With regard to CE, the annual global burden amounts to 188,000 new cases every year and leads to 184,000 DALYs (42).

Forms of echinococcosis

Echinococcosis can appear in four different forms: cystic echinococcosis (CE), alveolar echinococcosis (AE), polycystic echinococcosis (PE), and unicystic echinococcosis (UE), caused by *E. granulosus*, *E. multilocularis, E. vogeli, and E. oligarthra*, respectively. All of the species share the same common definitive hosts, which are the canids, except *E. oligarthra,* which has members of the family Felidae as the definitive host (43). Definitive hosts harbor adult worms in the small intestine and release the embryonated eggs of the parasite in the environment with feces. *Echinococcus granulosus* and *E. multilocularis* enjoy a wide range of intermediate hosts, including bovines, which harbor the larval stages of the parasite in their visceral organs after ingestion of the embryonated eggs (44, 45). Humans and monkeys serve as aberrant hosts for all species, and diseases may progress from asymptomatic to severe clinical diseases, which can eventually cause death (27, 46). The disease pattern of echinococcosis is usually asymptomatic in livestock, and the diagnosis is generally made on necropsy findings in the abattoir; however, it has serious implications regarding the condemnation of the carcasses. In human CE, clinical signs include asthenia, weight loss, epigastric pain, hepatomegaly, and cholestatic jaundice (47). It may become a fatal disease when the cysts rupture and their fluid contents and protoscoleces

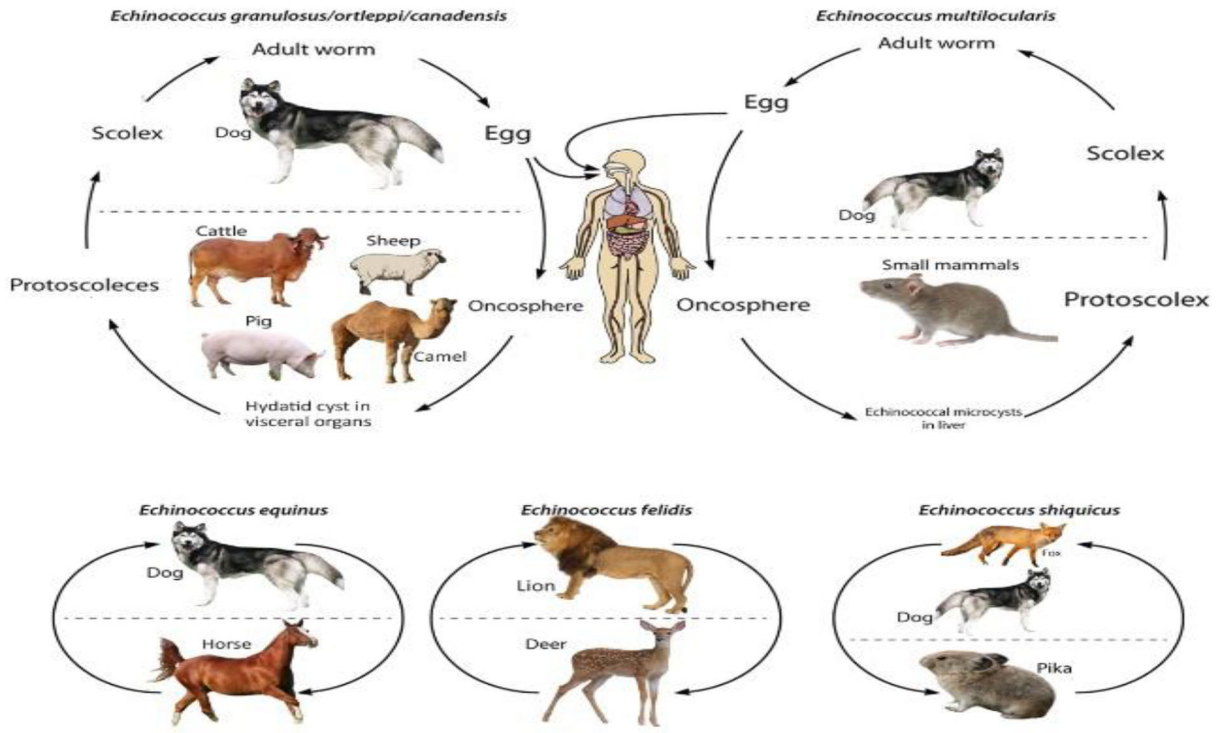

FIGURE 2
Life cycle of *Echinococcus* species.

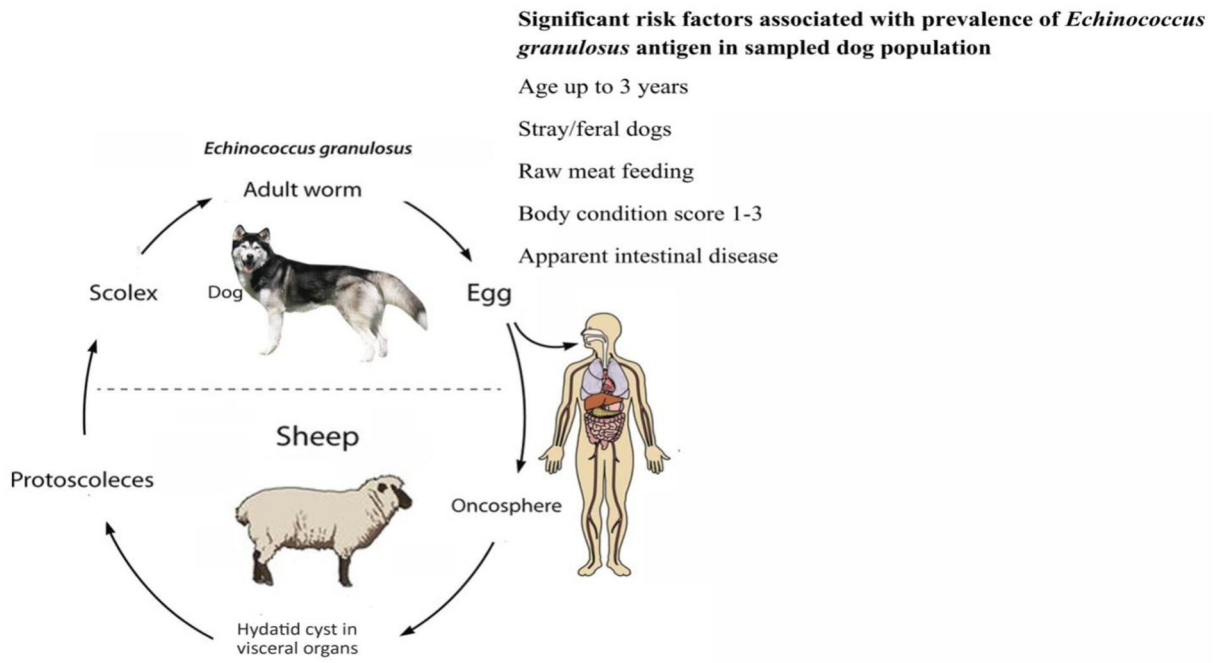

FIGURE 3
Risk factors for *Echinococcus granulosus* in dogs.

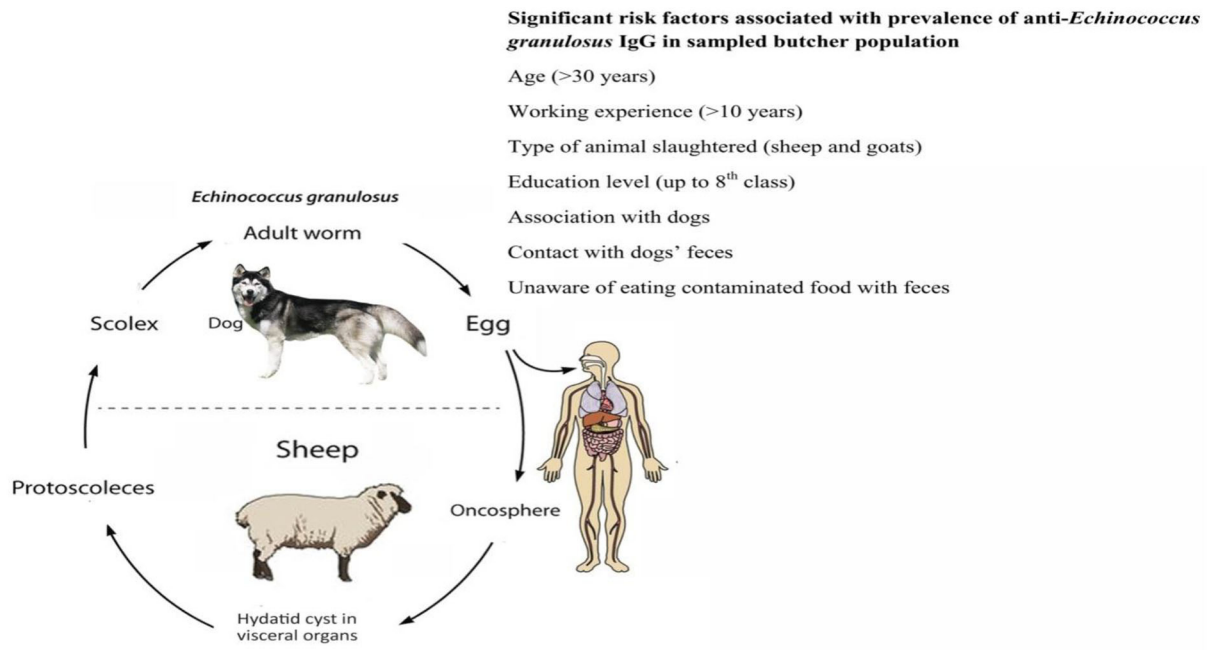

FIGURE 4
Risk factors for *Echinococcus granulosus* in butchers.

are drained into the peritoneal cavity, leading to anaphylactic shock (48).

Sequencing of mitochondrial cytochrome-c oxidase and NADH-dehydrogenase genes has revealed at least 10 genotypes (G1-G10) of CE (49). *Echinococcus granulosus sensu lato* species complex is a major veterinary and medical concern causing cystic echinococcosis (CE) in the target species across the globe. Genotyping results have indicated that *E. granulosus* sensu stricto (G1 common sheep strain, G2 Tasmania sheep strain, and G3 buffalo strain), *E. equinus* (G4 horse strain), *E. ortleppi* (G5 cattle strain), and *E. canadensis* (G6 camel strain, G7 pig strain, G8 cervid strain, G9 human strain, and G10 Fennoscandian cervid strain) are the members of this species complex (50–53). Most of the cysts are formed in the liver (70%) and the lungs (20%), while up to 10% are found in other body tissues like the brain, kidneys, marrow cavities of the bones, and ocular orbits. The chances of the formation of cysts in the central nervous system are only 1–2% of the total *E. granulosus* infections (54, 55). Apart from economic concerns, hydatidosis is a major public health issue as the hydatid cysts grow as unilocular fluid-filled bladders in the internal organs like the lungs and liver of humans (56).

Diagnostic methods

There are a number of diagnostic techniques for echinococcosis, including ultrasonography, serological tests, e.g., enzyme-linked immunosorbent assay, immunoelectrophoresis, immunoblotting, indirect fluorescence antibody test (IFAT), and latex agglutination test (LAT), (57) and molecular methods, e.g., loop-mediated isothermal amplification (LAMP)-based techniques, and polymerase chain reaction (PCR). Each of these has its own advantages and limitations (58, 59).

For the diagnosis of *Echinococcus* infection, post-mortem analysis of the small intestine and arecoline purgation are reliable tools. Intestinal scraping, fecal sedimentation, and fecal centrifugal flotation methods have high sensitivity and are good indicators of worm burden in the host intestine. The morphology of the eggs of *E. granulosus*, *E. multilocularis,* and *Taenia* species resembles each other, suggesting that methods are not very sensitive and specific (60). The coproantigenic ELISA has also been another tool, as it can detect the *E. granulosus* antigen both in patent and prepatent stages. The sensitivity of this ELISA is higher for animals with a high infection burden. Another diagnostic tool to access the infection is PCR, which has high specificity and sensitivity (61). Cerebral hydatidosis is a rare form of echinococcosis and accounts for only 2% of total cases. The incidence of this form is higher in children because of frequent contact with pet dogs. However, adults can also get the infection. It is important to distinguish neurohydatidosis from other intracranial cysts when making a diagnosis. In this peculiar form, serological testing is usually not useful as far as diagnostic tools are concerned. The only reliable diagnostic technique is

MRI to assess the size of the hydatid cyst and devise a plan to excise it (62) surgically.

Various techniques have been developed over the past 30 years to identify *Echinococcus* variants. However, several molecular techniques have been used in the past, such as PCR-RAPD (random amplification of polymorphic DNA), PCR-RFLP (restriction fragment length polymorphism), a Southern Blot approach, DNA fingerprinting, and dideoxy fingerprinting. PCR is the preferred method for parasite identification, molecular epidemiology research, and confirmation purposes (63).

Nested PCR and multiplex PCR are frequently used for the differential detection of different *Echinococcus* species. PCRs that are followed by sequencing help detect variations within species and genotypes (64). While sequencing is a time-consuming and skill-intensive method, it is also the most accurate method for identifying the species of *Echinococcus* and finding genetic variation.

Recently, approaches like loop-mediated isothermal amplification (LAMP) that are low-cost and simple to use have been created and tested (65). Due to the fact that DNA may be amplified using a straightforward water bath without the use of complicated instruments, LAMP is an ideal technology to utilize in low-resource settings where alveolar and cystic echinococcosis are common. However, because of its high sensitivity, this system can be tricked into giving false positives.

Contemporary methods using single-locus or multilocus microsatellite analysis have been developed to explore the genetic diversity, the population structure of parasites, and the geographic relatedness of *Echinococcus* species (66, 67).

For the identification of parasitic diseases, real-time PCR (qPCR) has a number of advantages over traditional PCR, including improved sensitivity and specificity, a shorter reaction time, and quantitative estimation of the amount of DNA in the sample (68, 69).

Treatment and control measures

Researchers have long been interested in finding ways to break the life cycle of the Echinococcus species in order to decrease the spread of illness in both animals and people. Using anthelmintics and creating awareness among the masses about slaughter hygiene is one of the most important strategies to reduce the prevalence of echinococcosis. Limited treatment regimens are available for curing patients suffering from echinococcosis, and the focus is on giving benzimidazoles that have only parastiostatic activity. Oral administration over a long time has also led to the development of resistance to varying degrees in the metacestode stages of *Echinococcus* (47). Amphotericin-B, nitazoxanide, and isoflavones have also been reported to possess anti-*Echinococcus* activity, but the efficacy of these chemotherapeutic agents is not much higher (70). Imatinib, an anticancer agent, has been found highly effective in killing protoscoleces and metacestodes *in vitro* at a concentration of 25 μM. This drug was evaluated after keenly investigating the signaling pathways of *Echinococcus* (71). Pharmacotherapy poses different threats because of drug residues in milk and meat, which can lead to health issues for the end consumer. In addition, low dosage or frequent administration of anthelmintics leads to parasite drug resistance (36). Despite these limitations and drawbacks, vaccination remains a suitable and long-term solution against *Echinococcus* infection. Vaccine development is a crucial phase, and attention must be paid to developing multifunctional vaccines considering the circulating serotypes and genotypes of *E. granulosus* (72). Identifying the strain is key to implementing control strategies to curtail disease transmission. *Echinococcus* spp. continue to be a global problem despite our extensive knowledge of the organism, its control techniques, vaccine research, and clinical case management (73).

Eradication of this notorious infection has been a challenge for both developed and developing countries (74). However, the community's involvement in controlling the incidence of the disease is of high value. Zhang et al. proposed a number of strategies to decrease the number of future cases. Promotion and awareness seminars about the epidemiology of the disease, inspection of animal offal at the slaughterhouses, proper disposal, and condemnation of carcasses with cysts, monthly deworming of dogs with praziquantel, and ultrasonography of suspected patients and their treatment are important to highlight here (75). Killing stray dogs to prevent the shedding of embryonated eggs in the environment is another prevention strategy that was adopted in the past. A complete ban on home-slaughtering practices and rodent control programs must be institutionalized, as *E. multilocularis* targets rodents as its intermediate host (76). One alternative is to hire a Hydatid Disease Control Officer (HDCO), whose responsibilities would include registering dogs, dosing them monthly with dewormers, and educating the public about echinococcosis control programs (75).

Prevalence of echinococcosis

Europe

Echinococcus granulosus infection is endemic in Southeast European countries, e.g., Bulgaria and Romania, and *E. multilocularis* infection is hyperendemic in France, Germany, Turkey, and Switzerland (77). G1 (the sheep strain) and G4 (the horse strain) are endemic in the United Kingdom and can be found in sheep, dogs, and horse/foxhounds, respectively (78, 79). Liver lesions due to *E. multilocularis* were revealed upon necropsy of nutria (*Myocastor coypus*) in a French wildlife park. There was evidence of contamination of the interior and

exterior of the park, with fox feces contacting *E. multilocularis*. Later, the intestines of five other foxes shot in the park were found to harbor *E. multilocularis*. A coprological analysis of other definitive hosts like wildcats, bears, and wolves showed a 5.3% prevalence (80). An epidemiological study in Turkey between 2006 and 2010 revealed that CE is endemic in Turkey.

Of the 166 buffaloes inspected after slaughter, the organs of 10.24% of the buffaloes had CE. Females and older animals had a prevalence rate of 21.66 and 37.93% compared to males and younger animals, with prevalence rates of 3.77 and 4.38%, respectively. A statistically significant difference between sex and age was observed. There were hydatid cysts in both the liver and the lungs; however, the lungs were the predominant site of encystations, with 47.06% of all cyst formation detected, compared to the liver, which had 29.41% of all cyst cases. G1, G2, and G3 genotypes were found after performing mitochondrial *cox*1 sequencing analyses. Another study was conducted in Ankara, Turkey, to compare fecal sedimentation technique and fecal centrifugal flotation to harvest taeniid eggs from the feces, identify *E. granulosus* eggs through PCR, and determine the prevalence of *E. granulosus* infection in the study area. Of 100 fecal samples, 27 had eggs. *Echinococcus granulosus*-specific PCR was carried out on the positive samples, and 14 samples were positive, resulting in a prevalence of 51.85% (50). The sedimentation technique was found to be significantly better than the flotation method, as the earlier one detected eggs in 27 samples while the latter one detected only 10 samples as positive for the eggs.

An epidemiological study was conducted in 16 counties in Romania to determine the prevalence of echinococcosis in sheep and cattle in the country. Out of 643 sheep and 1,878 cattle examined for hydatidosis, 421 sheep and 754 cattle tested positive for the cysts, giving an overall prevalence of 65.6 and 40.1%, respectively. Germinal layers were collected, and DNA was extracted to obtain an idea of the genetic diversity in the isolates. 12S ribosomal DNA and cytochrome c oxidase were used as genetic markers. The genetic diversity analysis results showed a dominance of the G1 genotype in the *E. granulosus sensu stricto* complex (81). A similar type of genetic diversity analysis was performed in Serbia to find which genotypes prevail in intermediate hosts (sheep and cattle) of the *E. granulosus sensu stricto* complex. The sequence analysis of the cytochrome c oxidase subunit 1 (*cox*1) mitochondrial gene from germinal layers of hydatid cysts showed that G2 and G3 are prevalent in the country (82).

North and South America

In Canada, only *E. canadensis* and *E. multilocularis* have been found to be prevalent, which cause CE and AE, respectively. Both of these parasitic species were confirmed to be circulating between wild canids like wolves and foxes, which serve as definitive hosts, deer, moose, and small mammals like rodents, which serve as intermediate hosts (23, 83, 84). The USA is not endemic to echinococcosis. This infection is hard to manage and treat because of the parasite's multiorgan cyst formation potential and the high recurrence rate (85). CE has been reported in many countries in South America, including Brazil, Argentina, Chile, Peru, and Uruguay. Studies in Chile confirmed that CE is prevalent in both livestock and animals (86, 87).

Asia

No detailed data are available with regard to the prevalence of echinococcosis in Afghanistan (88). A solitary study conducted in 1988 showed that 73% of stray dogs in Kabul harbored *E. granulosus* (89). However, CE clinical disease has been reported in Afghan immigrants and US soldiers who returned to the USA after performing duties in Afghanistan (90). Eighty percent of the Afghans work in agriculture or with animals, and many also have their own pets, like dogs. The population of stray dogs was found to be 10 times greater than that of domestic dogs. Poor law enforcement, political instability, and limited control strategies implementation are the factors for disseminated cases of CE and AE (75).

Echinococcosis is still underdiagnosed in Pakistan. Because of their limited scope, the few published reports on the disease's current state in the country cannot be considered indicative of the problem nationally. A total of 106 cases were reported to Agha Khan Hospital, Karachi, from 1995 to 2006, and 21 of them were Afghan refugees (91). The high prevalence of echinococcosis in Afghanistan and extensive free-border movement from Afghanistan into Pakistan highlight the need for conducting an extensive epidemiological study in Pakistan to assess the current status of the disease in Pakistan (92). Almost a three-decade-old study is available in the literature about the prevalence and serological investigation of echinococcosis in Islamabad, Pakistan. According to that study, hydatidosis was observed in 58.9, 38.90, and 33.06% of the slaughtered camels, cattle, and buffalo at a local slaughterhouse. Most of the cysts observed were infertile, while the specificity, sensitivity, and efficiency of indirect hemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) were low (93).

Almost 466 million people live in Central Asian countries and are at risk of suffering from echinococcosis. Occupational exposure to farmers, herdsmen, and farmers is very high. The semi-nomadic lifestyle of the inhabitants of these areas and the raising of sheep and cattle together with dogs make the environment conducive for the *Echinococcus* species to complete their life cycle (75).

Echinococcosis is highly endemic in China, covering more than 21 provinces that comprise 87% of the total geographical area of the country. The copro-antigen ELISA, copro-DNA PCR, and necropsy of the dogs strongly suggested a high prevalence

of both CE and AE in the dogs in China. As far as prevalence in cattle and sheep is concerned, CE in sheep and cattle was 50% and 44%, respectively (94).

After the fall of the Soviet Union, the number of human echinococcosis cases in Mongolia started to rise due to declining health facilities and poor dog deworming programs (95). Human CE is widely distributed in 12 provinces of Mongolia, with 10 of them adjoining Russia and China (91, 96). There is no echinococcosis control program institutionalized in Mongolia (96).

The dissolution of the Soviet Union led to the re-emergence of both human CE and AE in Kazakhstan, which are still endemic in the country due to several factors (97). Of them, nomadic lifestyles and the adoption of obsolete breeding practices shut down large farms; the establishment of small farms; the abandonment of dog deworming practices, and improper disposal of animal carcasses are important factors leading to the endemicity of echinococcosis (98).

Kyrgyzstan, Tajikistan, and Uzbekistan are endemic to human CE and AE (41, 99). The prevalence of CE in sheep and cattle in all three countries has been found to be alarming. Stray dogs and wild canines, including foxes, were found to be infected with *E. granulosus* (100). The control measures in these three countries are almost identical to those in Kazakhstan because they share similar geographical patterns, socioeconomic backgrounds, and literacy levels.

Iran is a camel-rich country with more than 1.5 million camels. CE is endemic in humans in Iran (101). Low-level endemicity was observed in northern and western Iran's sheep, goat, cattle, and camel populations (102, 103). Fecal samples from dogs and jackals were also found to be positive for containing *E. granulosus* DNA. Effective control measures were implemented in Kerman from 1991 to 1994, involving killing stray dogs and deworming pets and sheepdogs. These efforts were found to be highly effective as they decreased the incidence of the disease. An epidemiological study was conducted in five different regions of the country. Four hundred and thirty-eight dromedaries were examined, of which 135 were positive, resulting in a prevalence of up to 30.82%. The highest number of cysts were found in the lungs. The age group older than 15 years had the highest prevalence (31).

Africa

An epidemiological study was conducted to assess the prevalence of *Echinococcus granulosus* in client-owned dogs in the Sidi Kacem province of Morocco. The reason for choosing this province was that its climatic diversity, geographic conditions, and social system make it a model representative of the country. Dogs from both rural and urban areas were included in the study. Arecoline hydrobromide was administered orally to the dogs as a purgative. Of the 273 dogs included in the study, the feces of 224 dogs had *E. granulosus* worms, resulting in an overall prevalence of 82.1%. Compared to urban-owned dogs (18.8%), dogs in rural areas had a high prevalence (38%). A comparison was made between the prevalence of echinococcosis in dogs that had access to the slaughterhouse and those that did not. Results showed that dogs with access to slaughterhouses had a higher prevalence (62.7%) as compared to dogs (29.1%) that did not have such access (104).

Apart from infecting dogs as definitive hosts and omnivores and herbivores as intermediate hosts, *Echinococcus* also infects wild animals. The first confirmed case of echinococcosis caused by *E. felidis* was reported in a lion in South Africa about 80 years ago. Molecular characterization was performed on the eggs and archived feces (105). Hyena (*Crocuta crocuta*) also serves as a definitive host for *E. felidis* (106). *Echinococcus fields* have also been found to cause echinococcosis in hippos. Six hippopotami were investigated for cysts, and three were found to have hepatic cysts. The morphology of rostellar hooks helped identify *E. felidis,* and confirmation was performed through nuclear and mitochondrial DNA sequencing (107).

Echinococcus multilocularis enjoys a sylvatic life cycle. The first confirmed report appeared in 2011, when a red fox was found to be the definitive host for *E. multilocularis*. This study aimed to identify rodent species that serve as intermediate hosts of *E. multilocularis*. Liver samples from 1,566 rodents were collected and examined for *E. multilocularis*-specific lesions. Samples were subjected to PCR and sequencing to identify the pathogen. Tissues were examined histologically for *E. granulosus*-specific lesions. *Microtus agrestis* (1/187), *Arvicola amphibious* (8/439), *Myodes glareolus* (0/655), and *Apodemus spp*. (0/285) had a lesion size of more than 6 mm, which is specific to *E. granulosus* (108).

The magnitude of *Echinococcus* in north African countries is widespread because of the high population of stray dogs, which consume the carcasses of infected ruminants and camels and thus take up the cysts from such condemned carcasses (109). The majority of cases of hydatidosis in Tanzania are due to the *Echinococcus granulosus* G1 genotype. This study investigated the genetic relationship between the localization of E. granulosa G1 hydatid cysts in the liver and lungs of humans, sheep, and cattle. Host species and localization differentiation were two factors responsible for genetic differentiation, as determined by single-strand conformation polymorphism and allozyme variation (110).

Eight hundred and thirty-two fecal samples from six conservation areas in Kenya were collected to determine the prevalence of *E. granulosus* in wild mammals, including jackals, hyenas, leopards, lions, and wild dogs. A total of 120 samples were positive, containing taeniid eggs, showing an overall prevalence of 14.4%. A total of 1160 eggs were collected and subjected to restriction fragment length polymorphisms—polymerase chain reaction (RFLP-PCR) of the gene *nad*1, and sequencing was performed later. Of these samples, 26

were of *E. felidis,* and 12 were of *E. granulosus sensu stricto* complex (106).

Advancements in molecular taxonomy of *Echinococcus* species

The field of molecular epidemiology (ME) has altered our understanding of the spread of infectious diseases. This area of epidemiology provides the tools that can characterize the etiological agents of infectious illnesses. Furthermore, molecular epidemiology is useful for surveillance tasks, particularly when investigating emerging diseases. Molecular epidemiology is becoming increasingly important in the context of disease emergence and conservation for both human and domestic animal health, as well as for infectious agents in wildlife (111).

The parasite cestode *Echinococcus* is the cause of echinococcosis, which has two types—cystic and alveolar—and affects people worldwide. When investigating the epidemiology of infectious diseases, it is crucial to comprehend their etiology. It is important to understand how different host species are involved in the transmission of echinococcosis because this has been a problem in many endemic regions. This is even more important when various *Echinococcus* species, "strains," or genotypes are present. Molecular epidemiology has been extremely helpful in understanding the wide genetic and phenotypic variability exhibited between the species present within the *Echinococcus* genus (112, 113).

Formally identifying a species requires effective communication, which is essential when the targeted species have public health significance and necessitate coordinated control measures. Regarding *Echinococcus*, there have long been taxonomic and nomenclatural ambiguities, especially at the species level. This occurred due to the lack of significant morphological features and the frequent overriding of the importance of host occurrence by taxonomic considerations (111). *Echinococcus* transmission ecology in areas with a variety of affected host species has been a topic of study for some time. Fortunately, there is widespread agreement that ten species of *Echinococcus* should be recognized based on morphological, genetic, and ecological factors. Molecular approaches usually confirm original taxonomic assumptions and, more importantly, the validity of certain morphological traits. They have also been instrumental in resolving taxonomic issues.

Echinococcus granulosus

Echinococcus granulosus is the species most frequently responsible for cystic echinococcosis in humans and has the broadest spectrum of intermediate hosts (112, 113). In some regions, like Australia, it also impacts wildlife, although cycles involving cattle keep it in balance most of the time. In areas with a range of intermediate hosts that can be sensitive to other species besides *E. granulosus, E. granulosus* is also common. Because they allow for species identification from metacestode stages, molecular tools are essential in these situations. For instance, in some areas of Europe and the Middle East, livestock may contract *E. granulosus*, *E. ortleppi*, and *E. intermedius*, which also pose a risk to humans (112–114). It is necessary to ascertain whether distinct species cohabit in such situations to avoid impeding control strategies and focused public wellbeing measures. In many gulf countries, *E. granulosus* and *E. intermedius* are both kept sympatrically in cycles, with dogs acting as the final hosts and camelids and ovines serving as the intermediate hosts (114). Either species may occasionally infect people in mixed infestations (115). Camels are the main intermediate host for E. intermedius and, to a lesser extent, *E. granulosus* in a number of Middle Eastern regions. *E. granulosus* was the only species found in both animals and people, according to a ME investigation in Iran's Mazandaran Province. Additionally, *E. granulosus* was only found in sheep and camels, according to a ME study in Riyadh, Saudi Arabia (116). Cattle are thought to have a minor role in the spread of *E. granulosus*. However, recent investigations in Sudan and Ethiopia revealed that cattle serve as the most significant intermediate hosts for *E. granulosus*, *E. ortleppi*, and *E. intermedius* (117, 118).

Previous studies used host preference and phenotypic variations to discriminate between the genotypes G1, G2, and G3 of the E. granulosus species (111). Despite the need to maintain genotypic recognition in light of the phenotypic features that have epidemiological significance, current molecular characterization at a number of loci has not provided any evidence that G2 and G3 require species delimitation (119, 120).

Echinococcus ortleppi and *E. equinus*

Dogs serve as the final hosts for *E. ortleppi* and *E. equinus*, whereas cattle and horses serve as the intermediate and final hosts (111). Both of these species demonstrate high host selectivity for both types of hosts. However, only *E. ortleppi* can infect humans. Dogs' high host specificity likely explains why both species are sporadic and do not have a wide geographic distribution since they rarely have access to the metacestode stages in horses and cattle, especially since public health care has progressed in the endemic regions. However, according to recent ME studies, both species are still spreading over the African and South American continents, some regions of Europe, and Turkey (121, 122). Interestingly, the first ME investigation on echinococcosis carried out in Bhutan (123) discovered that both *E. ortleppi* and *E. granulosus* were locally transmitted.

TABLE 1 Overview of prevalent genotypes of *Echinococcus* species and molecular markers that were investigated in different countries.

Sr. No.	Country	Marker	Genotype	References
1	Bulgaria	*nad*1	G1	(129)
2	China	*cox*1	G1 and G6	(130)
3	Italy	*rrnS*	G1 and G3	(131)
4	Chile	*cox*1	G1 and G6	(86)
5	Italy	*cox*1 *nad*1	G1, G3, G4 and G5	(132)
6	Turkey	*cox*1	G1 and G3	(133)
7	Peru	*cox*1 and *ef1a*	G1, G6 and G7	(134)
8	Iran	*cox*1	G1 and G3	(135)
9	Brazil	*cox*1 and *12S rRNA*	G1, G3 and G5	(136)
10	Iran	*cox*1 *nad*1	G1, G3 and G6	(137)
11	Australia	*cox*1, *nad*1 and *rrnS*	G1 and G3	(138)
12	Iran	*cox*1, *nad*1, *atp*6, and 12S *rRNA*	G1	(139)
13	Brazil	*cox*1	G1 and G5	(140)
14	Iran	*cox*1 and *nad*1	G1 and G3	(141)
15	Iran	*ITS*1	G1	(142)
16	Palestine	*cox*1	G1	(143)
17	China	*cox*1 and *nad*1	G1-G3 complex G6-G10 complex	(144)
19	India	*cox*1	G1, G3 and G6	(145)
20	Russia		G1, G6, G8 and G10	(146)
21	Romania	*cox*1 and 12S *rRNA*	G1 and G3	(80)
22	Chile	*cox*1 and *nad*1	G1, G3 and G4	(85)
23	Iran	*cox*1	G1, G3 and G6	(147)
24	China	*nad*2	G1	(148)
25	China	*cox*1, *cyt*b and *nad*1	G1	(149)
26	China	*cyt*b	G1	(150)
28	China	*cox*2	G1 and G6	(151)
29	Egypt	*nad*1 and *cox*1	G1, G5 and G6	(152)
30	Iran	*cox*1	G1 and G3	(153)
31	Iran	*ITS*1 and *cox*1	G1 and G6	(154)
32	Greece	*cox*1 and *nad*1	G1	(155)
33	Iran	*cox1*	G3	(156)
34	Iran	*cox*1 and *nad*1	G1, G3 and G6	(157)
35	Serbia	*cox*1	G1, G3	(81)
36	Iran	*cox*1	G1, G3 and G6	(158)
37	Bangladesh	*cox*1 and 12S *rRNA*	G1 and G3	(159)
38	Iran	*nad*1 and *cox*1	G1, G3, G5 and G6	(160)
39	Iran	*nad*1 and *cox*1	G1, G3 and G6	(161)
40	Iraq	*rrnS cox*1	G1 and G3	(22)
41	Sudan	*nad*1	G1, G5 and G6	(117)
42	Saudi Arabia	*cox*1	G1 and G3	(116)
43	Turkey		G1	(162)
44	Chile	*cox*1	G1 and G3	(163)
45	Iran	*ITS*1 and *cox*1	G1 and G3	(164)
46	China	12S *rRNA* and *cox*1	G1	(165)
47	Iran	*cox*1	G1 and G3	(166)
48	Nigeria	*nad*1 and *cox*1	G6/G7	(167)
49	Iran	*cox1*	G1 and G3	(168)

(Continued)

TABLE 1 (Continued)

Sr. No.	Country	Marker	Genotype	References
50	Iran	*nad*1 and *cox*1	G1 and G3	(169)
51	China	*cox*1	G1 and G3	(170)
52	Italy	*nad*2 and *nad*5	G7	(171)
53	Uzbekistan	*nad*1 and *cox*1	G1, G3 and G4	(172)
54	Pakistan	*nad*1 and *cox*1	G1, G3 and G5	(173)
55	Iran	*nad*1 and *cox*1	G1, and G3	(174)
56	Pakistan	*cox*1	G1, G3 and G6	(175)
57	Turkey	*cox*1	G6/G7	(176)
58	Iran	*nad*5	G1,and G3	(177)
59	Iran	*cox*1	G1	(178)
60	Mozambique	*cox*1 and *nad*1	G5	(179)
61	Iran	*cox*1 and *nad*1	G1, and G3	(180)

TABLE 2 Different markers were used to investigate the prevalence of *Echinococcus* species and a corresponding number of studies covered in this review.

Sr. No.	Genetic marker	No. of studies
1	*nad*1	22
2	*rrnS*	3
3	*cox*1	49
4	*ef1a*	1
5	12S *RNA*	5
6	*atp*6	1
7	*ITS*1	3
8	*nad*2	2
9	*Cytb*	2
10	16S *RNA*	1
11	*cox*2	1
12	*nad*5	2

Echinococcus canadensis and *E. intermedius*

It has been known for a long time that *E. granulosus* is distinct from other *Echinococcus* species and is maintained in cycles that include domestic pigs and cervids as intermediate hosts (111). According to previous ME findings (124), the four distinct *Echinococcus* genotypes (G6, 7, 8, and 10) are sustained in these life cycles. These studies added to the morphological descriptions of the adult parasites that originated in cervids and pigs. Nomenclatural issues have made it difficult to fully understand these forms' transmission cycles, despite molecular techniques' value in demonstrating the genetic distinctiveness of these forms.

There are two subspecies of the species *E. canadensis*: G8 and G10 (111). Initially, it was proposed to include the genotypes G6 and G7 in the species *E. canadensis* (125). Unfortunately, this did not reflect their ecological or geographical distribution. (124). According to molecular techniques (124, 126), the four genotypes now clearly represent two species. Sequences of G6 and G7 wildlife isolates are notably different from those of G8 and G10, and those of the latter were remarkably diverse from one another (122). Phylogenetic analysis revealed that the G6/G7 and G8/G10 groups should be considered as two discrete species, *E. intermedia* and *E. canadensis* (126). This was previously advocated (124) because the name *E. intermedius* had already been proposed for the species infecting pigs and camels.

In North America and Scandinavia, both genotypes of *E. canadensis* primarily infect wolves and other cervids, with occasional human infections (114). On the other hand, the majority of E. intermedius infections are found in the Middle East, Africa, and Europe, typically in regions where *E. granulosus* is also common. E. intermedius infections are also sustained in domestic cycles and are communicable to humans (114). The known geographic range of *E. intermedius* will likely expand as more ME surveillance is done. For instance, according to current data from various West African countries, the genotype G6 of *E. intermedius* is the most prevalent species due to the widespread use of camels (127) and is, therefore, the CE strain that poses the greatest risk to the local population's health. According to the authors of research on the illness in wild canids in Quebec and Maine (128), coyotes are more likely than wolves to contaminate urban green spaces and peri-urban habitats, according to the authors of research on the illness in wild canids in Quebec and Maine (128). This is true even though both wolves and coyotes have been shown to be hosts for *E. canadensis*. Overview about prevalent genotypes of *Echinococcus* species and molecular markers investigated in different countries and different markers used to investigate prevalence of *Echinococcus*

species and corresponding number of studies covered in this review are mentioned in Tables 1, 2, respectively.

Conclusion

The taxonomy of *Echinococcus* has mostly been elucidated due to molecular science. As a result, a useful and educational lexicon for use in epidemiological studies has been created. We must now have the resources available to carry out epidemiological studies. These are useful in clarifying life cycles and transmission patterns in endemic areas, as previously discussed. This will be especially important in regions with several transmission cycles and the potential for mixed infections. Molecular techniques will increasingly inform and direct public health initiatives regarding clinical treatment. Those diagnosed with echinococcosis whose *Echinococcus* species of infection is unclear or who may have infections from many *Echinococcus* species are most likely to benefit. The discovery of genotypic variation within a species and its relationship with virulence foreshadows the creation of markers for clinical use.

Author contributions

Conceptualization: MA and AA. Data curation and writing—original draft preparation: MA. Reviewing and editing: AA. Both 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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Evaluation of Bcl-2 as a marker for chronic kidney disease prediction in cats

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Chronic kidney disease (CKD) is a frequent condition in elderly cats. Bcl-2 is linked to kidney disease through the processes of apoptosis and fibrosis. The purpose of this study is to examine Bcl-2 levels in CKD and clinically healthy age-matched cats in order to evaluate the relationship between Bcl-2 levels, signalment, and blood parameters in cats with CKD. The circulating levels of Bcl-2 were determined using an immunoassay in twenty-four CKD cats and eleven clinically healthy age-matched cats by the utilization of the general linear model (GLM), Pearson correlation, principal component analysis (PCA), ROC curves, the Cox hazard model, and Kaplan–Meier survival analysis. These were all conducted in order to explore Bcl-2 levels and their connection with other variables. The Bcl-2 immunohistochemical intensity was graded in each glomerulus and tubulointerstitium. McNemar's test was performed in order to compare the expression of Bcl-2 in the two renal tissue sites. The circulating Bcl-2 of CKD cats was significantly lower than those of clinically healthy age-matched cats ($P = 0.034$). The presence of circulating Bcl-2 ($P < 0.01$) and the severity of CKD ($P = 0.02$) were both linked with the survival time of cats with CKD. The area under the curve (AUC) of Bcl-2 for detection of CKD was 0.723. In cats, decreased circulating Bcl-2 was associated with increased blood BUN, creatinine levels, and CKD severity. Bcl-2 protein expression was reduced in the renal tissues of CKD cats as the disease progressed, resulting in a decrease in their survival time. This study demonstrated that Bcl-2 may be effective in diagnosing feline CKD.

KEYWORDS

Bcl-2, biomarker, cat, chronic kidney disease, prediction

Introduction

Chronic kidney disease (CKD) is a common disease in older cats. CKD can affect cats of various ages and breeds (1). The prevalence of feline CKD was 1.9% in the United States (2), 20% in Australia (3), as well as 0.63 and 2.37% in Bangkok and Chiang Mai, Thailand, respectively (4, 5). Feline CKD operates in four stages based on serum

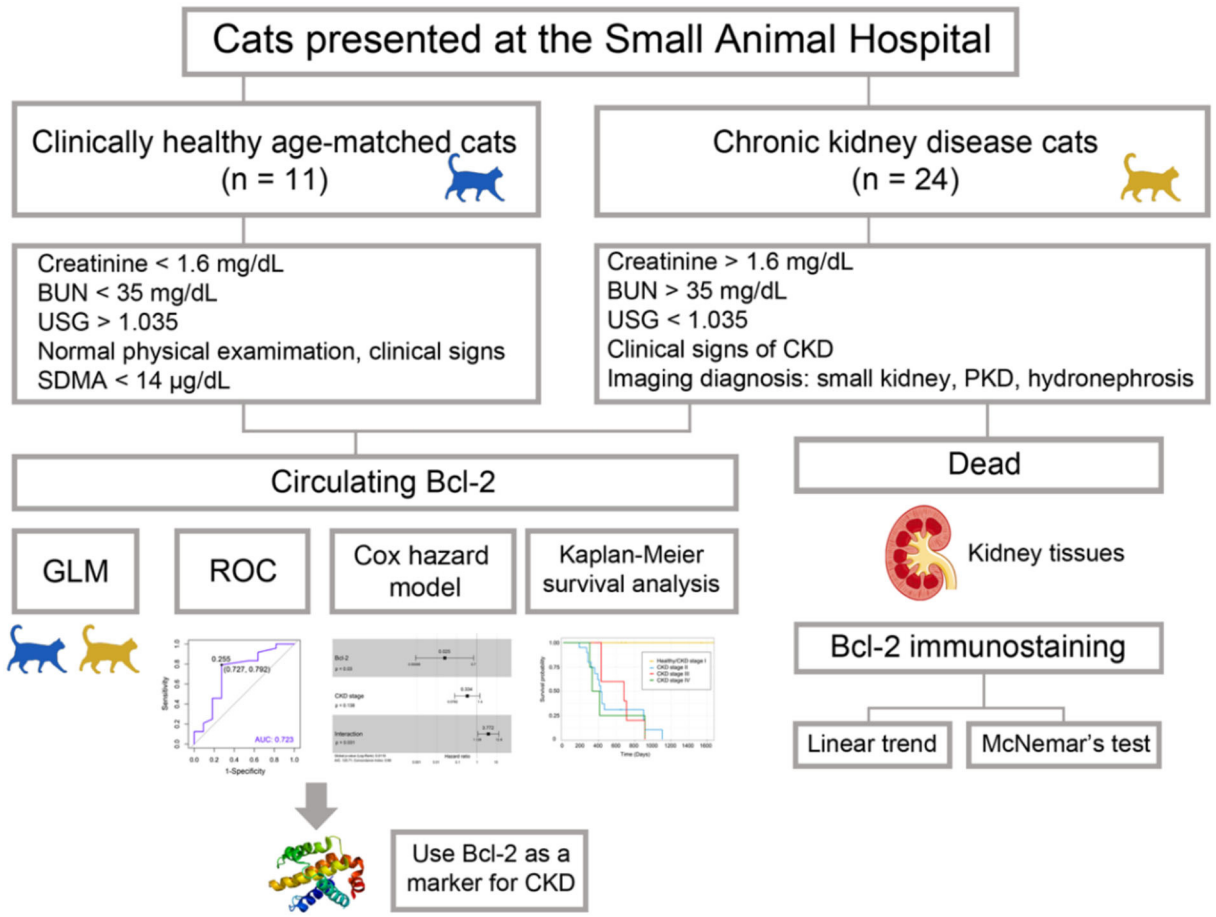

FIGURE 1
The workflow of the experiment. The goal of Bcl-2 studies is to determine the efficacy of Bcl-2 as a biomarker in feline chronic renal disease.

creatinine and symmetric dimethylarginine (SDMA) (6). Additionally, sub-staging is possible due to proteinuria and hypertension.

The glomerular filtration rate (GFR) is used to assess renal function. Inulin clearance is the gold standard for the purposes of measuring GFR. However, this method is impractical and time-consuming. Although serum creatinine remains a criterion for determining GFR, it is normal until GRF is reduced by nearly 75%. Moreover, it can be influenced by non-renal factors, such as muscle mass.

The Bcl-2 family of proteins plays a critical role in regulating the apoptosis process. The Bcl-2 family proteins are regulated by the intrinsic pathway of apoptosis, which can be activated by DNA damage, oxidative stress, radiation, oncogene activation, and nutrient deprivation. There are three classes of Bcl-2 family proteins—anti-apoptosis, pro-apoptosis, and BH3-only proteins—that bind and regulate the anti-apoptotic in order to promote apoptosis (7). Bcl-2 is an anti-apoptotic gene that has an inverse correlation with Bax, as shown in the previous study of unilateral ureteral obstruction (UUO) subjects, whereby it was shown that Bcl-2 levels will decrease during UUO (8, 9). Bcl-2 levels of patients with end-stage renal disease, who were maintained on hemodialysis, underwent decreased expression when compared to the CKD group (10). A lack of Bcl-2 will increase the expression of Bax, which promotes the release of cytochrome C and mitochondrial outer membrane fragmentation. Further, the expression of Bax will also lead to caspase-3 activation and cell death (7, 8). In another study of humans, it was found that a deposition of myofibroblasts at the interstitial area after cell death can induce irreversible kidney damage. Moreover, kidney fibrosis is known to also result in chronic kidney disease (11).

However, there have been a few studies that have investigated Bcl-2 levels in cats with CKD. Thus, this study aimed to compare the Bcl-2 levels between CKD and clinically healthy age-matched cats. This was performed in order to determine the correlation among the Bcl-2 levels, signalment, and blood parameters of cats with CKD, as well as to determine the Bcl-2 level in the kidney

tissues of CKD cats—in both quantitative and qualitative aspects *via* immunohistochemistry techniques.

Materials and methods

Animals and sample collection

Twenty-four CKD and 11 clinically normal age-matched cats were included. The CKD cats were presented at the Small Animal Hospital, Faculty of Veterinary Medicine, Chiang Mai University. There were no restrictions on gender or breed for these CKD cats. However, the age must be ≥5 years old with serum creatinine levels >1.6 mg/dl; a urine specific gravity at <1.035; and blood urea nitrogen levels >35 mg/dl, or with abnormal kidney structure that is revealed by ultrasound, such as in the small kidney, polycystic kidney, and hydronephrosis. Moreover, CKD cats must have azotemia or abnormal kidney structure for more than 3 months before being included in the study. They were also excluded if they had hyperthyroidism, other thyroid-related problems, or if they were fed a phosphorous-restricted diet. In addition, 11 clinically normal age-matched cats were examined and none were found to have any signs of CKD. Furthermore, individuals must pass a routine physical examination and additional blood chemistry tests in order to be eligible. All the clinically normal age-matched cats had serum SDMA levels <14 μg/dL, which was within the reference interval. In addition, the clinically normal age-matched cats were randomly selected without regard to gender or breed. The exclusion criteria of CKD and healthy cats were set as those cats that possessed other systemic diseases and/or retrovirus infections. The following ethics committee approved all cats for animal experimentation: the Faculty of Veterinary Medicine, Chiang Mai University No. S25/2563.

Blood and urine samples were collected from both groups. Blood samples were collected from the cephalic or saphenous vein. They were stored in EDTA tubes (0.5 ml) and heparin tubes (1 ml) for the purposes of analyzing complete blood count and blood chemistry. One ml of each blood sample was collected in VACUETTE® serum tubes (Greiner Bio-One, Kremsmünster, Austria) in order to obtain serum for the purposes of determining the circulating Bcl-2. Serum samples were stored at −20°C for further analysis. The cystocentesis procedure was used to obtain urine samples for urinalysis analysis. Seventeen kidney tissues were obtained from cats that died during the study from CKD at the Small Animal Hospital, Faculty of Veterinary Medicine, Chiang Mai University. These kidney tissues were analyzed for the purposes of evaluating the

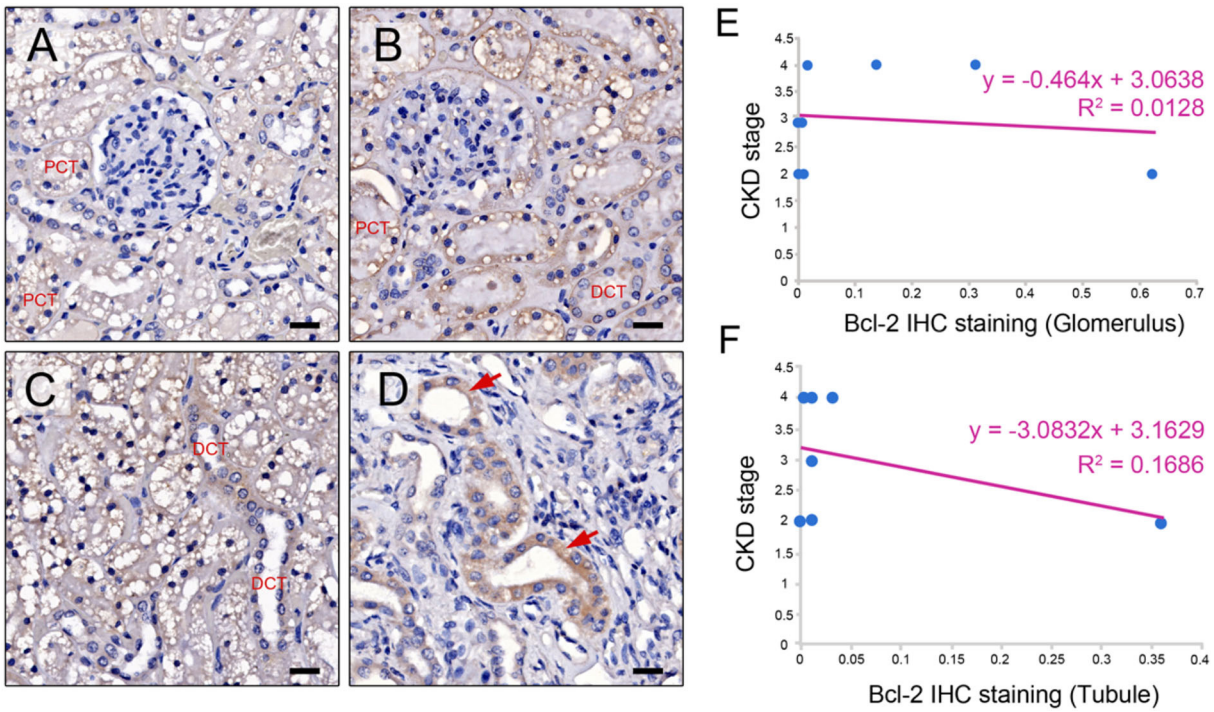

FIGURE 2
Bcl-2 protein expression. Representative IHC images illustrating the range of staining intensity for Bcl-2 in the Bowman's capsule, the proximal convoluted tubule (PCT), distal convoluted tubule (DCT) **(A, B)**, the distal convoluted tubule (DCT) **(C)**, and the collecting duct in the cats with CKD (red arrows) **(D)**. Original magnification 400×. Scale bar = 20 μm. CKD stage and Bcl-2 staining are correlated at the glomerulus and **(E)** and tubule **(F)**.

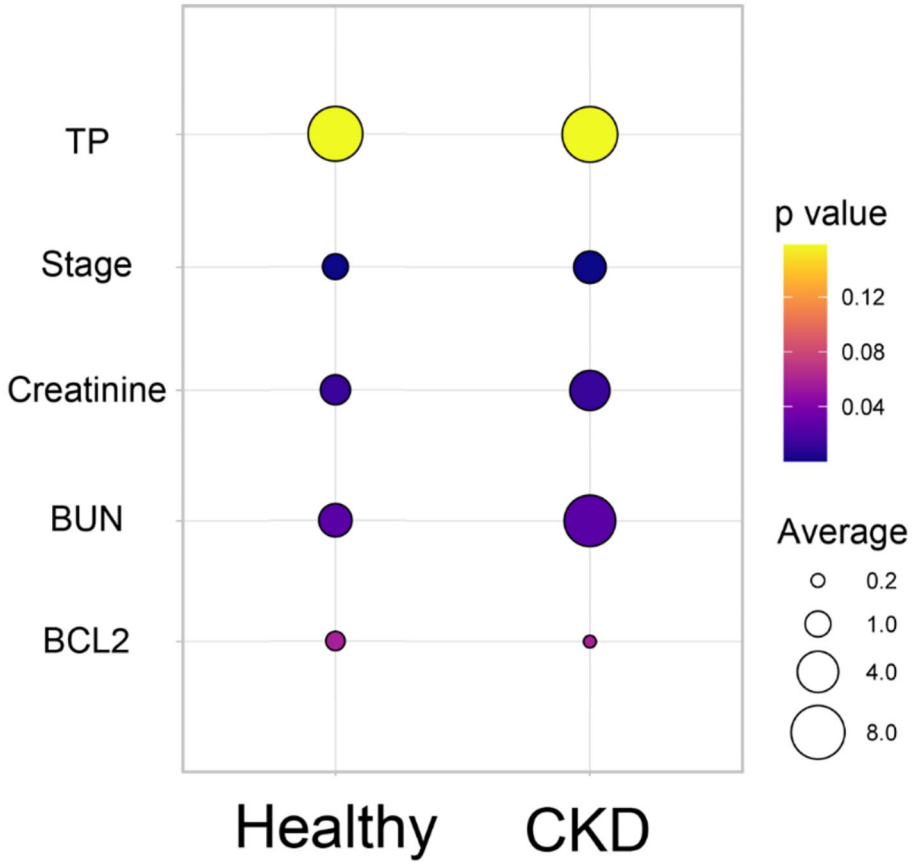

FIGURE 3
Bubble plot of the comparison of the average blood parameters between the clinically healthy age-matched and CKD cats. The average of each blood parameter is shown in different circle sizes. The *p*-value of the different averages of each group is shown in the different colors.

TABLE 1 Mean ± standard deviations (SDs) of other parameters and relative Bcl-2 abundance between clinically normal age-matched and chronic kidney disease cats.

Parameter	Unit	Clinically normal cats ($n = 11$)	CKD cats ($n = 24$)	*P*-value
Age	years	8.85 ± 2.26	9.76 ± 3.86	0.412
Body weight	kg	4.60 ± 1.03	4.46 ± 1.24	0.754
Systolic blood pressure	mmHg	146.67 ± 5.77	161.78 ± 27.57	0.155
BUN	mg/dL	21.21 ± 2.73	71.72 ± 70.05**	**0.002**
Creatinine	mg/dL	1.51 ± 0.09	3.68 ± 2.58**	**<0.001**
Albumin	mg/dL	3.34 ± 0.22	3.23 ± 0.32	0.236
Phosphorous	mg/dL	3.38 ± 1.02	5.30 ± 4.01	0.067
Hematocrit	%	42.44 ± 3.91	32.41 ± 7.11**	**<0.001**
Relative Bcl-2 abundance		0.43 ± 0.16	0.19 ± 0.02*	**0.034**

CKD, Chronic Kidney Disease; BUN, blood urea nitrogen.
**$P < 0.01$; *$P < 0.05$.
The bold values refer to the parameters with a *P*-value less than 0.05.

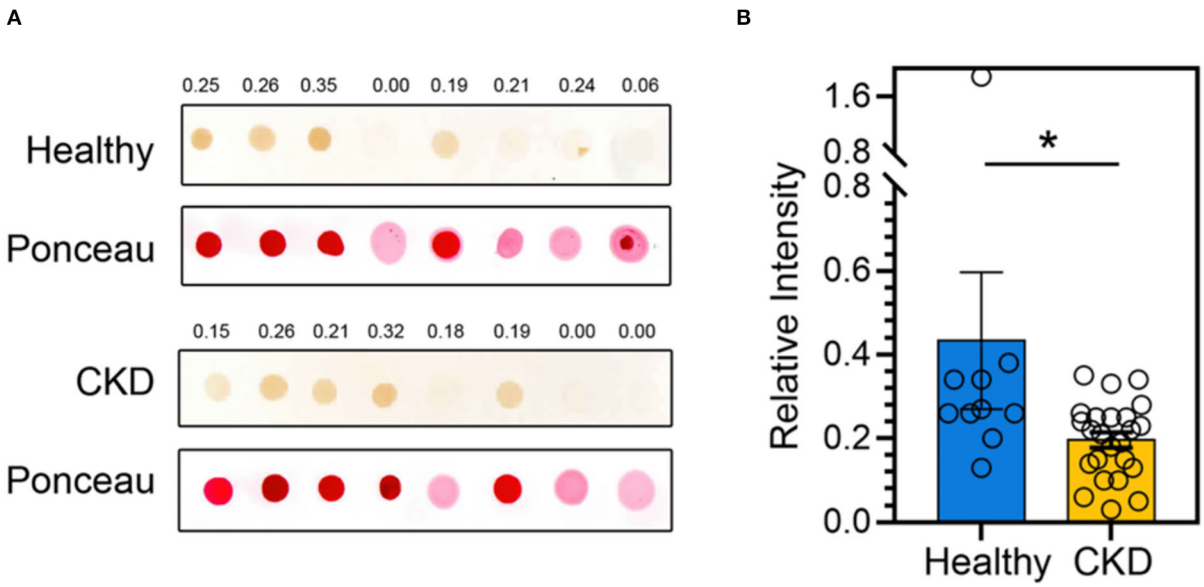

FIGURE 4
(A) A representative dot blot of the Bcl-2 abundance of clinically normal age-matched (upper panel) and CKD cats (lower panel). Ponceau S staining is used in order to control the amount of protein loaded onto membranes. Relative Bcl-2 intensities are indicated at the top of each panel. **(B)** The bar graph depicts the mean and the standard error of the mean (SEM) of relative Bcl-2 dot intensity of clinically normal age-matched ($n = 11$) and CKD cats ($n = 24$). $*P < 0.05$. Statistical significance was determined using a generalized linear model (GLM).

Bcl-2 immunostaining intensity. The conceptual framework of our investigation is shown schematically in Figure 1.

Dot blot analysis

Cat sera were diluted with PBS (1:1) and incubated at 95°C for 5 min. The PVDF membrane was soaked using methanol and placed on soaked Whatman filter paper. Three µl of diluted serum was dropped onto the PVDF (Bio-Rad, Hercules, CA, USA) and dried. Then, it was blocked with a blocking buffer for 30 min at room temperature. After that, the PVDF was incubated with a primary mouse monoclonal anti-Bcl-2 antibody (BioLegend, San Diego, CA, USA) (1:1,000 dilution) within a blocking buffer at room temperature for 2 h. It was then incubated with HRP secondary goat anti-mouse IgG (BioLegend) (1:2,000 dilution) in a blocking buffer for 45 min at room temperature. Finally, the membrane was stained with a DAB substrate and dried at room temperature. Another PVDF membrane was prepared for the purposes of determining the total protein in each serum. This PVDF has been stained with Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) staining for 10 min at room temperature. The Image Studio Lite™ program (LI-COR, Lincoln, NE, USA) was used for the purposes of quantifying the densitometry of proteins, which were in the ratio of Bcl-2/Ponceau S (12, 13). Each typical dot blot was converted to grayscale before protein densitometry was quantified.

Immunohistochemistry

Kidney tissues were obtained from the autopsy unit in the Faculty of Veterinary Medicine, Chiang Mai University. Each slide was deparaffinated *via* xylene for 10 min. Then, it was rehydrated by absolute ethanol, 90% ethanol, 70% ethanol, and 50% ethanol for 5 min/each step. Next, each slide was warmed with a citrate buffer for the purposes of antigen retrieval. The slices were arranged in a container with citric buffer at a pH of 6.0 and incubated in the microwave, 700 w for 20 min. After that, the slides were blocked with endogenous peroxidase in 3% hydrogen peroxide for 5 min and washed with PBST three times for 5 min each. The 200 µl of 2.5% bovine serum albumin was added onto each slide and incubated for 5 min. The primary antibody was applied *via* dropping 200 µl of a primary antibody that was diluted with 1% PBS to the sections of the slides and then incubating at 37 °C for 2 h. The slide was incubated with a primary mouse monoclonal anti-Bcl-2 antibody (BioLegend) (1:200 dilution) in a blocking buffer at 37 °C for 2 h and then all slides were washed with PBS, three times for 5 min. The 200 µl of normal goat serum (1:5) was added at room temperature for 30 min and washed with PBS, three times for 5 min. Then, 200 µl of HRP secondary goat anti-mouse IgG (BioLegend) (1:200 dilution) in a blocking buffer was added for 45 min at room temperature and then washed with PBS, three times for 5 min. DAB substrate was used for the purposes of staining for 5 min, as well as terminating the reaction by rinsing with

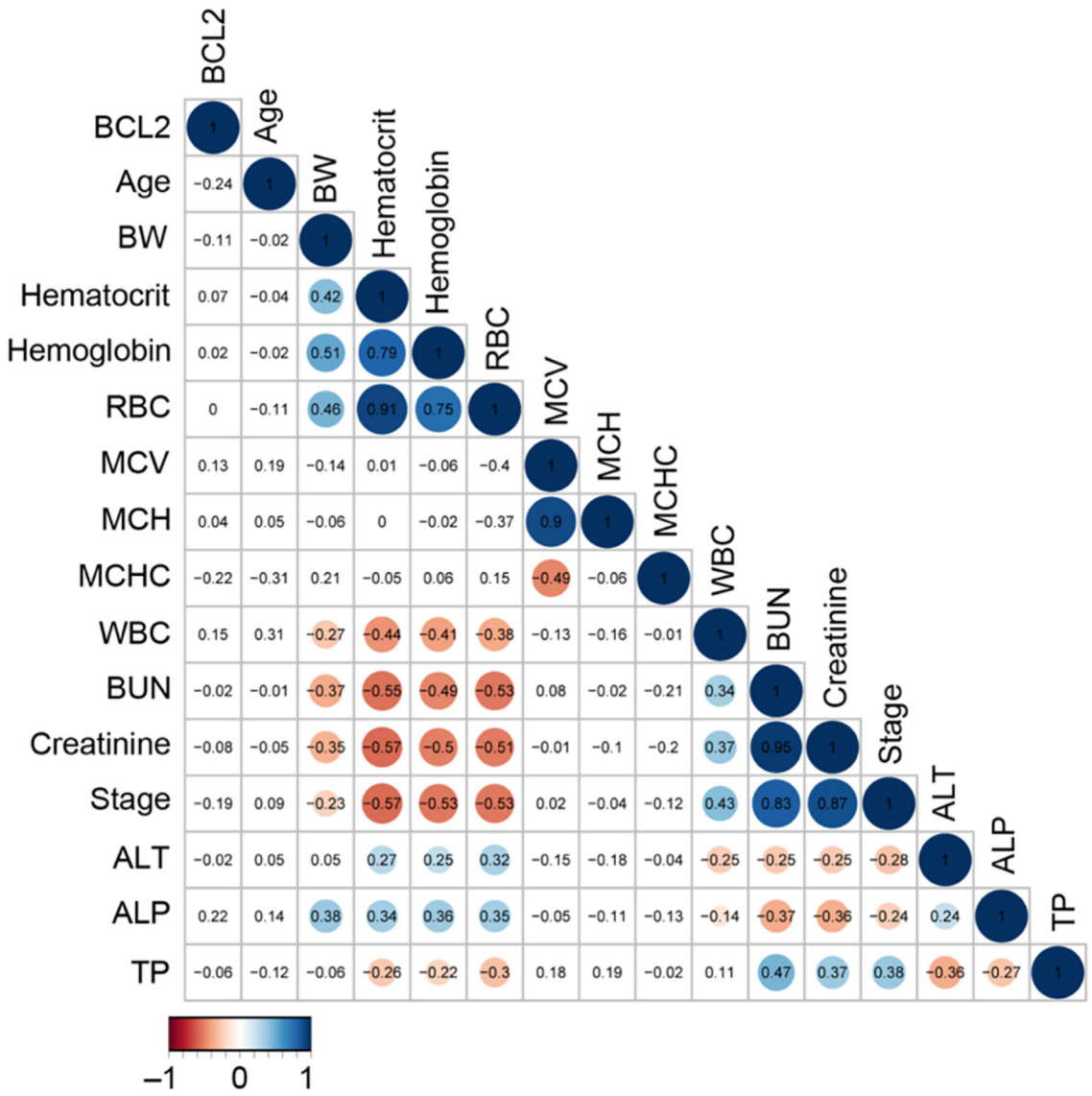

FIGURE 5
The correlation analysis of circulating Bcl-2 with other blood parameters. The correlation matrix's colored circles represent the correlation coefficients for each pair of Bcl-2 in the relevant cell-matrix.

running tap water. The slides were stained with hematoxylin for 10 s, lithium for 10 s, and then washed with distilled water. Finally, the process of mounting and covering the slides was performed. Negative control was conducted *via* replacing the primary antibody with the normal mouse serum (1:200). The immunostaining was visualized and scanned with a Pannoramic MIDI Slide Scanner (3D HISTECH, Budapest, Hungary). The scoring basis was semi-quantitative and was scored from 0 to 2 (14), as follows: grade 0 is no staining; grade 0.5 is trace staining; grade 1 is weak staining; and grade 2 is strong staining. The following equation was utilized in order to determine the score:

$$\text{Intensity score} = [(0.5\times N_{0.5}) + (1\times N_1) + (2\times N_2)]/N$$

Statistical analyses

The circulating Bcl-2 levels between CKD and clinically normal age-matched cats were compared using the general linear model (GLM). The correlation among the circulating

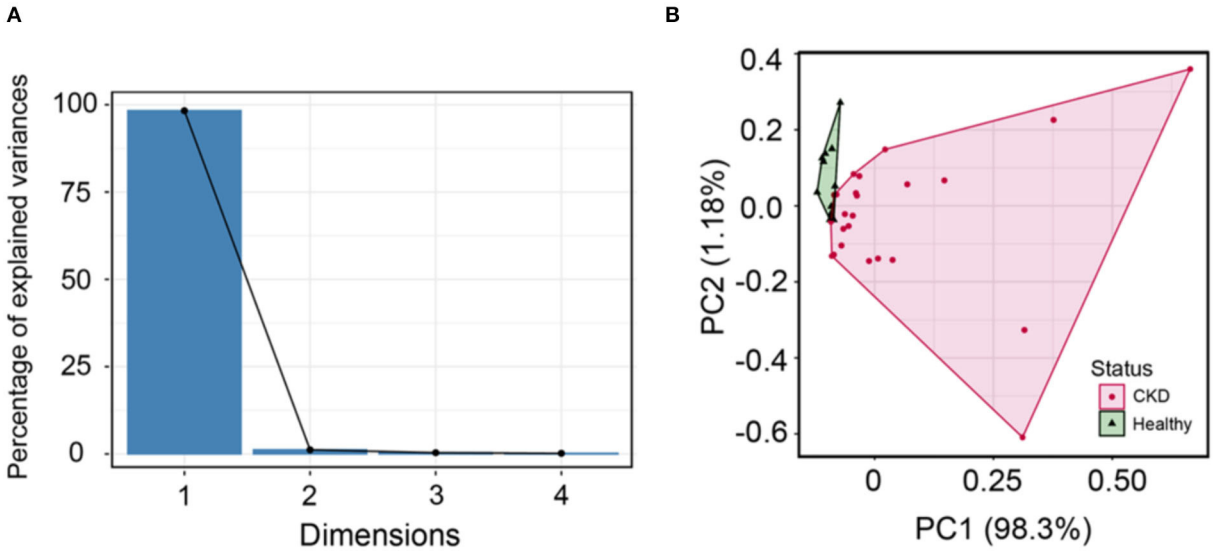

FIGURE 6
(A) The scree plot shows dimensions and percentage of variance, as explained by each dimension (PC). **(B)** Principal component analysis can distinguish between normal age-matched cats (green) and CKD (pink) detected when using cluster analysis.

Bcl-2, signalment, and blood parameters was determined using Pearson's correlation method. The association between the survival time of the CKD cats and the potential variables was investigated using the Cox hazard model and Kaplan–Meier survival analysis. The area under the curve of Bcl-2 for the purposes of detecting CKD was achieved through using the receiver operating characteristic. In regard to statistical testing and graphical presentations, the following R packages were used: cluster, corrplot, factoextra, ggfortify, ggplot2, pROC, RColorBrewer, survival, survminer, and viridis. In order to identify factors linked with the progression of CKD, we employed a Cox proportional hazard model. Moreover, a backward-stepwise Cox regression analysis was performed on all variables with a $p < 0.20$. The linear trend estimates the intercepts and slopes of mean trends for the CKD stage and the Bcl-2 IHC staining. Furthermore, McNemar's test was used for comparing the Bcl-2 expression of the two locations of the renal tissues; the result with $P < 0.05$ was considered significant. The GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) or R packages (The R Foundation) were used to visualize the data acquired from statistical analysis.

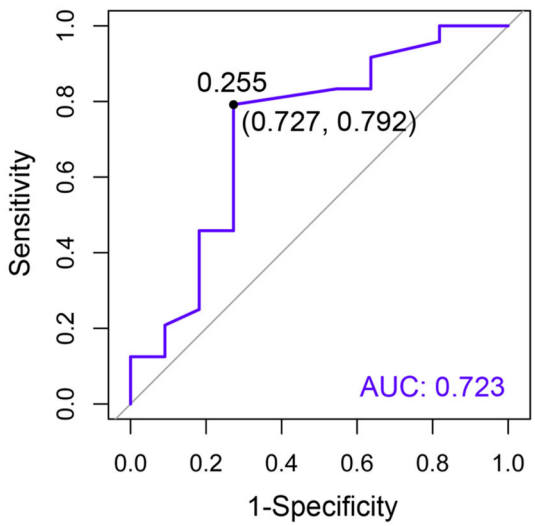

FIGURE 7
The receiver operating characteristic (ROC) curve of circulating Bcl-2 for the purposes of CKD diagnosis. The specificity (x-axis) is displayed vs. the sensitivity (y-axis). Moreover, AUC is an area under the ROC curve.

Results

The Bcl-2 immunostaining was detected within the CKD cat glomerular and tubular epithelium cells of the renal tissues ($n = 17$) (Figures 2A–D). When examined at the glomerular sites, Bcl-2 protein was found in the Bowman's capsule, the proximal convoluted tubule (PCT), and the distal convoluted tubule (DCT), as shown in Figures 2A, B. The Bcl-2 immunostaining was also identified in the distal convoluted tubule and the collecting duct at renal tubules (Figures 2C, D). There was no difference in the mean intensity of Bcl-2 staining at the glomerular (0.2 ± 0.27) and tubulointerstitium (0.19 ± 0.32).

TABLE 2 The parameters that are associated with CKD progression (hazard ratio > 1).

Parameter	Beta	HR	LowerCI	UpperCI	Wald.test	*P* value
BCL2	−1.1	0.33	0.097	1.1	3.3	0.071
Sex	0.11	1.1	0.48	2.6	0.07	0.79
Age	0.057	1.1	0.95	1.2	1.1	0.3
BW	0.062	1.1	0.77	1.5	0.14	0.71
Hct	−0.0061	0.99	0.94	1.1	0.05	0.83
BUN	0.0049	1	1	1	2.9	0.089
Creatinine	0.1	1.1	0.97	1.3	2.3	0.13
Stage	0.49	1.6	1.1	2.5	5.3	0.022
ALT	−0.029	0.97	0.94	1	2.7	0.1
ALP	0.0023	1	0.97	1	0.02	0.89
TP	0.45	1.6	0.83	3	1.9	0.17
Albumin	−0.27	0.76	0.17	3.5	0.12	0.73

ALP, Alkaline phosphatase; ALT, Alanine transaminase; BUN, blood urea nitrogen; BW, Body weight; CI, confidence interval; CKD, Chronic Kidney Disease; Hct, Hematocrit; TP, Total protein.

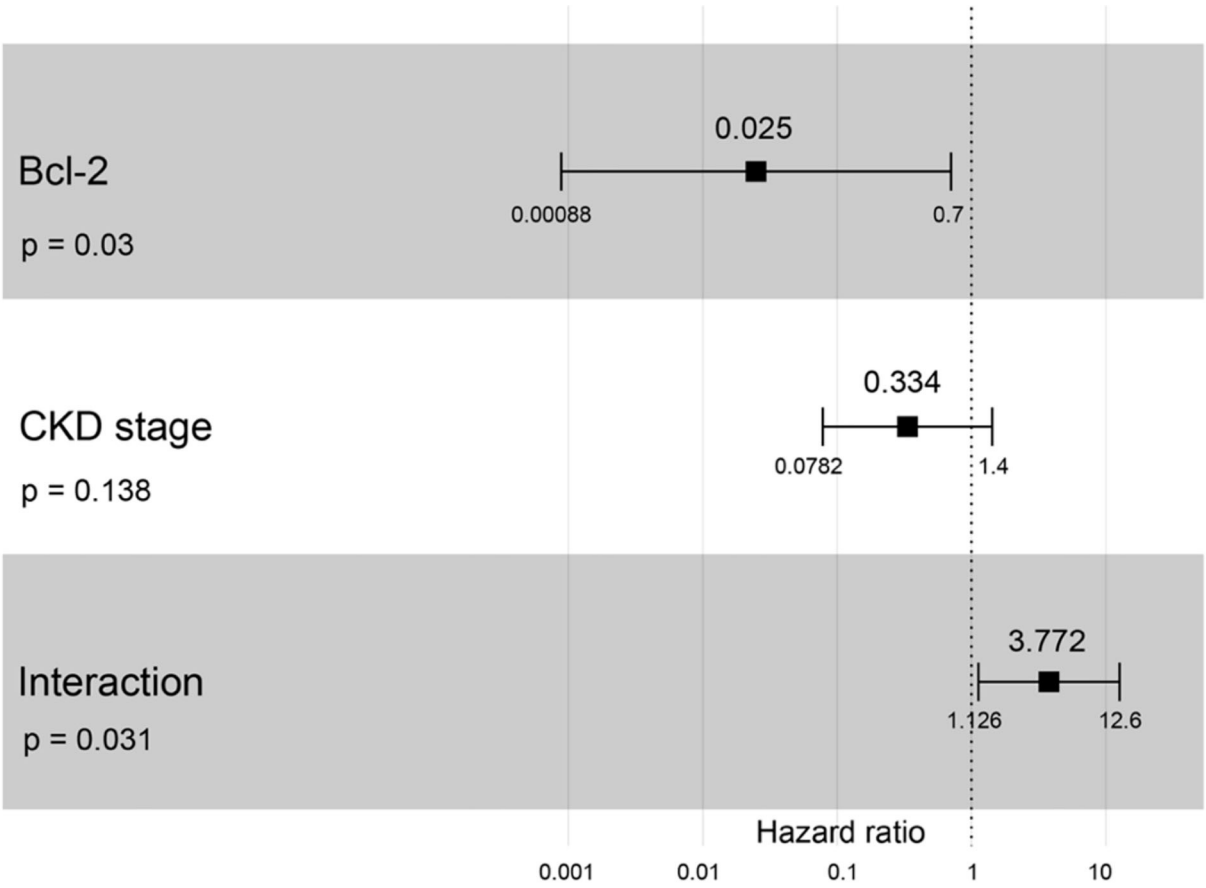

FIGURE 8
Forest plot output for the interaction of Bcl-2 with CKD stage is covariate with CKD progression based on hazard ratios (HRs) as determined *via* Cox regression analysis. Squares represent hazard ratios. Bars represent 95% confidence intervals.

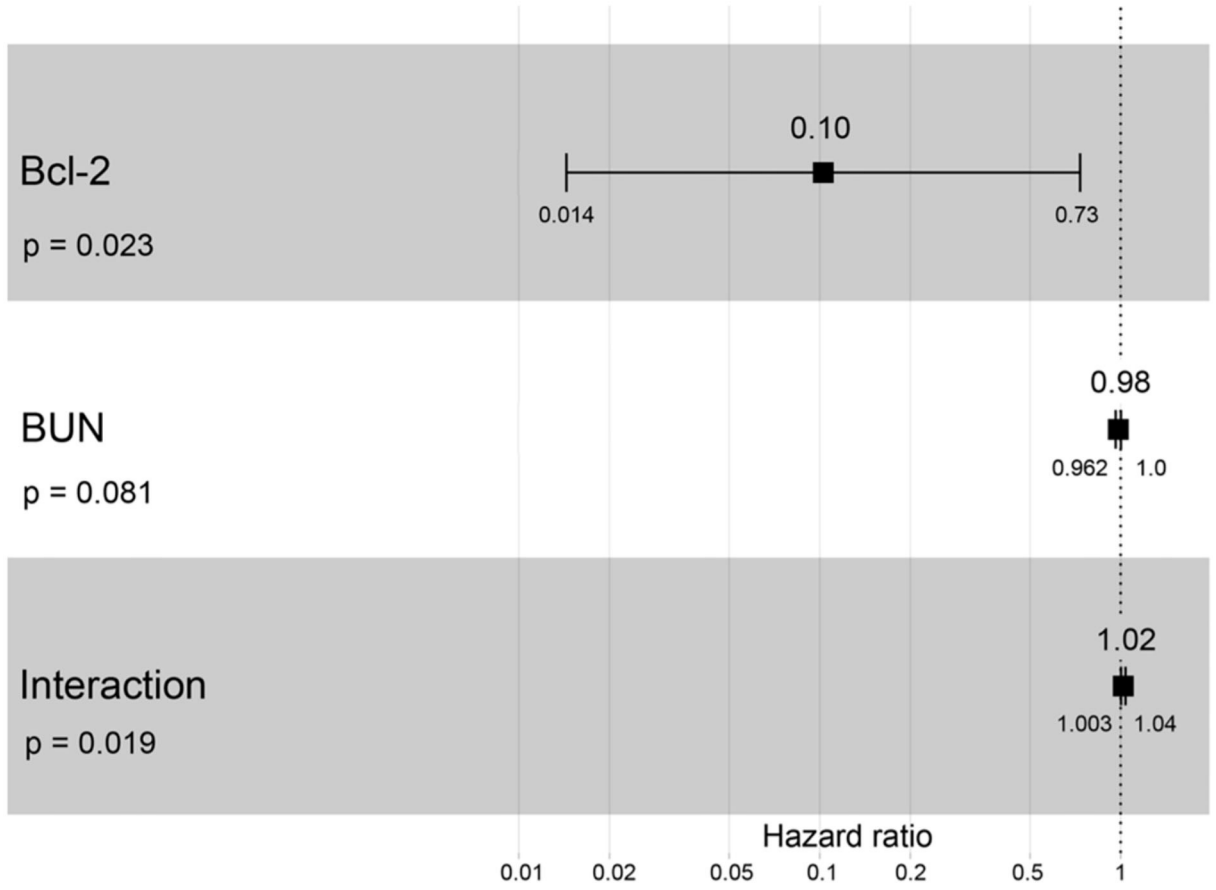

FIGURE 9
Forest plot output for the interaction of Bcl-2 with BUN that is covariate with the CKD progression based on hazard ratios (HRs) *via* Cox regression analysis. Squares represent hazard ratios. Bars represent 95% confidence intervals.

The CKD stage and Bcl-2 staining had a negative connection. Furthermore, in the advanced stage of CKD, both glomerular and tubular Bcl-2 expression was reduced (Figures 2E, F). McNemar's test revealed that the renal tissues of the CKD cats contained Bcl-2 protein staining in the same proportion at the glomerular and tubular sites. As a result, Bcl-2 labeling at both sites did not affect disease progression. Furthermore, a negative control was demonstrated by substituting the primary antibody with normal mouse serum (Supplementary Figure 1).

According to the comparison results of the average blood parameters between the clinically healthy age-matched and CKD cats, the CKD stage ($P = 0.0001$) was statistically different between the clinically normal age-matched and CKD cats, followed by creatinine ($P = 0.02$), BUN ($P = 0.02$), and Bcl-2 ($P = 0.05$) (Figure 3). The hematocrit of CKD cats was considerably lower than that of clinically normal age-matched cats ($P < 0.01$). Moreover, Bcl-2 levels in CKD cats were significantly lower than in clinically normal age-matched cats ($P = 0.034$); please refer to Table 1 and Figure 4A for more detail. In addition, Figure 4B depicts the Bcl-2 protein intensity in clinically normal age-matched and CKD cats.

The CKD stage was a positive correlation with BUN ($r = 0.83$), creatinine ($r = 0.87$), and leukocyte ($r = 0.43$), but a negative correlation with RBC ($r = -0.53$), hematocrit ($r = -0.57$), and hemoglobin ($r = -0.53$) (Figure 5). In order to analyze the principal component analysis (PCA), factors that interacted with CKD progression (such as circulating Bcl-2, blood urea nitrogen (BUN), creatinine, and kidney disease stage) were examined.

A scree plot was employed in order to determine the number of dimensions to conduct in PCA. According to the scree plot, just two components (PC1 and PC2) were utilized to correlate variation in each component (Figure 6A). Further, PCA can be conducted with these two components. The circulating Bcl-2 can distinguish between CKD and clinically normal age-matched cats using PCA (Figure 6B). Although there were no correlations between circulating Bcl-2 and other factors in

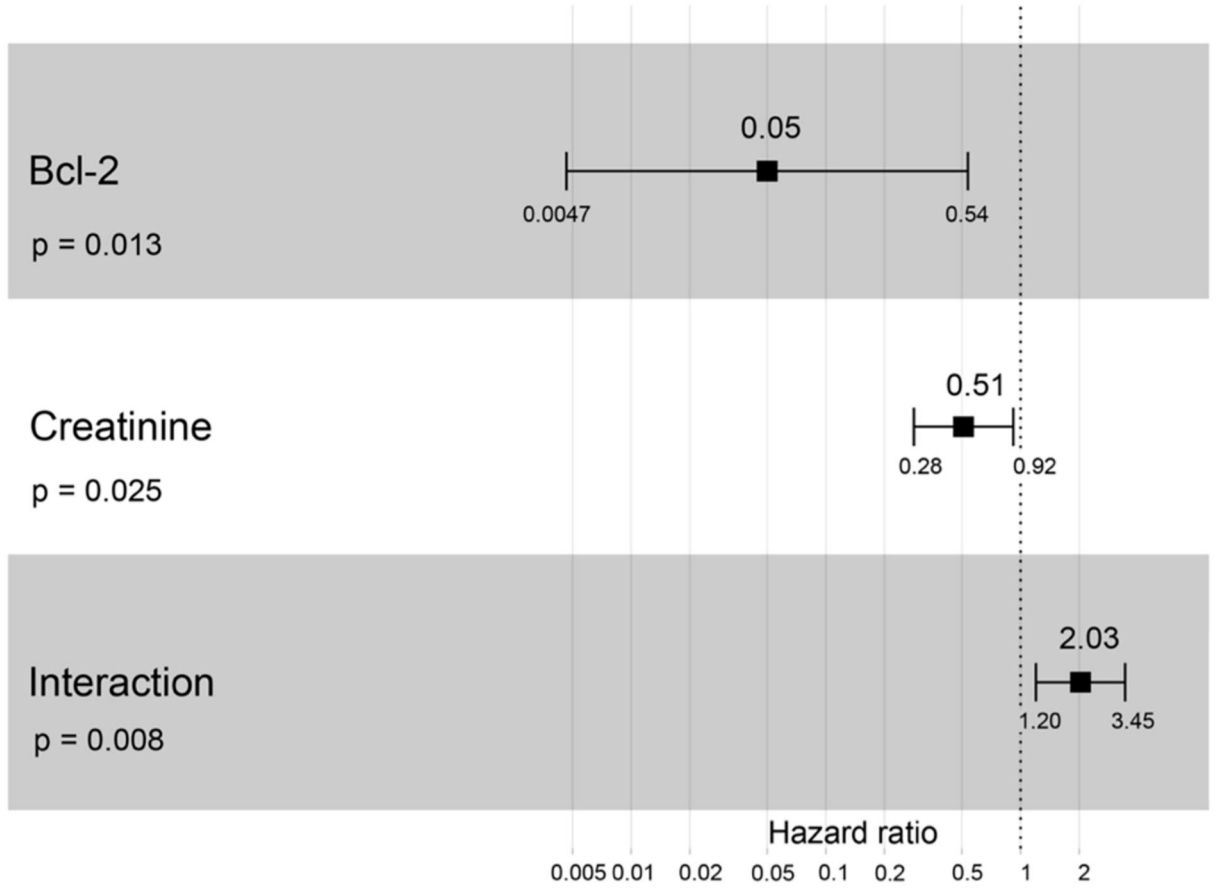

FIGURE 10
Forest plot output for the interaction of Bcl-2 with creatinine that is covariate with the CKD progression based on hazard ratios (HRs) *via* Cox regression analysis. Squares represent hazard ratios. Bars represent 95% confidence intervals.

correlation analysis, circulating Bcl-2 can distinguish between CKD and clinically normal age-matched cats *via* PCA.

The area under the curve (AUC) of circulating Bcl-2 for the detection of CKD was 0.723, indicating an acceptable predictor (Figure 7). The Youden index determined the cutoff threshold for circulating Bcl-2 in cats with CKD and clinically healthy age-matched cats in order to identify those at high risk. When the optimal value of the Youden index was 0.255, this feature was understood to possess optimal performance (sensitivity: 0.727; specificity: 0.792, Figure 7).

A hazard ratio greater than one, sex, older age, higher body weight, higher creatinine, higher CKD stage, and higher total protein resulted in an increased risk of developing CKD (Table 2). All variables were included in order to analyze the final models of the interaction between each factor and CKD progression. Figures 8–12 depict the final models of the association between Bcl-2 and other variables for CKD progression. All models indicated that circulating Bcl-2 without interaction with other factors was a risk for CKD progression (Figures 8–12). Moreover, serum creatinine without interaction with other factors in the model was at high risk for CKD progression (Figure 10). The final models of the association between Bcl-2 and CKD stage, as well as between Bcl-2 and serum creatinine had hazardous factors for CKD progression due to the fact these models had a hazard ratio of more than 1 (Figures 8, 10). However, the other final models include Bcl-2 interaction with serum BUN (Figure 9); Bcl-2 interaction with serum BUN and total protein (Figure 11); and Bcl-2 interaction with creatinine and total protein (Figure 12). All of these possessed a hazard ratio equal to 1. As such, these models were at no risk for CKD progression (Figures 9, 11, 12).

Clinically healthy age-matched cats will have the most extended lifespan (<1,600 days) from the date of diagnosis to the date of analysis. Likewise, cats with CKD stage II will live around 1,100 days, but cats with CKD stages III and IV will live to only about 900 days, $P = 0.02$ (Figure 13). Cats with CKD stage II had a higher survival time than cats with CKD stages III and IV. Moreover, clinically healthy age-matched cats

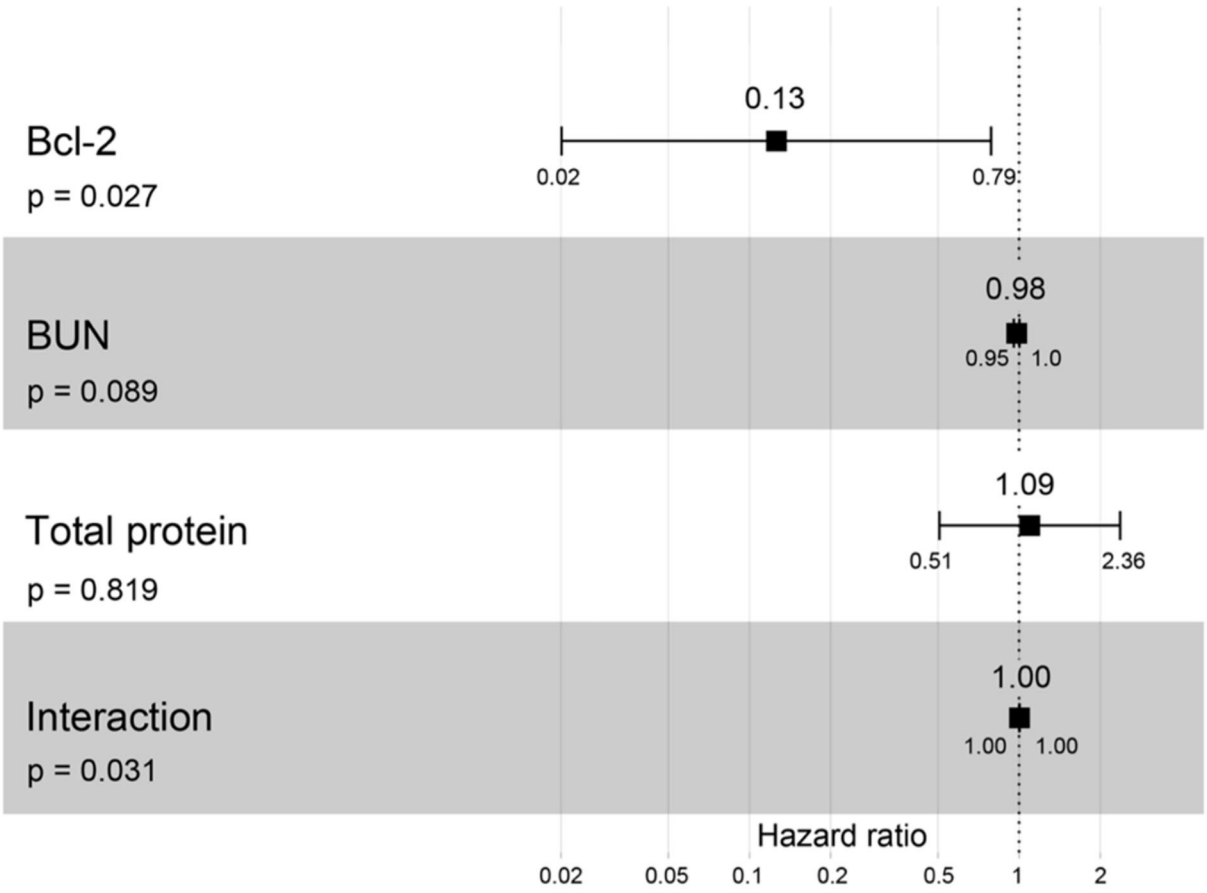

FIGURE 11
Forest plot output for the interaction of Bcl-2 with two different covariates (BUN and total protein) with CKD progression based on hazard ratios (HRs) *via* Cox regression analysis. Squares represent hazard ratios. Bars represent 95% confidence intervals.

had a significantly higher survival time than cats with CKD stages II–IV.

Discussion

The present study demonstrated that serum BUN and the creatinine concentration of CKD cats were significantly higher than those found in clinically normal age-matched cats. Other studies have also found that CKD cats increased BUN and creatinine (5, 15, 16). The hematocrit of CKD cats was considerably lower than that of clinically healthy age-matched cats, including the CKD cats that had a reduced blood hematocrit owing to erythropoietin deficiency (17, 18). In this study, the CKD stage correlated with leukocytosis. In addition, increased WBC levels in the Chinese population with normal kidney function can predict future declines in renal function (19). Moreover, the CKD stage possessed a negative correlation with body weight. Piyarungsri et al. (5) also demonstrated that most CKD cats are affected by weight loss and anorexia.

In addition, the CKD stage was negatively correlated with anemia. Erythropoietin deficiency in kidney insufficiency may explain this result (17, 20). In this study, the survival time of cats with the higher stage of CKD was lower than in the early stage. In agreement with the previous research, CKD cats with stage IIb possessed a higher survival time period than cats with stage III and stage IV CKD (21). Increased serum creatinine and BUN, proteinuria, and decreased PCV were all associated with influencing survival time in cats with CKD (22). As such, these results confirmed consolidated knowledge related to CKD.

The present study indicated Bcl-2 was weakly expressed in Bowman's capsule, proximal and distal convoluted tubule, and the collecting duct epithelium cells. The previous studies also reported similar results in other species (8, 9, 23–26). UUO studies demonstrated Bcl-2 expressed in the tubular cells of rats (8) and mice (9). The Bcl-2 was stained in proximal tubules, distal tubules, and interstitial cells in glomerulonephritis patients (23). Bcl-2 expression was strong in mildly dilated tubules and weak in severely dilated tubules of rats with ureteral

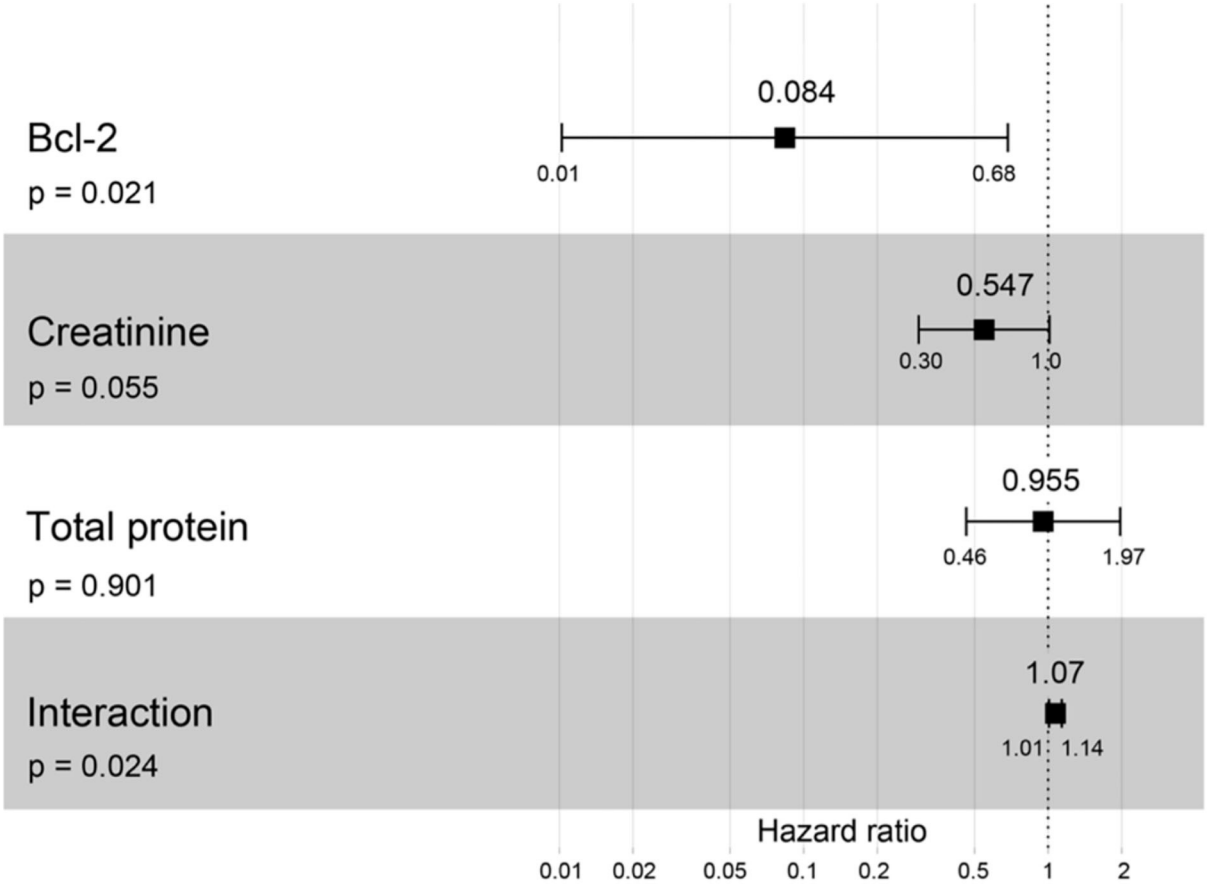

FIGURE 12
Forest plot output for the interaction of Bcl-2 with two different covariates (creatinine and total protein) with CKD progression based on hazard ratios (HRs) *via* Cox regression analysis. Squares represent hazard ratios. Bars represent 95% confidence intervals.

obstruction (24). Bcl-2 was increased in regard to staining within distal tubules but decreased in the proximal tubules of rats with acute ischemic renal failure (25). Moreover, the Bcl-2 expression had significantly decreased in the renal tissue of diabetic nephropathy patients (26).

The current study investigated whether the circulating Bcl-2 in the CKD cats was significantly lower than those found in clinically normal age-matched cats. In agreement with previous studies, decreased Bcl-2 was also detected in other species with kidney problems (8–10, 27). According to Zhang et al. (8), Bcl-2 immunostaining in the kidney tissues of rats with UUO was less detectable than in the control group. Gene expression levels of Bcl-2 significantly decreased in the renal tissue of monosodium glutamate (MSG)-induced rats compared to the control group (27). Consistent with a recent mouse study, Bcl-2 mRNA was substantially expressed in the UUO3 group but decreased in the UUO7 and UUO14 groups (9). Additionally, a prior investigation on human patients found that Bcl-2 levels were lower in patients with end-stage renal disease patients than in individuals with chronic kidney disease (10).

The Bcl-2 protein was one of the self-protection mechanisms induced by damaged cells (8). Another study reported that Bcl-2 preserve the mitochondrial membrane and binds to Bax in order to inhibit apoptosis in leukemias (28). In addition, wounded cells express both anti- and pro-apoptotic proteins, but injured cells will survive if an increase in Bcl-2 is achieved (9). The results of the present study substantiated Bcl2's anti-apoptotic involvement in chronic renal disease in cats. The involvement could be clarified by previous studies (27, 29, 30). The down-regulation of Bcl-2 plays a role in renal cell death (27). Decreased Bcl-2 may enhance ischemia/reperfusion-induced oxidant stress, as well as tubular apoptosis (29). Renal tubular apoptosis, by the decreasing in Bcl-2, was associated with tubular atrophy and chronic renal fibrosis (30). Thus, Bcl-2 may be related to kidney disease through apoptosis.

Although the current study indicated that circulating Bcl-2 did not relate to BUN and creatinine, lower circulating

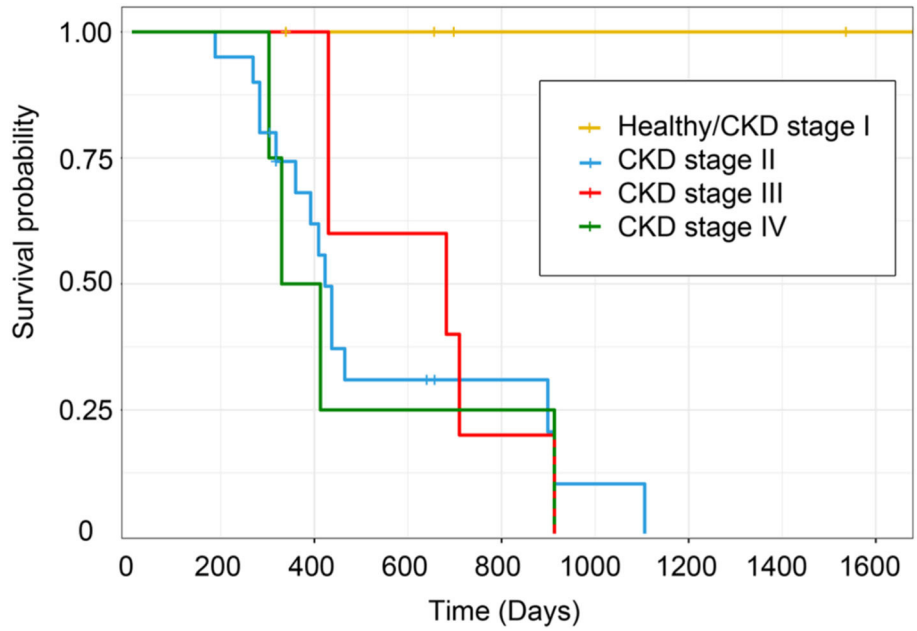

FIGURE 13
Kaplan–Meier survival estimates by CKD stage. The survival curve of each CKD stage shows the survival time based on the staging of CKD.

Bcl-2 with higher BUN, creatinine, and CKD severity was associated with CKD progression. The present study is a limited cohort size that may affect no correlation between circulating Bcl-2 and other parameters. However, more sample sizes in the previous studies reported the same trend as the present study. Forty patients with renal diseases possessed lower Bcl-2 gene expression in their blood samples than in 20 healthy individuals (10). As previously reported, there was no correlation between Bcl-2 positive cells and BUN or creatinine levels in glomerulonephritis patients (31). However, Bcl-2 was related to hypertension and glomerular hypertrophy, which could develop CKD (32). Further study is needed in order to include more clinically healthy cats and CKD cats.

Several studies also reported the AUC of Bcl-2 in other chronic diseases (33–35). Bcl-2 was a significant predictor in epithelial ovarian cancer patients (0.838) (33) and breast cancer patients (AUC = 0.841) (34). Moreover, the Bcl-2/Bax ratio may be a differentiator for human osteoarthritis (AUC = 0.673) (35). This study indicated that the AUC of Bcl-2 for predicting cats with CKD was 0.723, which is a reasonable discrimination ability (36). Thus, it could be evaluated that Bcl-2 has a prediction ability for CKD in cats.

Conclusions

In conclusion, Bcl-2 levels in CKD cats were considerably lower than those in clinically normal age-matched cats. The AUC analysis of Bcl-2 demonstrated that it adequately predicted CKD in cats. Moreover, in cats, decreased Bcl-2 expression was associated with increased BUN, creatinine, and CKD severity. Unfortunately, the immunostaining intensity of Bcl-2 was weak in the kidney tissues of cats with CKD. This study revealed that Bcl-2 could help with distinguishing between cats with chronic kidney disease and healthy cats. However, the present study is a small sample size, is not fully validated, and has no longitudinal follow-up study. Further study is required in order to include a more substantial sample size, to investigate the validation of these results, to follow up the longitudinal results, to perform on Bcl-2 immunostaining in kidney tissues from cats that died from other diseases, and to better determine the relationship between Bcl-2 levels in kidney tissue and Bcl-2 levels in the blood of live cats.

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 Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai,

Thailand (reference No. S25/2563, date of approval: 17 July 2020). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

KPi and PC designed the study, were involved in the supervision, and edited the manuscript. KPi and PPi recruited cases and organized the sample collection. PC and PPa performed the statistical analysis. KPr provided helpful suggestions and performed the immunohistochemistry. PPi, KPi, and PC performed the assays. PPi wrote the first draft of the manuscript. KPi was involved in funding acquisition. All authors contributed to the article and approved 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.

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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.2022.1043848/full#supplementary-material

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Complete genome analysis of *Bacillus subtilis* derived from yaks and its probiotic characteristics

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Probiotics have attracted attention due to their multiple health benefits to the host. Yaks inhabiting the Tibetan plateau exhibit excellent disease resistance and tolerance, which may be associated with their inner probiotics. Currently, research on probiotics mainly focuses on their positive effects on the host, but information regarding their genome remains unclear. To reveal the potential functional genes of *Bacillus subtilis* isolated from yaks, we sequenced its whole genome. Results indicated that the genomic length of *Bacillus subtilis* was 866,044,638 bp, with 4,429 coding genes. The genome of this bacteria was composed of one chromosome and one plasmid with lengths of 4,214,774 and 54,527 bp, respectively. Moreover, *Bacillus subtilis* contained 86 tRNAs, 27 rRNAs (9 16S_rRNA, 9 23S_rRNA, and 9 5S_rRNA), and 114 other ncRNA. KEGG annotation indicated that most genes in *Bacillus subtilis* were associated with biosynthesis of amino acids, carbon metabolism, purine metabolism, pyrimidine metabolism, and ABC transporters. GO annotation demonstrated that most genes in *Bacillus subtilis* were related to nucleic acid binding transcription factor activity, transporter activity, antioxidant activity, and biological adhesion. EggNOG uncovered that most genes in *Bacillus subtilis* were related to energy production and conversion, amino acid transport and metabolism, carbohydrate transport and metabolism. CAZy annotation found glycoside hydrolases (33.65%), glycosyl transferases (22.11%), polysaccharide lyases (3.84%), carbohydrate esterases (14.42%), auxiliary activities (3.36%), and carbohydrate-binding modules (22.59%). In conclusion, this study investigated the genome and genetic properties of *Bacillus subtilis* derived from yaks, which contributed to understanding the potential prebiotic mechanism of probiotics from the genetic perspective.

KEYWORDS

yak, probiotics, *Bacillus subtilis*, genome, KEGG

Introduction

Yaks (*Bos grunniens*) are an ancient breed of the high-elevation mionectic environment of Tibetan plateau (3,000 above sea level) characterized by cold-resistance and strong anti-adversity (1–3). Early investigations showed that yaks have settled in the Qinghai-Tibet Plateau for thousands of years, which exhibited excellent adaptability to the harsh local environment (3, 4). Statistically, the population of yaks in China has reached 16 million, accounting for more than 90% of the world's total population (5, 6). Yaks are the important source of milk, meat and even vehicle for local herders, which plays an important roles in the agricultural and economic development of the Qinghai-Tibet Plateau (7, 8). Therefore, any disease appeared in the yaks may cause huge economical losses to the herdsmen on the cold plateau. However, the yaks especially in juvenile populations prone to gastrointestinal diseases because of the nutritional deficiency and harsh environment (9, 10). Increasing evidence indicated that environmental factors such as hypothermia and hypoxia could affect the evolution of species (11, 12). For instance, the gut microbiota was significantly different between human inhabiting Tibet Plateau and inland plain due to differences in living circumstances (12). Moreover, Xin et al. also revealed significant differences in the gut microbiota between yaks and other breeds of cattle (2). Consequently, yaks have evolved a unique gut microbial community structure with the environment.

Gut microbiota is a complicated and dynamic microecosystem that consists of ∼100 trillion microorganisms involving over 2,000 diverse species (13–15). Notably, some strains in the gut microbiota such as *Bacillus subtilis*, *Bacillus licheniformis*, *Pediococcus lactis*, etc. are recognized as probiotics (16–18). Probiotics are regarded as beneficial commensals that provide multiple health benefits to the host involving antibacterial activity, immunological regulation and intestinal barrier improvement (19–21). Moreover, recent publications on probiotics also demonstrated their properties to lower cholesterol, regulate gut microbiota and alleviate liver-related diseases (22–24). For instance, Sun et al. demonstrated the remarkable efficacy of *Bacillus subtilis* alleviated obese by stabilizing the gut microbiota in some exploratory mice studies (25). Likewise, Zhao et al. indicated that *Bacillus subtilis* could alleviate alcoholic liver disease by regulating gut microbiota and intestinal permeability (24). Although probiotics supplementation has been shown to be a promising strategy for alleviating diarrhea, growth retardation and liver-related diseases, studies regarding probiotics in yaks remains scarce.

Recently, high-throughput sequencing technologies, including metagenomes and metabolomes, have been successfully applied to analyze the complex structure of gut microbiota and their metabolites (26–28). In-depth dissection of gut microbial composition and structure is beneficial for understanding the importance and underlying mechanisms of gut microbiota in intestinal function and host health (29, 30). Additionally, high-throughput genomic technology can also conduct a comprehensive analysis of probiotic whole genome sequences, which contribute to exploring potential probiotic mechanisms (31, 32). Previous studies indicated that the genome of *Bacillus subtilis* derived from different sources such as tilapia and soil has been well-characterized and revealed the reason for their probiotic properties (33). However, until now, studies regarding the genome characteristics of *Bacillus subtilis* isolated from yaks remains scarce. Numerous studies indicated that the adaption to the extreme environment of the Tibet plateau may potentially be associated with gut microbiota and adapted microorganism, which may be dramatically affected by the structure of their genomes (1, 2). Consequently, we performed this study to investigate the genome characterization of *Bacillus subtilis* isolated from yaks.

Materials and methods

Bacterial strains

The 6 ± 2 yak calves were purchased and raised at free ranged area up to the age of 2 years. Afterwards, the *Bacillus subtilis* was isolated from their intestinal cecum after slaughtering. The *Bacillus subtilis* was inoculated into Luria-Bertani broth at 37°C for 24 h for multiplication culture (34). Subsequently, the bacterial solution was sent to Beijing Biomarker Technologies Co, LTD for whole-genome sequencing.

Genome DNA extraction

Prior to the bacterial DNA extraction, the bacterial solution needs to be centrifuged at 8,000 rpm for 40 min to obtain the bacterial pellet. Subsequently, the bacterial pellet was washed three times with sterile phosphate buffer as described above. The genomic DNA of bacterial pellet was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) based on the manufacturer's recommendations. Moreover, the agarose gel electrophoresis and quantitative of extracts were performed to guarantee its integrity, purity and concentration meet the subsequent analysis requirements.

Library construction and sequencing

BluePippin automatic nucleic acid recovery system was used to recycle and purify targeted DNA fragments. DNA samples were subjected to fragment, damage repair, end repair, magnetic bead purification and adapter ligation. The SQK-LSK109 ligation Sequencing Kit was applied to construct

DNA library. Subsequently, the Invitrogen Qubit 3.0 was used for measuring library concentration. The final library was performed sequencing using Illumina HiSeqTM 2000 sequencer.

Quality control and sequence assembly

The raw data obtained from sequencing needs to be quality controlled to remove low quality and short sequences and obtain available clean data. Subsequently, the statistical analysis of clean data were performed to obtain relevant information such as the total amount of data, the length of reads, and the distribution of quality values. The second- and third-generation data were assembled using Unicycler software and the reads were aligned to the assembled sequences. The distribution of sequencing depth was counted and the assembled sequence was distinguished from chromosomal sequence or plasmid sequence according to the sequence length and alignment, and whether the sequence was circular or not. (1) Preliminary assembly: the SPAdes was used to assemble the second-generation data into a frame diagram; (2) Create bridges: the miniasm and Racon was used to add three generations of data to the frame diagram, and the three generations of long read data was used to create bridges; (3) Bridge application: In the presence of conflict, the Unicycler was used to select a high-scoring bridge according to the quality score corresponding to the created bridge; (4) Looping judgment and final confirmation: the start_genes was used to start site correction and final confirmation, screen chromosome and plasmid sequences and assemble the chromosome sequence into a circular genome.

Bioinformatics analysis

Gene prediction

Coding gene predictions were performed on newly sequenced genomes using GeneMarkS (Version 4.17) software. tRNA, rRNA, and sRNA were predicted by tRNAscan-SE software (Version 1.3.1), rRNAmmer software (Version 1.2), and Rfam database. Interspersed and tandem repeat predictions were performed using the prediction software RepeatMasker (Version open-4.0.5) and TRF (Tandem Repeats Finder, Version 4.07b). IslandPath-DIOMB software (Version 0.2), phispy software (Version 2.3), and CRISPRdigger (Version 1.0) were used to predict gene islands, prophages, and CRISPR prediction on the sample genome, respectively.

Functional annotation

Predicted genes were BLAST aligned with various functional databases. Based on the BLAST result of each sequence, the alignment with the highest score is annotated (default identity $\geq$ 40%, coverage $\geq$ 40%). The predicted coding genes were annotated in the GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (Cluster of Orthologous Groups of proteins), NR (Non-Redundant Protein Database), TCDB (Transporter Classification Database), CAZy (Carbohydrate-Active EnZymes Database) function database database, and the pathogen-host interaction database was annotated. According to the above sequencing and bioinformatics analysis results, the genome circle map of *Bacillus subtilis* was drawn.

Results

Data acquisition and analysis

Quality control statistics for *Bacillus subtilis* are shown in Table 1. In this research, a total of 109,397 reads and 866,044,638 bases were obtained. The average length of the reads was 168,691 bp and the maximum length of the reads was 114,102 bp. The sequencing reads N50 length and N90 length were 14,073 and 3,043 bp, respectively. The average sequencing quality of reads was 11.12. Subsequently, statistical analysis of the length and data volume of the reads was performed and found that the reads were mainly concentrated in 10,000–20,000 bp (Figure 1).

Basic characteristics of genome

The basic information of the genome of *Bacillus subtilis* is shown in Figure 2. The genome of *Bacillus subtilis* consists of 1 chromosome and 1 plasmid and the chromosome and plasmid genome are circular. The length of chromosome and plasmid are 4,214,774 and 54,527 bp, respectively.

Coding gene and non-coding RNA

The gene encoding information of *Bacillus subtilis* is shown in Table 2. A total of 4,429 coding genes were predicted, and the total length of all coding genes was 3,751,617 bp. The length of coding genes ranges from 90 to 10,764 bp, with an average length of 847 bp. The majority of genes are between 100 and 999 bp in length (Figure 3). *Bacillus subtilis* contains 86 tRNAs, 27 rRNAs (9 16S_rRNA, 9 23S_rRNA and 9 5S_rRNA), and 114 other ncRNA (Supplementary Table 1).

Genomic island and prophage prediction

Twenty genomic islands were predicted in the genome of *Bacillus subtilis* (Table 3). The length of the predicted genomic islands are 4,915, 12,686, 9,247, 10,861, 39,818, 40,088, 6,130, 8,870, 24,920, 6,441, 10,096, 11,278, 6,245, 8,489, 18,551, 36,598, 36,110, 17,647, 5,638, and 10,887, respectively. Moreover, the

TABLE 1 The information of quality control of *Bacillus subtilis*.

Reads type	SeqNum	SumBase	N50Len	N90Len	MeanLen	MaxLen	MeanQual
Clean reads	109,397	866,044,638	14,073	3,043	7,916	114,102	11.12

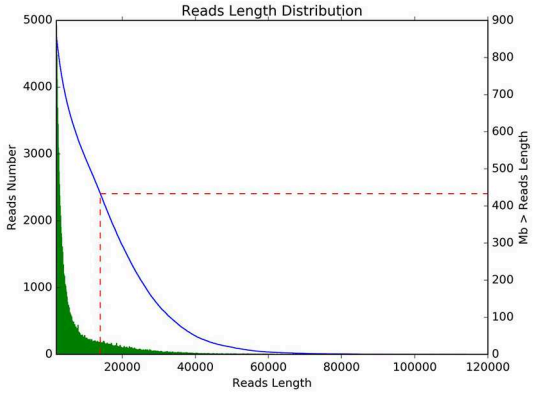

FIGURE 1
The length distribution of sequencing. The horizontal axis represents reads length, and the vertical axis represents reads number.

total and mean length of the genomic islands are 325,515 and 16,275, respectively (Supplementary Table 2). A total of 3 prophage were predicted with lengths of 8,848, 84,557, and 134,638, respectively (Table 4). Moreover, the total length and mean length of the predicted prophage are 228,043 and 76,014, respectively.

CRISPR predictive analysis

A total of 10 CRISPR were predicted and the average spacer length were 19, 19, 19, 45, 19, 38, 19, 24, 55, and 58, respectively (Table 5). The average repeat length of the predicted CRISPR were 21, 21, 21, 21, 38, 22, 22, 19, 26, and 34, respectively.

GO annotation results

Bacillus subtilis was performed for functional prediction using the GO database and the genic functions were divided into three categories, including biological processes, molecular functions, and cellular components (Figure 4). The number of genes involved in biological processes, molecular functions, and cellular components was 100, 200, and 300, respectively. In terms of cellular component, most genes are associated with extracellular region, cell, membrane, macromolecular complex, organelle, organelle part, virion part, membrane part, and cell part. In terms of molecular function, most genes are associated with transcription factor activity, protein binding, nucleic acid binding transcription factor activity, catalytic activity, signal transducer activity, structural molecule activity, transporter activity, binding, electron carrier activity, antioxidant activity, molecular transducer activity, and molecular function regulator. In terms of biological process, most genes are related to cell killing, metabolic process, cellular process, reproductive process, biological adhesion, signaling, developmental process, locomotion, single-organism process, response to stimulus, localization, multi-organism process, biological regulation, cellular component organization or biogenesis, and detoxification.

KEGG annotation results

In terms of metabolism, most genes are related to alanine, aspartate and glutamate metabolism, arginine and proline metabolism, cysteine and methionine metabolism, glycine, serine and threonine metabolism, histidine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, valine, leucine and isoleucine degradation, amino sugar and nucleotide sugar metabolism, butanoate metabolism, citrate cycle (TCA cycle), fructose and mannose metabolism, galactose metabolism, glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism, pentose and glucuronate interconversions, pentose phosphate pathway, propanoate metabolism, pyruvate metabolism, starch and sucrose metabolism, methane metabolism, nitrogen metabolism, oxidative phosphorylation, sulfur metabolism, 2-Oxocarboxylic acid metabolism, biosynthesis of amino acids, carbon metabolism, fatty acid biosynthesis, peptidoglycan biosynthesis, fatty acid metabolism, glycerolipid metabolism, glycerophospholipid metabolism, biotin metabolism, folate biosynthesis, pantothenate and CoA biosynthesis, selenocompound metabolism, purine metabolism, and pyrimidine metabolism (Figure 5). In terms of cellular processes, most genes are related to bacterial chemotaxis and flagellar assembly. In terms of environmental information processing, most genes are related to ABC transporters, phosphotransferase system (PTS) and two-component system. In terms of genetic information processing, most genes are related to protein export, DNA replication, homologous recombination, mismatch repair, aminoacyl-tRNA biosynthesis, and ribosome.

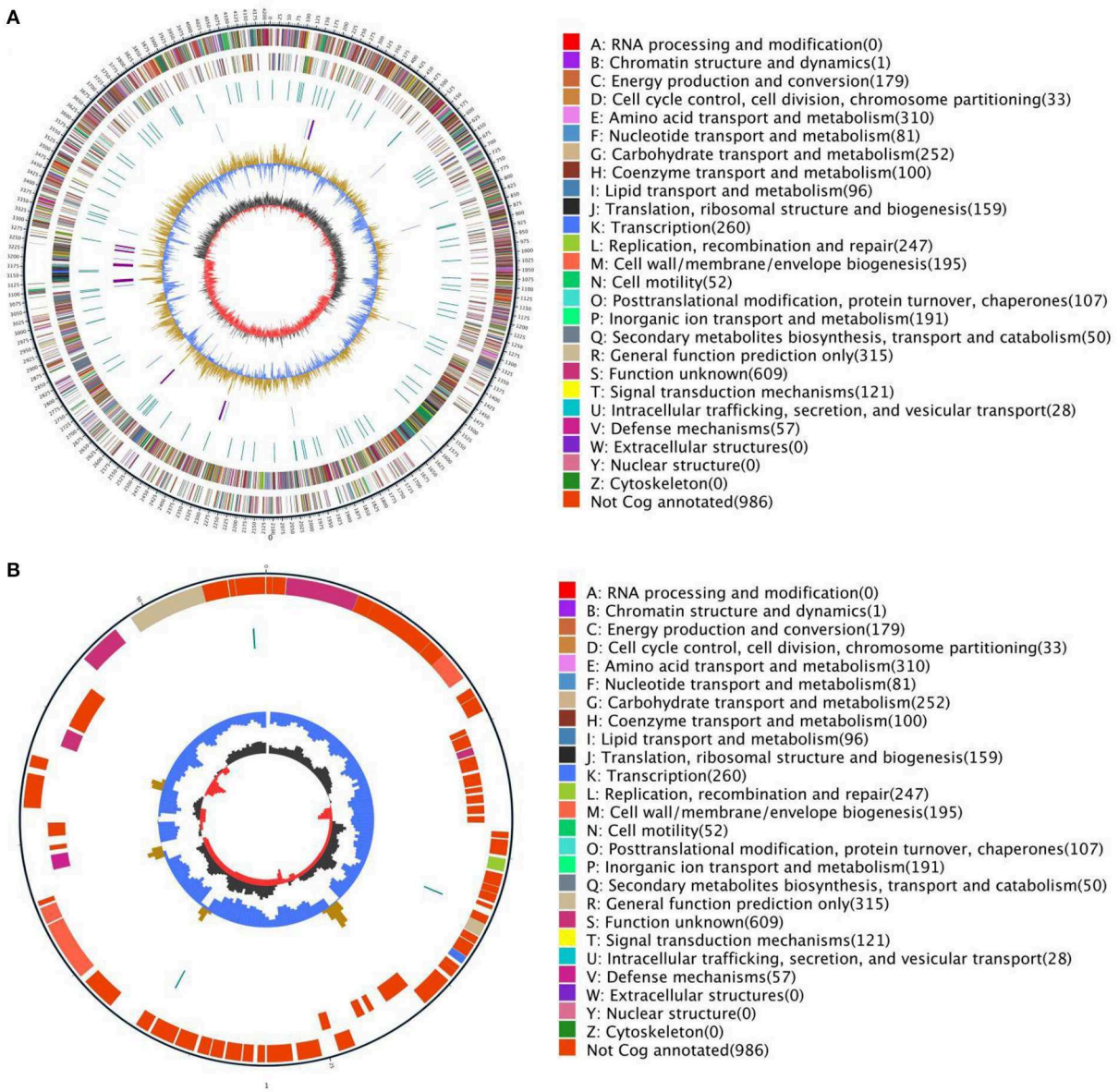

FIGURE 2
Circular genome map of *Bacillus subtilis* **(A)**. The genome circular maps of *Bacillus subtilis* plasmid **(B)**. The outermost circle is the mark of the genome size and each scale is 5 kb; the second and third circles are the genes on the positive and negative strands of the genome respectively, and different colors represent different COG functional classifications; the fourth circle is the repetitive sequence; the fifth circle is tRNA and rRNA, the blue is tRNA, and the purple is rRNA; the sixth circle is the GC content. The light yellow part indicates that the GC content in this area is higher than the average GC content of the genome. The higher the peak value, the greater the difference from the average GC content. The blue part indicates that the GC content of this region is lower than the average GC content of the genome; the innermost circle is GC-skew, the dark gray represents the area where the G content > C, and the red represents the area where the C content > G.

TABLE 2 The prediction results of coding gene.

Geneset number	Total length (bp)	Average (bp)	Max length (bp)	Min length (bp)
4,356	3,705,798	850	10,764	90

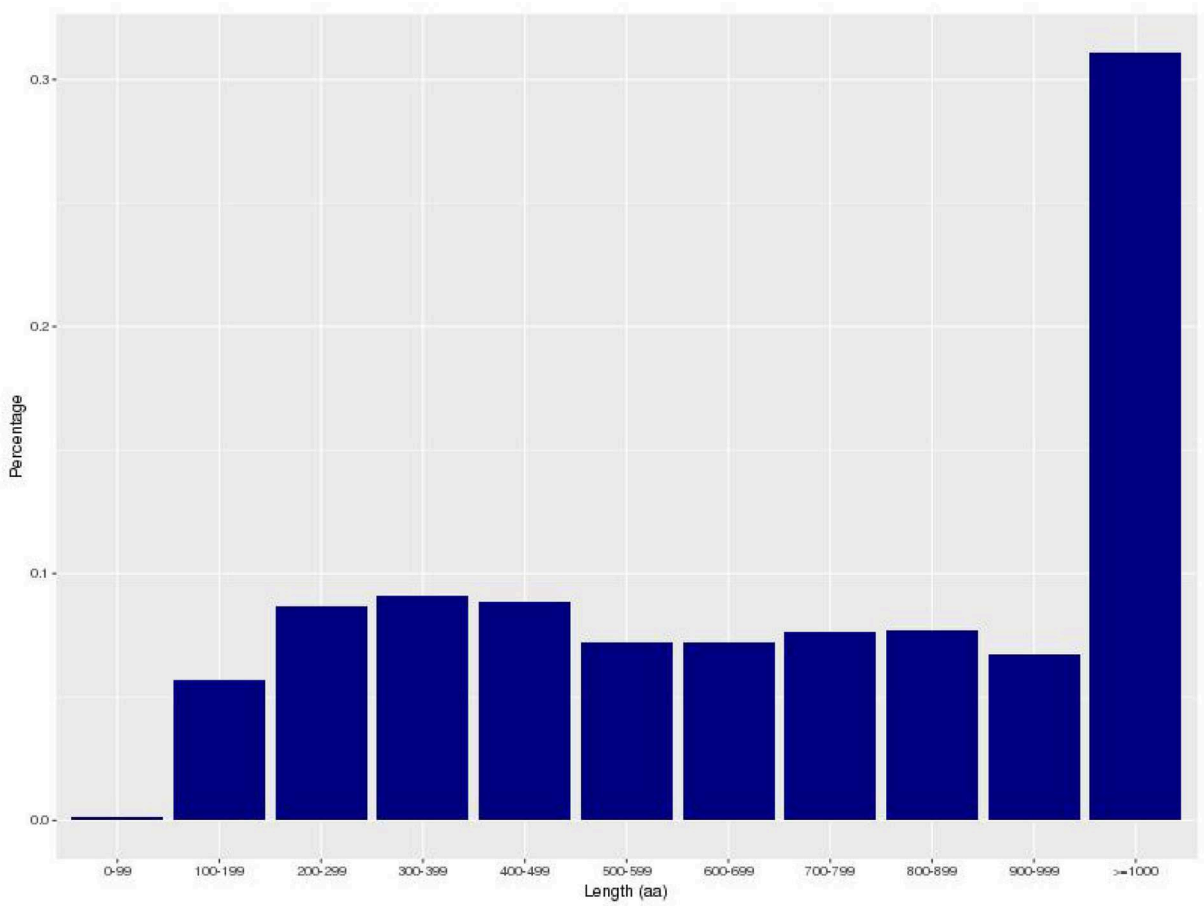

FIGURE 3
The map of coding gene length distribution. The horizontal axis represents the length of the protein sequence and the vertical axis represents the proportion.

EggNOG annotation results

EggNOG annotation results indicated that most genes are related to chromatin structure and dynamics (1–0.03%), energy production and conversion (179–5.1%), cell cycle control, cell division, chromosome partitioning (33–0.94%), amino acid transport and metabolism (310–8.84%), nucleotide transport and metabolism (81–2.31%), carbohydrate transport and metabolism (266–7.58%), coenzyme transport and metabolism (103–2.94%), lipid transport and metabolism (96–2.74%), translation, ribosomal structure and biogenesis (161–4.59%), transcription (260–7.41%), replication, recombination and repair (248–7.07%), cell wall/membrane/envelope biogenesis (200–5.7%), cell motility (52–1.48%), post-translational modification, protein turnover, chaperones (108–3.08%), inorganic ion transport and metabolism (199–5.67%), secondary metabolites biosynthesis, transport and catabolism (58–1.65%), general function prediction only (315–8.98%), function unknown (609–17.37%), signal transduction mechanisms (136–3.88%), intracellular trafficking, secretion, and vesicular transport (35–1%), and defense mechanisms (57–1.63%; Figure 6).

CAZy database annotation result

The CAZy database annotation results are shown in Figure 7. The results show that a total of six functions are annotated such as glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE), auxiliary activities (AA), and carbohydrate-binding modules (CBM). The proportion of annotated functions are 33.65, 22.11, 3.84, 14.42, 3.36, and 22.59%, respectively. Moreover, the number of genes involved in the annotated functions are 70, 46, 8, 30, 7, and 47, respectively.

TABLE 3 Genomic island prediction results statistics.

Genomic islands	Scaffold_id	Start	End	Length (bp)
Genomic_island_1	Contig00001	327,094	332,008	4,915
Genomic_island_2	Contig00001	357,791	370,476	12,686
Genomic_island_3	Contig00001	696,916	706,162	9,247
Genomic_island_4	Contig00001	708,313	719,173	10,861
Genomic_island_5	Contig00001	724,211	764,028	39,818
Genomic_island_6	Contig00001	1,102,910	1,142,997	40,088
Genomic_island_7	Contig00001	1,254,876	1,261,005	6,130
Genomic_island_8	Contig00001	1,273,090	1,281,959	8,870
Genomic_island_9	Contig00001	1,390,699	1,415,618	24,920
Genomic_island_10	Contig00001	1,438,503	1,444,943	6,441
Genomic_island_11	Contig00001	1,991,961	2,002,056	10,096
Genomic_island_12	Contig00001	2,090,597	2,101,874	11,278
Genomic_island_13	Contig00001	2,146,419	2,152,663	6,245
Genomic_island_14	Contig00001	2,604,742	2,613,230	8,489
Genomic_island_15	Contig00001	2,697,661	2,716,211	18,551
Genomic_island_16	Contig00001	3,053,174	3,089,771	36,598
Genomic_island_17	Contig00001	3,132,900	3,169,009	36,110
Genomic_island_18	Contig00001	3,360,721	3,378,367	17,647
Genomic_island_19	Contig00001	4,053,466	4,059,103	5,638
Genomic_island_20	Contig00001	4,142,648	4,153,534	10,887

TABLE 4 Statistics of prophage prediction results.

Prophage_id	Scaffold_id	Start	End	Length (bp)
Prophage_1	Contig00001	709,561	718,408	8,848
Prophage_2	Contig00001	1,050,842	1,135,398	84,557
Prophage_3	Contig00001	1,305,581	1,440,218	1,440,218

Discussion

Typically, yaks are mainly free-range on the Qinghai-Tibet Plateau and not supplemented with additional nutrients, which causes their slow growth and high disease incidence (35, 36). Additionally, juvenile yaks exhibit high rates of diarrhea because of the harsh environment, nutrient deprivation and immature gut microbial community, resulting in massive mortality and economic losses (9). Dietary intervention has long been regarded as a vital way to decrease morbidity rate and increase production performance, which has attracted growing attention (37, 38). As a recognized probiotic, *Bacillus subtilis* is widely applied in animal husbandry due to its strong stress resistance and positive effects on the host (39, 40). For instance, Zhang et al. demonstrated that *Bacillus subtilis* PB6 administration dramatically increased villus height and maintain intestinal mucosal barrier function of ducks (41). Analogously, Mohamed et al. found that *Bacillus subtilis* administration in the basal diet could dramatically enhance the growth performance of broiler chickens by regulating gut microbiota and intestinal morphology (42). However, most of the present studies focus on the beneficial mechanism of probiotics on the host and few research pay attention to the genomic characteristics of probiotics. Whole genome sequence analysis can more comprehensively evaluate the safety of *Bacillus subtilis* and gradually deepen from traditional phenotypic analysis to molecular mechanism and genetic characteristics (43, 44). To further understand of the genomic bioinformatics characteristics of *Bacillus subtilis* isolated from yaks, this study conducted the whole genome analysis to analyze the genome characteristics and functional predictions based on the previous research on the *in vitro* probiotic characteristics of *Bacillus subtilis*.

TABLE 5 Results of CRISPR prediction.

CRISPR id	Contig id	Start	End	RN	ARL (bp)	SN	ASL (bp)
CRISPR.1	Contig00001	738,657	738,717	2	21	1	19
CRISPR.2	Contig00001	1,126,406	1,126,466	2	21	1	19
CRISPR.3	Contig00001	1,983,791	1,983,851	2	21	1	19
CRISPR.4	Contig00001	2,518,060	2,518,146	2	21	1	45
CRISPR.5	Contig00001	2,518,351	2,518,445	2	38	1	19
CRISPR.6	Contig00001	2,751,179	2,751,440	5	22	4	38
CRISPR.7	Contig00001	2,963,697	2,963,759	2	22	1	19
CRISPR.8	Contig00001	3,805,753	3,806,029	7	19	6	24
CRISPR.9	Contig00001	3,808,304	3,808,572	4	26	3	55
CRISPR.10	Contig00002	32,279	32,404	2	34	1	58

RN, repeat number; ARL, Average repeat length; SN, Spacer number; ASL, average spacer length.

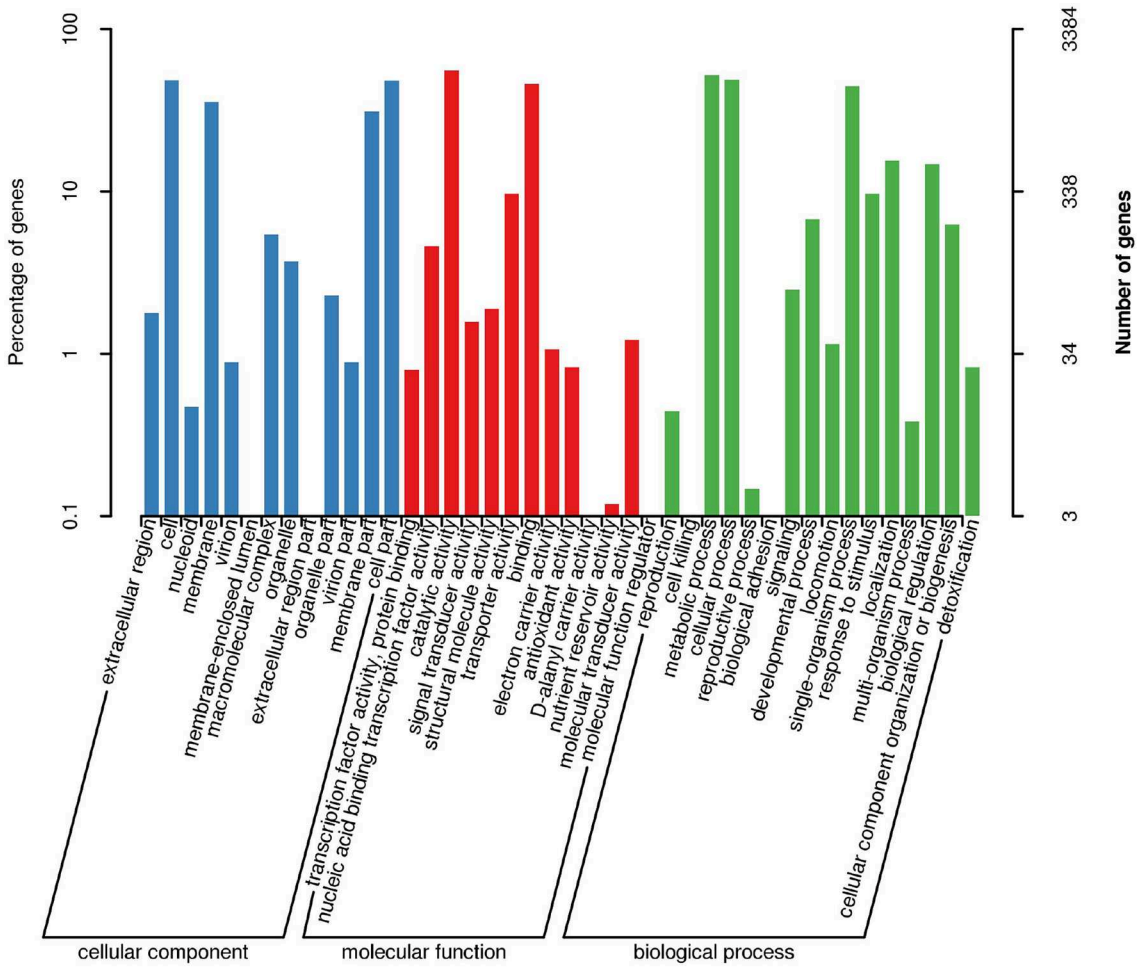

FIGURE 4
Functional classification results of GO annotation. The horizontal axis represents the content of each category of GO, the left side of the vertical axis represents the percentage of the number of genes, and the right represents the number of genes.

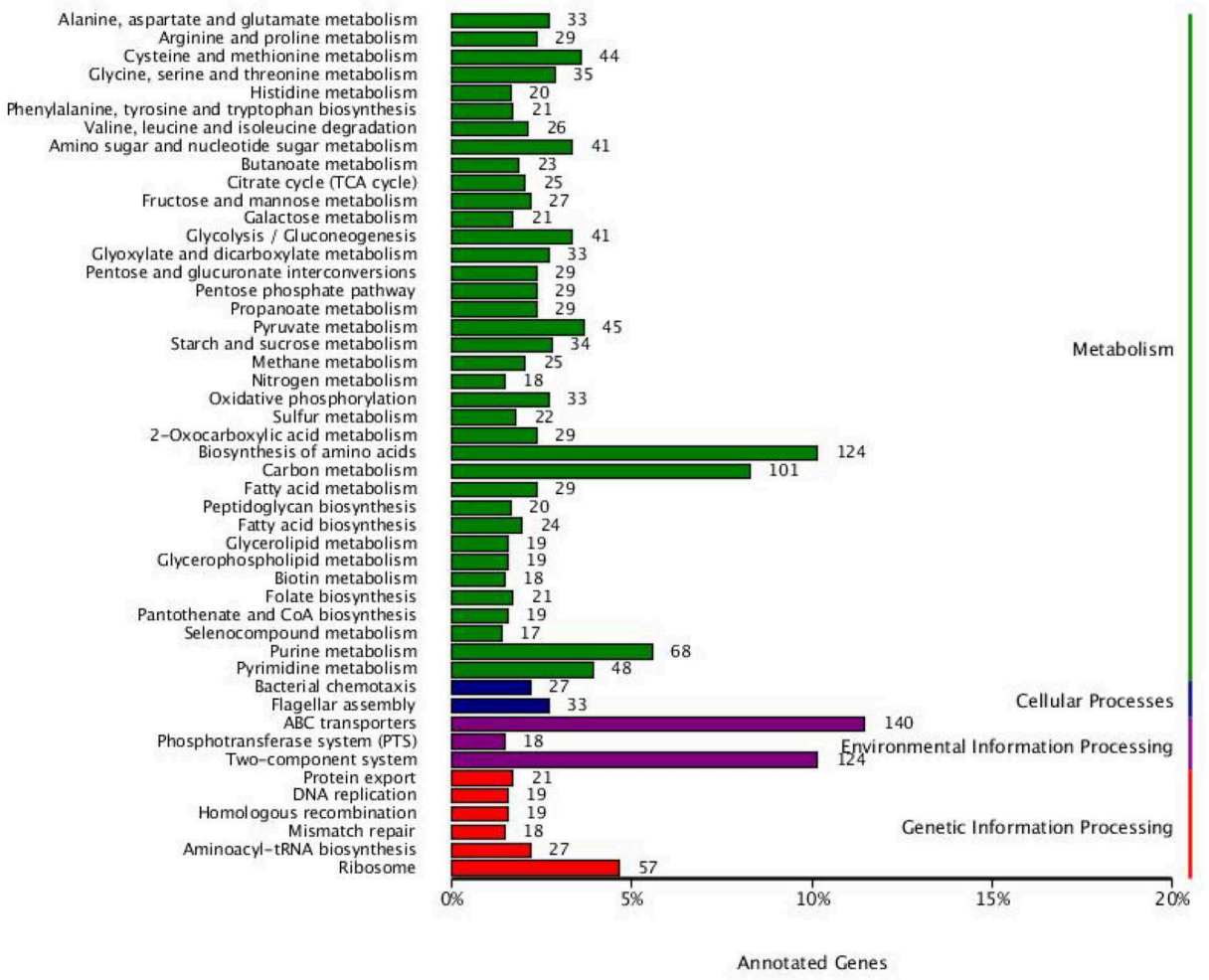

FIGURE 5
Functional classification results of KEGG annotation. The histogram displays the number of KEGG pathway genes involved and the colors signify various systems.

Carbohydrate-active enzymes are encoded by the gut microbial genomes of animals, which play a key regulatory role in carbohydrate breakdown. Early investigations indicated that carbohydrate-active enzymes were essential for host nutrient absorption (45). Glycoside hydrolases are an important class of enzymes that usually play a key role in the hydrolysis of glycosidic bonds of carbohydrates. Previous studies indicated that there are hundreds of different glycosyltransferases involved in the biosynthesis of disaccharides, oligosaccharides and polysaccharides, contributing to the formation of glycosidic bonds (46). In this study, the genome of *Bacillus subtilis* contains genes encoding GHs and GTs, and these active enzymes enable *Bacillus subtilis* to synthesize carbohydrates and metabolize sugars.

Amino acids are the basic constituent unit of proteins, which play key roles in energy conversion, protein biosynthesis and protein biosynthesis (47). For instance, arginine can participate in the ornithine cycle and convert the ammonia produced in the body into non-toxic urea through the ornithine cycle, thereby decreasing the blood ammonia concentration (48). Serine has been demonstrate to possess several metabolic functions including providing precursors for the synthesis of phosphoglycerides, neurotransmitters, sphingolipids, proteins and phosphatidylserine (49, 50). Tryptophan can participate in the renewal of plasma proteins in animals, promote the role of riboflavin and the synthesis of niacin and heme, which contributes to increasing the antibodies in the fetuses of pregnant animals and promote lactation in dairy cows and sows (51). Early surveys indicated that tryptophan deficiency caused growth arrest, weight loss, fat accumulation and testicular atrophy in breeding stock (52). Previous studies have shown that phenylalanine and tyrosine could synthesize important neurotransmitters and hormones involved in the body's glucose metabolism and fat metabolism (53). Numerous investigations

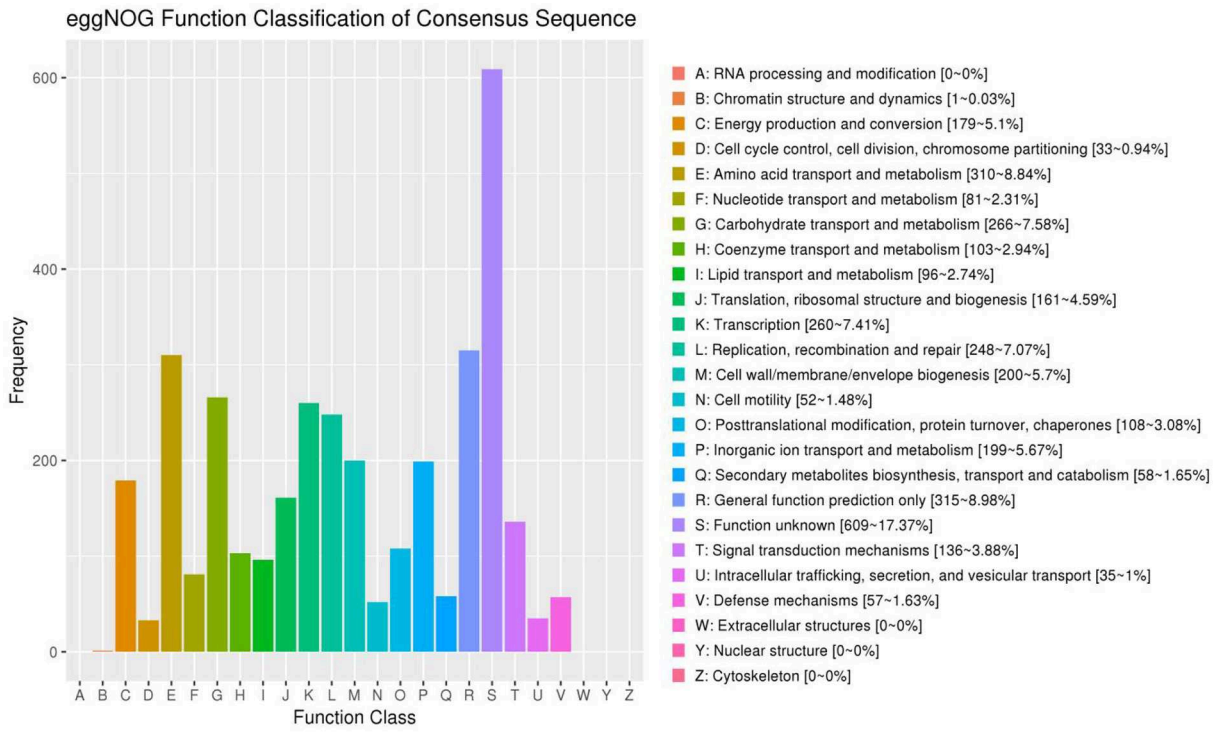

FIGURE 6
Functional classification results of eggNOG annotation. The horizontal axis represents the content of each category of eggNOG, and the vertical axis represents the relative proportion of the number of corresponding functional genes.

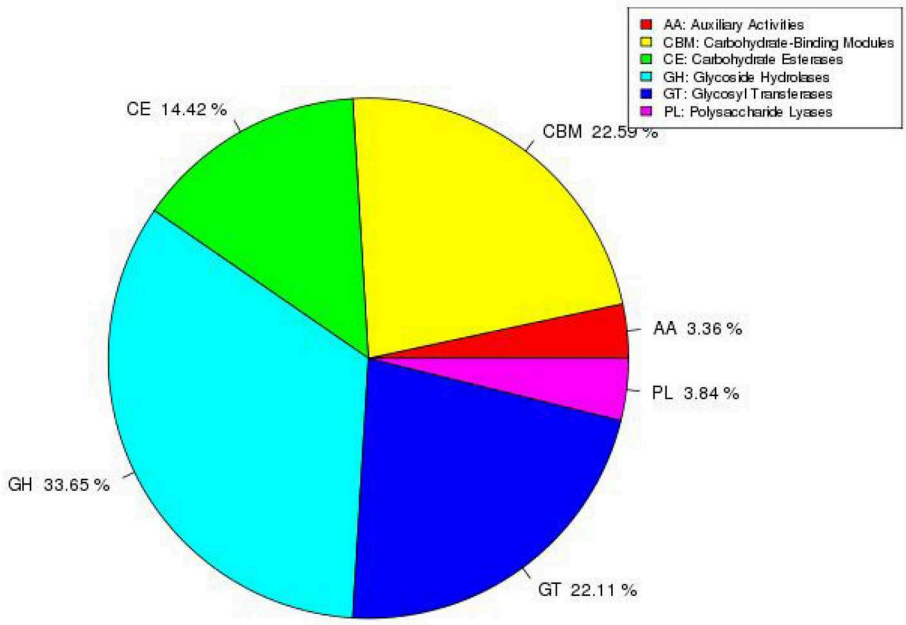

FIGURE 7
Distribution and scale diagram of carbohydrase.

indicated that gut microbiota played key roles in the metabolism and synthesis of amino acids (54). For instance, *Clostridium* in the intestine are key drivers of amino acid fermentation, whereas *Streptococcus* play a key role in the utilization of glutamate and tryptophan. In this study, we annotated a large number of genes related to amino acid metabolism in the genome of *Bacillus subtilis*, such as alanine, aspartate and glutamate metabolism, arginine and proline metabolism, cysteine and methionine metabolism, glycine, serine and threonine metabolism, histidine metabolism, etc., which may be one of the reasons for gut microbiota or probiotics regulate amino acid metabolism. Research on ABC transporters has demonstrate their essential roles in the transport of inorganic ions, monosaccharides, glycans, cholesterol, phospholipids, amino acids, peptides, proteins, toxins, drugs, antibiotics and heterologous substances, which are essential for cellular detoxification, membrane homeostasis, and lipid transport (55). Fatty acids exhibit multiple vital functions such as providing energy, maintaining the relative fluidity of cell membranes and normal physiological functions of cells, esterifying cholesterol to reduce blood cholesterol and triglyceride levels, improving brain cell activity, and enhancing memory (56). Notably, some members of fatty acids such as short-chain fatty acids (SCFAs: acetate, propionate, and butyrate) have attracted increasing attention due to their important roles in host health and metabolism (57). Early surveys on SCFAs provided evidence that they could regulate intestinal pH, intestinal permeability, gut microbiota and attenuate intestinal inflammation (58). Additionally, SCFAs have also been shown to play vital roles in cell proliferation, immune system and preventing intestinal leakage (59, 60). In this study, the genome of *Bacillus subtilis* contains genes encoding ABC transporters, fatty acid metabolism and fatty acid biosynthesis and this may be one of the reasons for probiotics exert beneficial effects on the host.

Biotin, also known as vitamin B7, can enhance the host immune response, infection resistance, maintain normal growth, and development (61, 62). Biotin can be used to treat diseases related to arteriosclerosis, stroke, lipid metabolism disorders, hypertension, coronary heart disease, and blood circulation disorders (63). Conversely, biotin deficiency can result in reproductive decline, skeletal dysplasia, stunted embryonic, and young child growth (63). Folic acid has been demonstrated to promote the formation of red blood cells and play important roles in protein synthesis, cell division, and growth (64, 65). Folic acid deficiency can cause megaloblastic anemia and affect hemoglobin production and cell maturation in red blood cells (65, 66). Currently, folic acid is widely used in animal husbandry due to various important physiological functions. For instance, folic acid administration in the basal diet significantly increase the serum immunoglobulin content, reduce the serum cholesterol and improve the apparent digestibility of dry matter, crude protein, and crude fat of chickens (67, 68). Moreover, folic acid supplementation can also improve the growth performance of piglets (69). In this study, we found that *Bacillus subtilis* contains genes related to folate biosynthesis and biotin metabolism, which is consistent with previous evidence that *Bacillus subtilis* can produce vitamins.

Oxidative stress was previously demonstrate to cause many chronic diseases such as aging, enteritis, atherosclerosis and even cancer (70–72). Moreover, oxidative stress can also decrease meat quality and fertility, seriously threatening livestock production (73, 74). Thus, decreasing oxidative stress is vital for ensuring human and animal health. Probiotics have been widely demonstrated to improve the host's antioxidant capacity by increasing the activity of antioxidant enzymes or activating antioxidant-related pathways (24, 75). Meanwhile, probiotics also have their own antioxidant systems. In this study, we observed the presence of antioxidant activity-related genes in *Bacillus subtilis*, which also validated the antioxidant properties of *Bacillus subtilis*.

In summary, this study first characterized the genome and genetic properties of *Bacillus subtilis* isolated from yaks. Results indicated that the genome of *Bacillus subtilis* contained genes related to anti-oxidation and biological adhesion. Moreover, we performed functional prediction on the genome and found that some genes were involved in amino acid metabolism, vitamin synthesis and metabolism, fatty acid metabolism, etc., which further confirmed that *Bacillus subtilis* isolated from yaks has good probiotic properties. This study revealed beneficial properties of *Bacillus subtilis* from the genomic perspective, which contributed to improving public awareness of *Bacillus subtilis* and providing a theoretical basis for the developing probiotic products. However, this study also has some limitations such as lack of comparative analysis with other bacterial genomes.

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 Ethics Committee of Huazhong Agricultural University.

Author contributions

AL and JL conceived and designed the experiments. YZ, ZL, MX, and LW contributed sample collection and reagents preparation. AL analyzed the data and wrote the manuscript. MW and MK revised the manuscript. All authors reviewed the manuscript. 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.2022.1099150/full#supplementary-material

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A review of foodborne *Toxoplasma gondii* with a special focus on its prevalence in Pakistan from 2000 to 2022

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Third-world countries have a higher prevalence of food-related disorders than developed nations. Millions of people in underdeveloped countries are seriously at risk from the potential water supply contamination with protozoan diseases. *Toxoplasma gondii* is one of the important protozoans causing diseases in livestock and humans. Despite the standard tests for diagnosing this parasite and different treatment methods, the spread of these parasites is uncontrollable and rising every year due to other management disorders. In this review, we summarize etiopathogenesis and prevalence in Pakistan. We looked for papers reporting the seroprevalence of *T. gondii* in people and animals between 2000 and 2022 in different databases: PubMed, Google Scholar, ScienceDirect, Scopus, and Web of Science. Data on the seroprevalence of *T. gondii* in Pakistan's domestic animals (sheep and goats, horses, donkeys, mules, cattle, and buffaloes), domestic pets (cats and dogs), poultry and rodents, and humans were gathered. According to the findings, sheep had an estimated pooled seroprevalence of *T. gondii* that varied from 11.20 to 26.50 %, and goats from 24.50 to 38.40%. Whereas in buffalo the opposite trend was followed, and the prevalence was observed is 0% in 2022, in horses, donkeys, and mules, only one study was reported according to which a high prevalence was observed in mules (28.60%) followed by donkeys (23.50%) and horses (23.50%), in cats 38.5% prevalence was observed in a recent study and in dogs 28.43% observed, and in humans from 22 to 60%. Human beings are found to be the most affected species showing high prevalence among all. According to our findings, animals and pets not only serve as a reservoir for the parasite but also serve as a direct route for human infection with *T. gondii*. The diagnostic techniques used in the observed studies were mostly serological testing whereas only a few studies have only been observed with molecular testing. To know the exact pattern of the disease for its control, the trend of molecular and advanced testing should be adopted as it is more reliable. Moreover, to decrease the transmission chances of *T. gondii* to humans, it is crucial to manage *T. gondii* infections in non-human species.

KEYWORDS

***Toxoplasma*, life cycle, transmission, symptoms, prevalence, diagnostic methods/prevention**

1. Introduction

Infections caused by food and water have attracted a lot of attention recently. The term "foodborne sickness" refers to a set of diseases that develop after consuming microbially or chemically contaminated food. Even contaminated water, utensils, as well as the hands of the diner can spread the disease. Third-world countries have a higher prevalence of food-related disorders than developed nations. Most people in the world still lack access to clean water and sanitary facilities, and households in rural areas where untreated water used for drinking, cooking, washing fruits, bathing, and swimming expose them to various pathogens including protozoan parasites (1, 2). Millions of people in underdeveloped countries are seriously at risk from the potential water supply contamination with protozoan diseases. There are many basic signs of food-related diseases, and gastrointestinal dysfunction is commonly used to diagnose them.

Parasites are capable of causing an acute, chronic, and debilitating type of diseases (3–6). In nature, parasitic protozoa may be found almost everywhere. In both developed and developing nations, they are accountable for epidemics and chronic poverty (7). Since certain parasites are zoonotic in nature and consequently exist in animals, their food and water prevalence should be considered a public health problem (8). The prevalence of food- and waterborne parasites has increased throughout time due to several past disease outbreaks linked to parasites. The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) published their worldwide risk rating of foodborne parasites (FBPs) in 2014 (2). It was followed in 2015 as a global burden related to foodborne pathogens (9). Despite being acknowledged as significant foodborne pathogens, parasites are still underappreciated when compared to bacterial and viral foodborne pathogens (10).

Toxoplasma spp is one of the important protozoans causing disease in livestock and humans (11–13). Across the world, this parasite has posed a serious threat. Despite the standard test for diagnosing this parasite and different treatment methods, the spread of these parasites is uncontrollable due to the other management disorders (14). This review summarizes etiopathogenesis, epidemiology in Pakistan from 2000 to 2022, and preventive measures for zoonotic toxoplasmosis.

2. Material and methods

2.1. Search technique

We searched databases (PubMed, Embase, Google Scholar, ScienceDirect, Scopus, ProQuest, and Web of Science) for articles reporting the seroprevalence of *T. gondii* in Pakistan from 2000 to 2022 to conduct this systematic review. The searches were limited to English-language articles. Electronic searches mostly employed the MeSH keywords (Human, Animal) AND (*Toxoplasma gondii* OR toxoplasmosis; Prevalence and seroprevalence OR serology).

For management, the citations were observed keenly. The final article choice was made after screening each article's title and abstract by eliminating duplicate records.

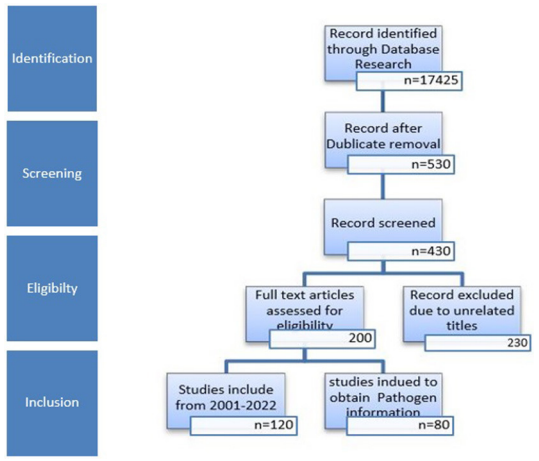

FIGURE 1
Overview of the number of studies from identification to inclusion.

2.2. Criteria for inclusion and exclusion

Using the titles as a guide, references were screened, and unnecessary and duplicate references were removed. A flow chart of the article identification, screening, eligibility, and inclusion criteria is shown in Figure 1. The last search was conducted on October 6th, 2022.

3. Toxoplasmosis

Toxoplasma gondii is a member of the phylum Apicomplexa, which is made up of a variety of protists, most of which are intracellular parasites capable of inflicting potentially life-threatening diseases in both humans and animals. Given its ability to infect virtually all warm-blooded vertebrates, *T. gondii* is the most prevalent. It is also thought to infect about one-third of the world's population of humans (15). According to the nation or region under consideration, seropositivity rates in the human population vary from <10 to over 90%, partly due to local socio-economic conditions and population patterns (16). For instance, there is a more

significant incidence in continental Europe, South America, and the United Kingdom than in the United States or the United Kingdom. Both wild and domestic animals have high seroprevalence, making them essential *T. gondii* reservoirs and sources for human contamination through meat intake (17). In addition to being an issue for human contamination, toxoplasmosis in farm animals has a significant negative impact on the livestock (on milk production and reproductive performance), which results in a high cost for the industry (18).

Only one species has been identified for the genus *Toxoplasma*, yet many clonal lineages have varying degrees of pathogenicity. Four primary clonal types I, II, III, and XII dominate the population pattern of *T. gondii* in Europe and North America (19–21). The most common strains in a wild and domestic context in Europe are type II (and type III, though to a lesser extent) (22, 23). Domestic isolates from North America are comparable to those from Europe (types II and III), while in the wild, strains from type XII prevail (24, 25). More contrast exists in other regions of the world. For instance, South America has a lot more genetic variety (26, 27), which suggests that recombination occurs more frequently there. Following *T. gondii* infection, the host's type, genetic makeup, and of course, the host's immune status all play a role in the development of the disease. Some species appear to be innately resistant to *T. gondii* infection. In contrast, others are highly susceptible, partly due to variables like their habitat's closeness to the parasite's definitive hosts (28). However, the host immune system and how parasite factors affect it continue to be one of the most critical factors affecting susceptibility to *T. gondii* (29–32).

4. Life cycle and routes of transmission of *Toxoplasma*

The life cycle of *T. gondii* includes both, asexual replication in a range of vertebrate hosts (intermediate hosts) and sexual replication in felids (definitive hosts). Felids consume the *T. gondii* by consuming encysted bradyzoites on infected intermediate hosts. Under the influence of digestive enzymes and acid, bradyzoites are liberated from cysts and enter the small intestine's epithelial cells. Although the parasite may spread throughout the body of the final host and cause clinical symptoms, this is uncommon (33). More typically, bradyzoites transform into schizonts in the intestine before reaching the merozoite stage (34). Merozoites differentiate into male and female gametes after a few cycles of asexual division. After that, male and female gametes combine to form diploid oocysts, which are enclosed in a solid, impenetrable wall. The millions of them contaminate the ecosystem that the felids excrete. The oocysts are resilient and survive in the environment, allowing them to spread (35, 36). Intermediate hosts consume sporulated oocysts by drinking or eating contaminated water and foods. Invading sporozoites quickly transform into the tachyzoite form inside a transitory parasitophorous vacuole (PV) that stays in the host cells (37). Tachyzoites are proliferative forms of toxoplasmosis that spread throughout the body and cause acute symptoms. They can move between tissues *via* blood vessels or the lymphatic system. At least when felids can prey on the intermediate host, this ensures parasite transmission to the final host to finish the cycle. Even when intermediate hosts aren't the felids who are often their prey, the parasites can still spread to new intermediate hosts through carnivory, keeping the parasite transmission cycle going without the necessity for sexual reproduction. The life cycle of *T. gondii* is shown in Figure 2.

Human infection can occur through food consumption, such as raw or undercooked meat containing cysts or vegetables, fruits, or water that has sporulated oocysts (38). To prevent foodborne toxoplasmosis, it is crucial to wash produce, cook meat properly, and adequately treat sewage or water (39). Congenital transfer of tachyzoites from a woman who is mainly infected to the growing fetus through the placenta is one of the alternative ways of transmission (40).

Congenital toxoplasmosis must be managed with precautions that restrict the mother's exposure to established transmission channels while pregnant and with quick identification and treatment beginning following infection. Although uncommon, blood transfusion (41) or organ transplantation (42) from sick donors are potential sources of contamination in people.

5. Clinical manifestations of toxoplasmosis

In immunocompetent people, toxoplasmosis can cause a minor, self-limiting disease or remain unnoticed in most cases (43). In pregnant women, congenital toxoplasmosis may develop because, during the parasite's dissemination phase, the parasite passes through the placenta and infect the growing fetus. Depending on the gestational stage at the time of maternal infection, it might result in varying degrees of neurological, ophthalmic, or systemic damage. For instance, a maternal illness in the first trimester may result in more severe symptoms (44). Although some of them can also happen later in life, hydrocephalus, mental retardation, epilepsy, and blindness are the most significant sequelae for newborns (45).

Even though acute acquired infection can occur, immunodeficiency in adults can also result in severe toxoplasmosis.

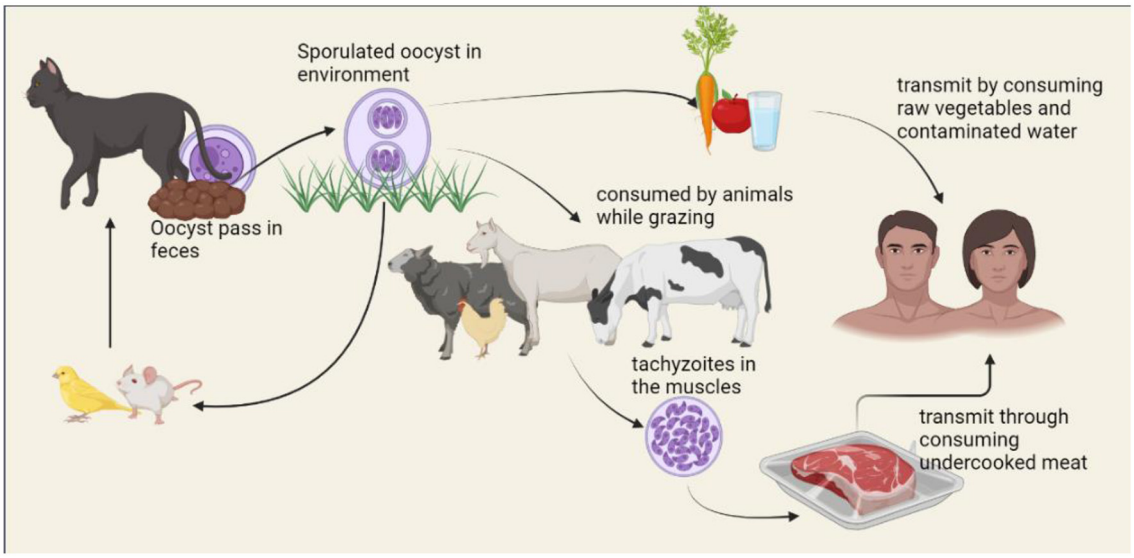

FIGURE 2
Life cycle of *Toxoplasma gondii*.

People who have impaired immune systems or immunosuppression [such as those with HIV (46), cancer patients (47), or transplant recipients] are particularly vulnerable. Toxoplasmic encephalitis may be the most severe outcome because it causes significant tissue damage and inflammation when toxoplasmosis from parasites ensconced in the central nervous system returns (48). Left untreated, this cerebral toxoplasmosis can be potentially fatal and frequently manifest as headache, fever, ataxia, or seizures. Acute toxoplasmosis has harmful effects, but chronic toxoplasmosis—the parasite's long-term persistence in the body as tissue cysts—may also have significant effects on behavioral changes and psychiatric problems, especially because it affects the central nervous system (49).

6. Prevalence of toxoplasmosis in humans and animals of Pakistan

Human studies that assessed the seroprevalence of *T. gondii* among the various individual groups listed in Table 1 have been published in Pakistan. Seroprevalence of *T. gondii* in dogs and cats, small ruminants, large ruminants, equines and camels, and poultry are mentioned in Tables 2–6, respectively. Pregnant women's seroprevalence has received the majority of attention in research (50–52), followed by patients with illnesses (53), The sociodemographic information, epidemiological profile, potential risk factors for transmitting the *T. gondii* infection, and the source of detection and diagnostic approach is also the focus of these investigations. Variable seroprevalence levels have been observed with a rise in percentage between 2001 and 2022.

The procedure used in the publications is often the collection of a blood sample from the population and testing it for anti-*toxoplasma* antibodies in the sera. The ELISA, latex agglutination (LA) test, and other serological assays were often employed (54, 55). Although their sensitivity and specificity varied, commercial test kits were employed, and the results were occasionally inconclusive.

In the food chain, which serves as a source of nutrition for humans and other animals, animals play a crucial role. The bradyzoite cyst is present in the body tissues of animals, and the parasite then transmits to new hosts by eating a raw or undercooked piece of the infected tissues (56). This situation raises the risk of zoonotic infection by foodborne pathogens since a particular group of humans, such as hunters, butchers, and consumers may get infected by ingesting domestic or wild meat (57). Only wild and domestic cats excrete the oocyst infective stage, which may infect humans when eaten in tainted food, water, or vegetables, which is especially noteworthy (58).

According to an analysis of the records currently available over the previous 20 years (2001–2022), domestic animals in Pakistan have a relatively high and rising seroprevalence rate of *T. gondii* as a zoonotic infection, except for buffalo, cats,

TABLE 1 Reported prevalence of *Toxoplasma gondii* in human population of Pakistan.

Species	Area of study	Prevalence rate (%)	Source of detection	No. of samples tested	Diagnostic approach	Age of animal	Year of study	References
Human	Lahore	22%	Serum	150	LAT	<15 years to >40 years	N/A	(59)
	Kohat, Khyber-Pakhtunkhwa	14.4%	Serum	180 pregnant	ELISA	N/A	June–September 2007	(60)
	Lahore	11.33%	Serum	300	LAT	Not mentioned for rats ≥45 years in humans	2012	(61)
	Rajanpur, Bahawalnagar, and Multan	29.45%	Serum	550	LAT	1–70 years	2010	(62)
	Khyber-Pakhtunkhwa	65.71%	Serum	420	ELISA	10 to >51 years	N/A	(63)
	Multan	19.4% pregnant women 15.2% non-pregnant women	Serum	232 pregnant women 171 non-pregnant women	ELISA	20–40 years	2017	(64)
	Khyber-Pakhtunkhwa	1.32%	Serum	150 pregnant women	ELISA	20–40 years	February–November 2015	(65)
	Charsadda	21%	Serum	300	LAT	15–75 years	May–July 2017	(66)
	Sub-Tropical Areas	20.37%	Serum	1,659	ELISA	<10 to >40 months	N/A	(67)
	Punjab	7.42%	Serum	593	ELISA	<20 to >40 years	January 1–December 31, 2017	(68)
	Bahawalpur	21.2% farmer 6.8% non-farmer	Serum	160 farmer 160 non-farmer	LAT	N/A	May 2016–April 2017	(69)
	Swat	25.92%	Serum	216	Lateral flow chromatographic immune-assay	31–40 years	June–September 2016	(70)
	Khyber-Pakhtunkhwa	40.6%	Serum	360	ELISA	16–40 years	N/A	(53)
	Peshawar	21.3%	Serum	94 Pregnant women	ICT	21–53 years	September–December 2017	(52)
	Sahiwal	24.5%	Serum	200	ELISA	N/A	May–November 2020	(71)
	KPK	39.94%	Serum	425	ELISA	15–50 years	N/A	(72)
	Khanewal	52%	Serum	200	ELISA	N/A	May–November 2020	(71)

and dogs. The comparison is shown in Figure 3. Although the prevalence level may have decreased and increased in different species, caution is advised.

All the data mentioned in the previous study of Figure 1 is collected from the year 2022 except for equines, dogs, and rats because the previous study performed in equine was in the year

TABLE 2 Reported prevalence of *Toxoplasma gondii* in cats and dogs in Pakistan.

Species	Area of study	Prevalence rate (%)	Source of detection	No. of samples tested	Diagnostic approach	Age of animal	Year of study	References
Cats	Faisalabad	60%	Serum	10	LAT	6 months to >4 years	N/A	(73)
	Lahore	56% cats	Serum	50	LAT	6 months	N/A	(59)
	Sub-tropical Arid parts	26.43% cats	Serum	420	ELISA	1–2 years	January–December 2012	(74)
	Lahore	2.3%	Feces	470	PCR	N/A	June 2013–May 2014	(75)
	KPK	25.4% cats	Serum and blood	50	PCR	2–4 years	N/A	(76)
	KPK	74.6%	Serum and blood	147	ELISA	2–4 years	N/A	(76)
	KPK	2.50%	Feces	40	Centrifugal sedimentation leading to PCR	N/A	January–December 2019	(77)
	Sahiwal and Khanewal	6.5%	Feces	200	Floatation/ sedimentation	N/A	May–November 2020	(71)
	KPK	12.22%	Serum	40	LAT	N/A	January–December 2019	(77)
	Sahiwal and Khanewal	38.46%	Feces	13	PCR	N/A	May–November 2020	(71)
Dogs	Faisalabad	50% dogs	Serum	40	LAT	6 months to >4 years	N/A	(73)
	Lahore	39% dogs	Serum	100	LAT	6 months to >7 years	N/A	(59)
	Lahore	46.88%	Serum	305	LAT	6 months to >4 years	N/A	(78)
	Sub-tropical Arid parts	28.43% Dogs	Serum	408	ELISA	1–2 years	January–December 2012	(74)

2015, and for rat, it was in 2012. Whereas in dogs, the last study was performed in 2014 in Pakistan.

7. Methods used for the detection of *T. gondii*

Examining the levels of immunoglobulin G (IgG), immunoglobulin M (IgM), and IgG avidity in a sample—typically serum from the blood of a particular host population—the serological test assesses the antibodies and calculates the seroprevalence of infection. Although it is the simplest and most straightforward test, it frequently yields false-positive or false-negative findings (106). Most of the studies in Pakistan have been diagnosed through Latex Agglutination Test (LAT). Cd4 mentioned in Table 1. The tests using molecular methods are reliable, perceptive, and accurate (107). Few studies have been reported and mentioned in Table 1, which have been diagnosed with Molecular methods. They use a variety of samples to find a specific gene of interest that is unique to this particular organism. Numerous techniques are routinely used, including loop-mediated amplification (LAMP), quantitative PCR, and traditional polymerase chain reaction (PCR) (108). This technique is seldom employed in histological procedures. It is primarily concerned with identifying the bradyzoite stage in. tissues such as the heart, liver, and brain. The bradyzoite stage is primarily detected in tissues, including the heart, liver, and brain (109). Before being

TABLE 3 Reported prevalence of *Toxoplasma gondii* in small ruminants (sheep and goats) in Pakistan.

Species	Area of study	Prevalence rate (%)	Source of detection	No. of samples tested	Diagnostic approach	Age of animal	Year of study	References
Sheep	Rahim Yar Khan	11.2%	Serum	90	LAT	N/A	2006–2007	(79)
	Mardan	44.13% sheep	Serum	290	IHA	1–2 years	N/A	(80)
	Pothwar Region	18.16% sheep	Serum	413	ELISA	1–3 years	September 2011–December 2012	(81)
	Southern Punjab	37.31%	Serum	335	LAT	N/A	May 2012–April 2013	(82)
	Northeast Punjab	26.2% sheep	Serum	470	ELISA	1–3 years	January–December 2013	(83)
	Multan	34.02%	Serum	288	LAT	4–73 months	April 2012–June 2013	(84)
	Khanewal	33.01%	Serum	212	LAT	4–73 months	April 2012–June 2013	(84)
	Cholistan desert (Punjab)	37.31%	Serum	335	LAT	1 to >25 months	N/A	(84)
	Cholistan desert (Punjab)	29.13%	Serum	865	LAT	1 to >25 months	N/A	(74)
	Multan	44.80%	Serum	125	LAT	<1 to > 2 years	N/A	(85)
	Charsadda	40.55% sheep	Serum	143	LAT	1–4 years	N/A	(86)
	Dera Gazi Khan	23% ELISA 25% LAT	Serum	103	ELISA and LAT	8–42 months	N/A	(87)
	Bahawalpur	36.25% sheep	Serum	160	LAT	N/A	May 2016–April 2017	(69)
	Peshawar	49% sheep	Serum	360	IHT	<1 to <2 years	N/A	(88)
	Khyber-Pakhtunkhwa	52.69%	Serum	167	ELISA	1 to > 3 years	2018–2020	(54)
	Jhang	31.49% sheep	Serum	181	LAT	<12 to >24 months	N/A	(90)
	Sahiwal	23.5%	Serum	1,000	ELISA	N/A	May–November 2020	(71)
	Khanewal	26.5%	Serum	1,000	ELISA	N/A	May–November 2020	(71)
Goats	Rahim Yar Khan	24.5%	Serum	110	LAT	N/A	2006–2007	(79)
	Mardan	42.28% goats	Serum	350	IHA	1–2 years	N/A	(80)

(Continued)

TABLE 3 (Continued)

Species	Area of study	Prevalence rate (%)	Source of detection	No. of samples tested	Diagnostic approach	Age of animal	Year of study	References
	Pothwar Region	14.32% goats	Serum	419	ELISA	1–3 years	September 2011–December 2012	(81)
	Northeast Punjab	42.8% goat	Serum	530	ELISA	1–3 years	January–December 2013	(83)
	Multan	40.80	Serum	125	LAT	<1 to >2 years	N/A	(85)
	Charsadda	41.61%	Serum	149	LAT	1 to >3 years	N/A	(65)
	Bahawalpur	28.1% goat	Serum	160	LAT	N/A	May 2016–April 2017	(69)
	Dera Gazi Khan	32.67% ELISA 35.64% LAT goat	Serum	101	ELISA and LAT	8–42 months	N/A	(87)
	Khyber Pakhtunkhwa	7.9%	Serum	70	ELISA	1 month to <2 years	2018–2020	(54)
	Dera Ghazi Khan	10%	Serum	410	LAT	1–3 years	6 months	(77)
	Khyber-Pakhtunkhwa	18.25 %	Serum	126	ELISA	1 to >3 years	2018–2020	(54)
	Faisalabad	33.59%	Serum	384	LAT	<2 to >5 years	October 2016–March 2017	(91)
	Peshawar	45.7% goats	Serum	420	IHA	<1 to <2 years	N/A	(88)
	Faisalabad	53.15%	Serum	380	LAT	1–6 years	N/A	(89)
	Khanewal	5.3%	Blood	898	PCR	1–3 years	March 2019–February 2020	(92)
	Faisalabad	17.9%	Serum	240	LAT	<1 to >3 years	September 2016, February 2017	(93)
	Khanewal	29.2%	Serum	1,000	ELISA	N/A	May–November 2020	(71)
	Jhang	36.52% goat	Serum	219	LAT	<12 months to >24 months	N/A	(90)
	Sahiwal	38.4%	Serum	1,000	ELISA	N/A	May–November 2020	(71)

examined under a microscope, such tissues are mounted on a glass slide and stained with hematoxylin and eosin (H&E). Another method of evaluating suspected samples, such as cat feces, liver, lung, and brain homogenates of intermediate hosts by inoculation and then testing the animal for the presence of an infection, is bioassay/*in vivo* using an animal model (mice/rat) (110). The test is costly and time-consuming, but it is an accurate approach to assessing the sustainability and pathogenicity of the various strains. Through the establishment of an enclosed environment where suspected specimens, such as blood, are cultured in a medium, the *in vitro*/tissue culture technique removes the usage of animals (111). Microscopy is used to assess the sample's motility or viability for the tissue culture endpoint. Most intuitive findings that can identify the parasite's morphology nevertheless rely heavily on microscopy as their foundation. Other tests like tissue culture and histology consistently rely on it because of its adaptability (112).

TABLE 4 Reported prevalence of *Toxoplasma gondii* in large ruminants (cattle and buffalo) in Pakistan.

Species	Area of study	Prevalence rate (%)	Source of detection	No. of samples tested	Diagnostic approach	Age of animal	Year of study	References
Cattle	Northern Punjab	19.75% cattle	Serum	400	ELISA	>24 to >48 months	January–December 2012	(94)
	Charsadda	55.39%	Serum	139	LAT	1 to >5 years	N/A	(65)
	Khyber Pakhtunkhwa	13%	Serum	100	ELISA	1 month to <2 years	2018–2020	(54)
	Khyber-Pakhtunkhwa	18 %	Serum	100	ELISA	1 to >3 years	2018–2020	(54)
	Rajanpur	12.2% cattle	Blood	190	PCR	≥5 years to <5 years	July–October 2019	(95)
	Pakistan	29.75%	Serum	90	ELISA	N/A	N/A	(96)
	Pakistan	35.75%	Serum	400	LAT	>5 years	N/A	(96)
Buffalo	Northern Punjab	15.16% buffalo	Serum	422	ELISA	>24 to >48 months	January–December 2012	(94)
	Charsadda	17.32% Buffalo	Serum	127	LAT	1–4 years	N/A	(86)
	KPK	15.51 %	Serum	58	ELISA	1 to >3 years	2018–2020	(54)
	Rajanpur	0% Buffalo	Blood	120	PCR	≥5 to <5 years	July–October, 2019	(95)

8. Comparison of serological techniques for *T. gondii* antibody detection in Pakistan

All studies employed convenient sampling to gather data, and two serological tests—the Latex Agglutination Test (LAT) and Enzyme-Linked Immunosorbent Assay (ELISA)—are mostly used to assess the outcomes based on the detection of IgG, IgM, and avidity test of T. *gondii* antibodies (Table 1) (96, 99). Most studies did not follow established procedures for collecting and processing specimens, and most did not have information on the control group. However, since a different company produced each ELISA and LAT test, it was challenging to evaluate and confirm each assay's specificities and sensitivities. Except for a few recent studies, further PCR validation of the data was not done (75, 77, 95, 100). Because of differences in the experimental design and the commercial kits utilized, some of the results are thus disputed.

9. Prevention of toxoplasmosis

The foundation for the current strategies employed to control *T. gondii* infection has been supplied by the exponential growth in our understanding of *T. gondii* biology, epidemiology, and ecology during the past few decades. Limiting contact with available transmission channels and minimizing exposure to the parasite's infectious phases are the main goals of preventative interventions.

As previously indicated, people contract *T. gondii* either by eating or drinking raw or undercooked meat with parasite cysts on it or by drinking water contaminated with oocysts deposited in cat feces. Additionally, eating raw shellfish can result in illness (106).

Therefore, seronegative should only consume fully cooked meat, refrain from consuming raw shellfish, carefully wash their hands after coming into contact with raw meat, avoid gardening and soil handling without gloves, and thoroughly clean fruits and vegetables.

People who take care of the litter box should make it a habit to wear disposable gloves and wash their hands thoroughly with antiseptic. Seronegative should refrain from adopting or handling stray cats, and cats should stay indoors whenever feasible. They should also not be fed raw or undercooked meat. The suggestions mentioned above for preventing *T. gondii* infection also apply to people in other particular at-risk groups. Soon after HIV diagnosis, standardized guidelines advise testing all for serological signs of prior *T. gondii* infection (107).

Primary prophylaxis should be given to those who are also seropositive for *T. gondii* and have peripheral blood CD4 T cell levels of 100/L (107). People receiving

TABLE 5 Reported prevalence of *Toxoplasma gondii* in equines and camels of Pakistan.

Species	Area of study	Prevalence rate (%)	Source of detection	No. of samples tested	Diagnostic approach	Age of animal	Year of study	References
Equines	Faisalabad, Lahore, and Gujranwala	Horses 23.5%	Serum	183 horses	LAT	≥5 years to <10 years	N/A	(101)
	Faisalabad, Lahore, and Gujranwala	Mule 28.6%	Serum	14 mules	LAT	≥5 years to <10 years	N/A	(101)
	Faisalabad, Lahore, and Gujranwala	Donkey 58.7%	Serum	75 donkey	LAT	≥5 years to <10 years	N/A	(101)
Camels	Bahawalpur Region	10%	Serum	100 camels	LAT	1–15 years	N/A	(102)
	Bhawalpur, Punjab	17.9%	Serum	201 camel	LAT	1–>13 years	February–December, 2015	(103)
	Punjab	40.1%	Serum	897 one-humped camel	Indirect ELISA	3–7 years	July–August, 2016	(104)
	Mianwali district	38%	Serum	350 camels	Indirect ELISA	3–7 years	N/A	(105)

TABLE 6 Reported prevalence of *Toxoplasma gondii* in poultry and rats in Pakistan.

Species	Area of study	Prevalence rate (%)	Source of detection	No. of samples tested	Diagnostic approach	Age of animal	Year of study	References
Poultry	Mardan	18.85%	Serum	536	IHA	N/A	N/A	(96)
	Faisalabad	36.33%	Serum	300	LAT	<1 to >2 years	July 2011–June 2012	(97)
	Kasur	12.5 %	Serum	200 wild birds	LAT	N/A	N/A	(98)
	Upper dir and Peshawar	10.84	295 tissue samples	295	PCR	30 days to 2 years	N/A	(99)
	Upper Dir and Peshawar	26.6%	398 serum	398	ELISA	30 days to 2 years	N/A	(99)
	Punjab	38.3%	Brain sample	120 rock birds	PCR	N/A	July 2018–October 2018	(100)
Rats	Lahore	58.57% of rats	Serum	Rat 210	LAT	N/A	2012	(61)

IFA, indirect fluorescent assay; IHA, indirect hemagglutination test; ELISA, enzyme-linked immunosorbent assay; LAT, latex agglutination test; PCR, polymerase chain reaction.

cART who have had more than 200 CD4 T cells/L for 3 months can stop using primary prophylaxis without risk. When they reach more than 200 CD4 T cells/L for 6 months, PLWH who have undergone effective therapy for TE and are getting cART can stop receiving maintenance treatment (107). It is important to remember that despite these precautions, *T. gondii* infection cannot be entirely avoided.

10. Conclusion

Infectious diseases of animals including parasitic infestations pose significant threats to health and productivity potential of animals (113–116) which leads to heavy economic losses (117–122). Parasitic infections lead to chronic and debilitating types of diseases and have zoonotic implications as well (3, 123–126). Results of the current evaluation on

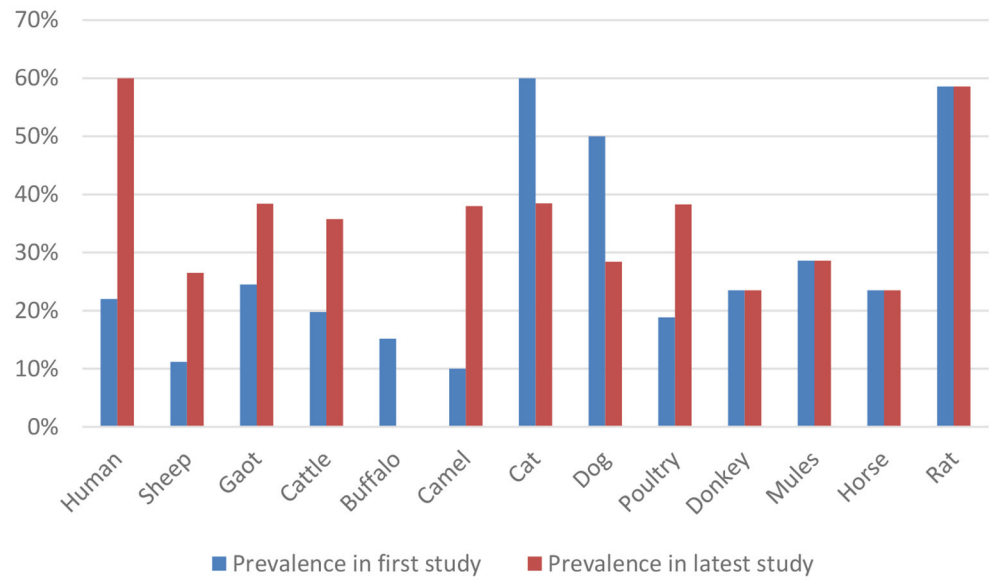

FIGURE 3
Comparison of prevalence rate of *Toxoplasma gondii* between the first and the latest study.

toxoplasmosis research in Pakistan from 2001 to 2022 revealed little information on animal seroprevalence in cases of humans, cattle, buffaloes, sheep, goats, cats, dogs, camels, and horses. In Pakistan, the seroprevalence among human females is rising. The frequency of toxoplasmosis in cattle, mainly chicken intended for human consumption, is also little understood. The *T. gondii* strain prevalent in Pakistan from HIV patients, pregnant women, livestock, and domestic cats has not yet been genetically characterized. Alarming reports of toxoplasmosis in the KPK population have been observed in humans. Despite the widespread occurrence and severe effects of toxoplasmosis, which are mostly seen in immunocompromised patients, there are significant flaws in the present control programs, particularly in the diagnostic resources available. The prevalence throughout the country is increasing every year. The majority of diagnostic procedures also frequently misdiagnose the illness in endemic regions. It is necessary to create molecular approaches that are sensitive, specific, straightforward to use, affordable, and high throughput because early detection is the most effective way to combat the illness. Researchers, healthcare professionals, veterinary professionals, and politicians can benefit from the current review on toxoplasmosis. Therefore, there is an urgent need to inform and educate the public about the risk factors for toxoplasmosis infection in humans and animals. That may be accomplished by running health-related advertisements and educational campaigns in regional newspapers, television, radio, and, more recently, social media platforms.

Data availability statement

All data supporting the conclusions of this article are included within the article.

Author contributions

WQ and AA worked on the development of this unique title of review, planned, designed, and structured the layout of the article. WQ wrote the article. AA reviewed the article. All authors finally approved this review article.

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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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In-vitro evaluation of immunomodulatory activity of sulphation-modified total ginsenosides derivative-3

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Background: Ginseng has been used in biomedicine to prevent and treat decreased physical and mental capacities. Total ginsenosides (TG) from ginseng root which have antitumor and immune-enhancing properties, are the principal active components of *Panax ginseng*, while the sulphation-modified TG derivative-3 (SMTG-d3) was expected to enhance the anticancer activity in conventional medicinal treatments.

Methods: The chlorosulphonic acid–pyridine technique, used for the sulfation modification of TG to improve their biological activity, and the infrared spectroscopic characteristics of TG and SMTG-d3 were investigated, and the effects of SMTG-d3 on immunocytes and cytokines relevant to tumor treatment were assessed. The MTT assay was used to assess the effect of TG and SMTG-d3 on the cytotoxicity and T-lymphocytic proliferation against mouse splenocytes. The LDH method was employed to evaluate NK activity induced by TG or SMTG-d3. The production levels of splenocytes-secreted IL-2 and IFN-γ and peritoneal macrophages-secreted TNF-α were determined using mouse ELISA kits.

Results and discussion: It showed that the ideal conditions for the sulfation modification of TG: the volume ratio of chlorosulfonic acid to pyridine lower than 1:2.5; controlled amount of chlorosulfonic acid; and a yield of 51.5% SMTG-d3 (2 h, $< 45°C$). SMTG-d3 showed two characteristic absorption peaks at 1,230 cm^{-1} and 810 cm^{-1}, indicating the formation of sulfuric acid esters and the presence of sulfuric acid groups. SMTG-d3 exhibited higher antitumor immunological activity than TG by promoting the proliferation of T lymphocytes and the production of IFN-γ and TNF-α, thus enhancing NK cell activity, and reducing cytotoxicity. The findings imply sulfated modification represents an effective method of enhancing the immunomodulatory activities of TG and could be used as the basis for developing new drug target compounds; SMTG-d3 can serve as an antitumor immunomodulator and can be considered an effective and prospective herbal formulation in clinical applications.

KEYWORDS

total ginsenosides (TG), sulphation derivative, immunological activity, antitumor, cytotoxicity

Introduction

The field of complementary/alternative medicine is taking new horizons in the recent era. Herbal medicines are considered to be good alternatives to allopatheic medicine (1, 2). Ginseng (genus Panax, family Araliaceae, Angiosperms, accepted scientific name: *Panax ginseng* C.A. Mey.) is a medicinal herb and an agricultural product that has widely been used in many traditional medicinal therapies in China, Korea, and Japan for thousands of years. Specifically, it has been used for prophylaxis and treatment of decreased physical and mental capacities, including tiredness, exhaustion, and weakness and during convalescence (3–5). Total ginsenosides (TG), dammarane-type triterpene saponins, are the main active components in the root of *P. ginseng*. The quality and composition of the active components depend on various factors, including plant species, cultivation method, age, and the section of the plant used (6, 7). It has been shown that ginseng can inhibit the proliferation and motility of cancer cell lines *in vitro*, and that it has anticarcinogenic properties in animal models; the anticarcinogenic effects of ginseng may in part be related to its ability to inhibit angiogenesis (8–11). Furthermore, anticancer effects of TG are thought to be mediated by an increase in the production of interferons and cytokines, which can activate natural killer (NK) cells and cytotoxic T cells, thereby causing lysis of tumor cells or inhibition of tumor development (12, 13). Notably, modulation of the immune response has been recognized as the pharmacological effect of ginseng, proving that it can modulate multiple immune cell types in terms of proliferation, phagocytic activity, cytokine expression, and antibody production (14–16). Studies performed on animal models have demonstrated that ginseng stimulates a significant immune response, protecting from both viral and bacterial infections, and enhances the protection conferred by vaccine treatment (17–19).

TG is composed of sapogenin and sugar chains. Because the aglycone is a relatively stable cycloalkane structure, it is hard to modify it for structural modification of the TG molecule, whereas sugar plays an essential role in the structure–activity relationship of TG, and its modification is more purposeful. Thus, the modification of TG in this study first focused on the sulfation modification of sugar or sugar chain. Additionally, according to Miyamoto et al. (20) there are sulfate groups on the sugar chain of sea *Cucumaria ehinata* saponins, and the anticancer activity of sea cucumber saponins is related to the sulfate groups. Because the structure of sea cucumber saponins is so similar to that of TG, we believed that modification of TG by sulfation would alter their biological activity. However, it is uncertain whether the sulphation modification of TG can enhance their immunological activity even further. Thus, increasing attention has been placed on the molecular modification and structure–activity relationship of TG. The current study focused on the chlorosulphonic acid–pyridine technique for the sulfation modification of TG to improve their biological activity; and the infrared spectroscopic characteristics of TG and SMTG-d3; to investigate the effects of SMTG-d3 on immunocytes and cytokines relevant to tumor treatment were assessed. In this study, the effects of TG and sulphation-modified TG derivative-3 (SMTG-d3) on immune cells and cytokines relevant to tumor therapy were examined using mouse peripheral blood lymphocytes and mouse peritoneal macrophages cultured *in vitro*; and the alterations in immune activity for the sulfation-modified TG were assessed. This study can serve as a foundation for subsequent chemical modification, such as sulfation, to change the pharmacological activity of TG and monomers contained therein, resulting in saponin derivatives with higher biological activity and reduced toxicity.

Abbreviations: TG, Total ginsenosides; SMTG-d3, sulphation-modified TG derivative 3; NK cells, natural killer cells; LPS, Lipopolysaccharide; ConA, Concanavalin A; LDH; lactate dehydrogenase; MTT, methylthiazol tetrazolium; YAC-1 cells, YAC-1 mouse lymphoma cells.

Materials and methods

Chemicals and reagents

Total ginsenosides (TG) (purity: 95.60 %) were purchased from Hongjiu Biotech. Co., Ltd of Jilin Province (Changchun, China). Lipopolysaccharide (LPS), Concanavalin A (ConA), lactate dehydrogenase (LDH), and methylthiazol tetrazolium (MTT) were obtained from Sigma-Aldrich Co. (St. Louis, USA). Trypsin was obtained from Life Technologies, Inc. (Carlsbad, USA), and RPMI 1640 media and calf serum were from Gibco Co., (Grand Island, USA). Chlorosulphonic acid, pyridine, and other reagents were of analytical grade and were purchased from Tianjin Bodi Chemical Co., Ltd., (China). ELISA Kits for mouse IL-2, IFN-γ, and TNF-α were obtained from Wuhan Boster Bio. Tech., Ltd (Wuhan, China). YAC-1 mouse lymphoma cells (YAC-1 cells) were obtained from Shanghai Institute of Material Medica, CAS, China. All cells in this study were incubated in RPMI-1640 media containing calf serum (10%, v/v) and antibiotics (1%, v/v, penicillin + streptomycin), at 37°C in a humid atmosphere of 5% CO_2. For testing, 0.25% trypsin was used to collect adherent cells in the logarithmic growth phase, and a hemocytometer was used to count the cells. For *in vitro* cytotoxicity studies, cells were seeded into new culture dishes and grown to 80 % confluence before drug treatment.

Animals

Forty female BALB/c mice (18–22 g) with a clean grade (Certificate No. SCXK [G] 2015-001) were collected from Laboratory Animal Center, Lanzhou Veterinary Research Institute, CAAS (China). An ordinary housing facility was used and complied with the national standard, Laboratory Animal Requirements of Environment and Housing Facilities (GB 14925-2001). All animals were kept under standard environmental conditions (23–25°C; relative humidity: 50%; light/dark cycle: 12/12 h). All mice had one-week pre-experimental acclimation period and access to standard food and water ad libitum.

Sulphation modification of TG and infrared spectroscopy analysis

Sulphation-modified TG (SMTG) was prepared using the chlorosulphonic acid–pyridine method (21, 22). In the present study, according to the volume ratio of chlorosulfonic acid to pyridine, reaction temperature, and reaction time, nine treatment schemes were designed by three-factor and three-level orthogonal test method.

Nine derivatives were obtained, and nine different conditions were named in proper sequence from 1 to 9. Explicitly, TG and sulphation-modified TG derivative-3 (SMTG-d3) were used in the tests below. The yield of derivative was used as the investigation index. Yield (%) was calculated as follows: weight of products / weight of total ginsenosides × 100%.

Fourier-transform infrared (FT-IR) spectra were obtained *via* KBr tablets on a Nicolet Avatar FT-IR 360 and Nicolet FT-IR 170 SX infrared spectrophotometer (Thermo Fisher Scientific).

T-lymphocyte proliferation test

Cytotoxicity of TG and SMTG-d3 against mouse splenocytes was evaluated by conventional MTT assay as described previously (23, 24). A suspension of mouse splenocytes was resuspended at 2.0×10^6/mL with RPMI-1640 complete media. The cells were inoculated into 96-well plates with 100 μL per well, and treated with different concentrations of ConA-containing TG or SMTG-d3 (50 μL/well, at 5, 10, and 50 μg/mL concentrations). The final concentration of ConA in each well was 10 μg/mL. RPMI-1640 medium and ConA were applied to four wells, each for the negative control and ConA control; each sample had four replicate wells. The plates were incubated for 44 h at 37°C under 5% CO_2. We added 20 μL of MTT per well (5.0 mg/mL) and incubated for an additional 4 h at 37°C. Then, the supernatant was aspirated, and 100 μL/well of DMSO (dimethyl sulfoxide) was added to dissolve formazan crystals existing in the viable cells. The absorbance was determined as the index of splenic T-lymphocyte proliferation using a microplate reader at 570 nm (Thermo Multiskan Mk3, USA). In addition, the cytotoxic concentrations of the drug (TG or SMTG-d3 without ConA), were determined by observing the half-inhibitory concentration of the drug on mouse T lymphocytes, and the method was the same as above.

NK-cell activity test

YAC-1 cells were prepared at 1.0×10^6/mL, and NK activity induced by TG or SMTG-d3 was measured by the LDH approach (25). Namely, 100 μL of splenocytes treated with TG or SMTG-d3 (5, 10, and 50 μg/mL) was placed in 96-well plates (20 μL drugs + 80 μL splenocytes), and then 100 μL of YAC-1 cells was added (E/T = 50/1). Additionally, the target cell's spontaneous-release wells (100 μL YAC-1 cells + 100 μL RPMI-1640) and maximum release wells (100 μL YAC-1 cells + 1% NP-40) were set as controls; each sample had four replicate wells. The plates were incubated for 24 h at 37°C under 5 % CO_2. Then, supernatant (100 μL) was aspirated from each well, added to another culture plate, and pre-warmed at 37°C for 10 min. We then added 100 μL of LDH substrate solution freshly prepared into each well, allowed reaction at room temperature for 10–15 min (protected from light), and added 30 μL of citric acid stop solution (1.0 mol/L) into each well to stop the reaction. The absorbance was recorded using a microplate reader (570 nm).

The NK-cell activity, expressed as the percentage of release, was determined by the following formula based on the average values from the four wells: NK cells activity (%) = (values of experimental group – values of spontaneous-release group) / (values of maximum-release group – values of spontaneous-release group) × 100%.

Detection of levels of IL-2, IFN-γ, and TNF-α secretion

In axenic conditions, splenocyte suspensions (2.0×10^6/mL) and peritoneal macrophage suspensions (1.0×10^6/mL) from the mice were prepared and added to 24-well plates (850 μL/well); meanwhile, 50 μL/well of ConA (in which the final concentration was 5 μg/mL) or LPS (in which the final concentration was 10 μg/mL) were added; then, TG or SMTG-d3 (at 5, 10, and 50 μg/mL concentrations) were added (100 μL/well). The plates were incubated for 24 h at 37°C in 5% CO_2. Then, the contents of the wells were centrifuged, and the supernatants were collected (26, 27). The production levels of splenocytes-secreted IL-2 and IFN-γ, and peritoneal macrophages-secreted TNF-α, were detected using mouse ELISA kits based on the manufacturer's test instructions.

Statistics

The data analysis was performed using SPSS Statistics 25.0 (IBM, Chicago, USA). One-way analysis of variance (ANOVA) followed by *post hoc* multiple-comparison tests (Tukey, Duncan, and Dunnett two-sided), and two-tailed *t* test (Dunnett's T3) were performed to estimate the significance of differences between the groups of control and TG or SMTG-d3. Results were expressed as mean values and standard deviations (mean ± SD), and graphs were drawn using OriginPro 2017C 64-bit (OriginLab Corporation Northampton, MA, USA). In all cases, differences were considered statistically significant when P was lower than 0.05.

Results

Sulphation modification of TG and infrared spectroscopy

According to the yield index and the degree of substitution of sulfuric acid groups, the results showed that the order of the three factors acting on the sulfation modification of TG was as follows: the temperature of the esterification reaction, the ratio of chlorosulfonic acid to pyridine, and the reaction time. However, none of the three factors significantly affected the yield index. The ideal conditions for the sulfation modification of TG were as follows: the volume ratio of chlorosulfonic acid to pyridine lower than 1:2.5; controlled amount of chlorosulfonic acid; the reaction time of 2 h; the reaction temperature lower than 45°C; and the yield of SMTG-d3 reaching 51.5 %.

The FT-IR spectroscopy of TG and SMTG-d3 is shown in Figure 1. Six absorption bands at 1,638 cm^{-1}, 1,330 cm^{-1}, 1,254 cm^{-1}, 1,037 cm^{-1}, 921 cm^{-1}, and 851 cm^{-1} were the characteristic absorption bands of TG; there was a strong absorption peak at 3,400 cm^{-1}, indicating the existence of -OH groups; and the strong absorption at 935–1,044 cm^{-1}, which indicated the stretching vibration of C-O-C (sugar ring). Compared with the IR spectrum of TG, two new characteristic absorption bands appeared in the IR spectra of SMTG-d3, one at near 1,230 cm^{-1} (describing an

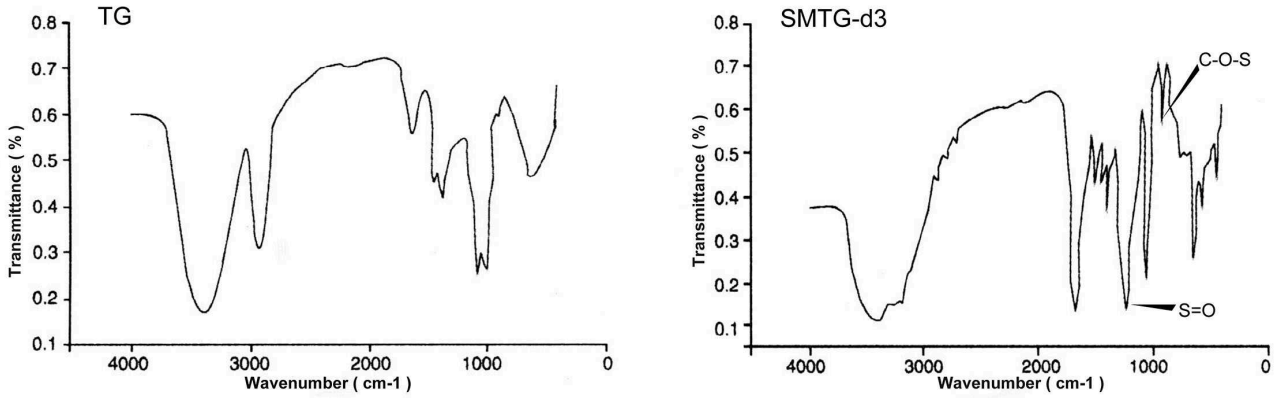

FIGURE 1
Fourier-transform infrared (FT-IR) spectroscopic analysis of TG and SMTG-d3 TG, total ginsenosides; SMTG-d3, sulphation-modified total ginsenosides derivative-3.

asymmetrical S=O stretching vibration) and the other at near 810 cm^{-1} (representing a symmetrical C-O-S vibration associated with C-O-SO_3 group), which were the characteristic absorption peaks of sulfuric acid bonds.

T-lymphocyte proliferation and NK-cell activity

The cytotoxicity assay of TG and SMTG-d3 on mouse T lymphocytes showed that TG significantly inhibited the proliferation of T lymphocytes at a concentration of 1,000 μg/mL ($P < 0.01$). In contrast, SMTG-d3 still had a strong promoting effect on the proliferation of T lymphocytes at the same concentration, and it significantly boosted the proliferation of splenocytes in the 50–1,000 μg/mL range ($P < 0.01$). Our findings demonstrated a considerable improvement in SMTG-d3 synergized with ConA to boost T-lymphocyte proliferation (Figures 2A, B).

Compared with the spontaneous-release group, both TG and SMTG-d3 significantly promoted the killing effect of NK cells on YAC-1 cells within the 5–50 μg/mL range ($P < 0.01$). Compared with the same concentration of TG, SMTG-d3 displayed more powerful capability ($P < 0.01$) (Figure 2C).

Levels of IL-2, IFN-γ, and TNF-α

According to the detection of the levels of cytokines, TG exhibited an inhibitory effect on the level of IL-2 secreted by mouse T lymphocytes in the 5–50 μg/mL range after co-culture with T lymphocytes for 24 h ($P < 0.01$). SMTG-d3 also showed a significant inhibitory effect at the concentration of 5 μg/mL and 50 μg/mL ($P < 0.01$), but at the concentration of 10 μg/mL, it demonstrated a promoting effect on the production of IL-2 ($P < 0.01$), that is, the effect of SMTG-d3 on IL-2 secretion was of bell-jar type. Moreover, the effects of TG and SMTG-d3 on IL-2 secretion at the same concentration also showed significant differences ($P < 0.01$) (Figure 2D). After TG and SMTG-d3 were co-cultured with T lymphocytes for 24 h, respectively, the levels of IFN-γ in the culture supernatant were measured. The results indicated that both TG and SMTG-d3 were able to significantly promote the secretion of IFN-γ by mouse T lymphocytes in the range from 5 μg/mL to 50 μg/mL ($P < 0.01$). In general, the enhancing capacity of SMTG-d3 on IFN-γ production was significantly better than that of TG at the same concentration ($P < 0.01$) (Figure 2E). After TG or SMTG-d3 were co-cultured with mouse peritoneal macrophages for 24 h, TNF-α secretion in the culture supernatant exhibited higher levels compared with the control ($P < 0.01$); and the SMTG-d3 promoted the secretion of TNF-α significantly better than TG at the same concentration ($P < 0.01$) (Figure 2F).

Discussion

The chemical modification research of natural active ingredients has now become a hot topic. Starting with the active ingredients of natural medicines, structural modification and systematic drug activity are studied to summarize the correlation between structure and activity (toxicity), which is then used as the basis for designing new drug target compounds (28–32). In a previous study, the sugar chain of TG was modified by chemical modification, and Marek's disease was employed as an animal model for the occurrence and development of viral tumors (33–36). The findings of immunological activity comparison revealed that there were fewer positive cells in the TG and its derivative drug groups than in the moroxydine group in chickens Marek's disease virus-infected; moreover, TG induced the apoptosis of MSB-1 cells (Marek's disease tumor cells) and inhibited the proliferation of MSB-1 cells. We noticed that sulfated polysaccharides often had better anti-virus, anti-tumor, and immune-improving activities (37–40), which suggests that the sulfation modification of TG should be investigated. TG are best known for their curative properties in cancer, neurodegenerative disorders, and cardiovascular diseases, and is composed of sapogenins and sugar chains. According to the structure–activity relationship of TG, their biological activity is related to the number of sugar chains present, implying that sugar chains play an important role in TG (41, 42). Thus, it is critical to sulfate the sugar chain of TG, change and enhance the biological activity, and discover more active medications from it.

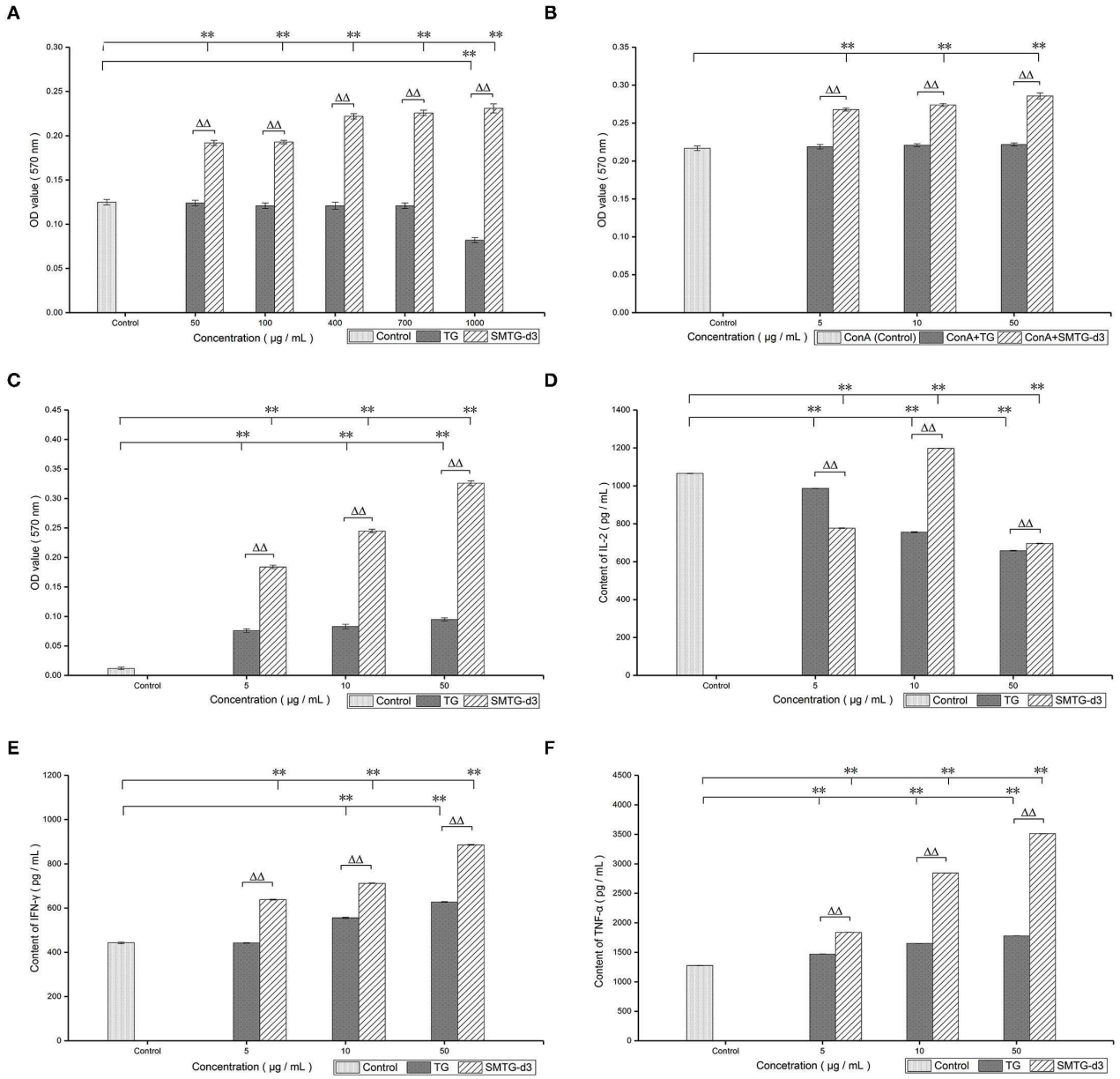

FIGURE 2
In vitro immunological activity of TG and SMTG-d3. Data are presented as mean ± SD. **(A)** Assay of cytotoxic concentration; **(B)** T-lymphocyte proliferation; **(C)** NK-cell activity; **(D)** IL-2 production; **(E)** IFN-γ production; **(F)** TNF-α production. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, compared with the TG group of the same concentration. TG, total ginsenosides; SMTG-d3, sulphation-modified total ginsenosides derivative-3.

In the present study, the chlorosulfonic acid–pyridine method was used to prepare sulfation-modified TG (SMTG) (21). Three main factors that affect the sulfation of polysaccharides were selected to facilitate the investigation and avoid too many experiments; these factors were the volume ratio of chlorosulfonic acid to pyridine, the reaction temperature, and the reaction time. We showed that the main factors affecting the sulfation degree of TG were the ratio of esterification agent, reaction temperature, and reaction time. We set the three levels each, and analyzed them according to the yield index through orthogonal design experiments. The results showed that the volume ratio of chlorosulfonic acid to pyridine was lower than 1:2.5; the amount of chlorosulfonic acid was controlled; the reaction time was 2 h; the reaction temperature was lower than 45°C; and the sulfation modification of TG was better. On this basis, the yield of preparing SMTG-d3 was >50%, and the effect was ideal.

The FT-IR spectrum was used to analyze the possible functional groups and bond types. Experimental results from the IR spectroscopy of TG showed that the band in the region of 3,400 cm^{-1} corresponded to the hydroxyl stretching vibration and indicated the existence of -OH groups; the band in the region of 2,920 cm^{-1} corresponded to a C-H stretching vibration; and the weak absorption bands at 1,460 cm^{-1} and 1,380 cm^{-1} corresponded to a -CH3 stretching vibration. The absorption at 1,640 cm^{-1} indicated the C=C stretching vibration; that at 1,150 cm^{-1} indicated

the C-O stretching vibration of C-O-C group; and that at 1,110 cm^{-1} indicated the O-H angular vibration, which illustrated the structure of TG. In addition to retaining the characteristic absorption peaks of TG, SMTG-d3 had absorption peaks at 1,230 cm^{-1} and 810 cm-1, corresponding to the S=O stretching vibration of OSO_3^- group and C-O-S stretching vibration, respectively. These two absorption peaks indicated that the sulphation modification had taken place in TG, which involved the formation of sulfuric acid esters and the existence of sulfuric acid groups in the molecule (Figure 1). These results showed that it was feasible to modify TG and even saponins by sulfation.

In this study, treatment with 1,000 μg/mL TG inhibited splenocyte proliferation ($P < 0.01$). However, SMTG-d3 significantly boosted the proliferation of splenocytes in the 50–1,000 μg / mL range ($P < 0.01$) (Figure 2A). These findings indicated that the sulphation modification markedly reduced the cytotoxicity of TG, and SMTG-d3 was effective for clinical practice at a lower dose. Lymphocytes, an essential component of the immune system, are involved in numerous disease-induced immunological responses. Tumor immunity is made up mostly of cellular and humoral immunity, with the former being superior to the latter (43–47). As a part of the immune response, activated T lymphocytes can secrete IFN-γ. Our findings revealed that both TG and SMTG-d3 boosted the proliferation of splenic T lymphocytes in the 5–50 μg/mL range, with SMTG-d3 producing a greater increase in T-lymphocytes proliferation than TG ($P < 0.01$) (Figure 2B). ConA stimulates T lymphocytes primarily as a mitogen; thus, treatment with ConA induces the cells to move into G_1 phase from G_0 phase (DNA presynthetic phase); otherwise, the cells remain in the G_0 phase. In this study, the proliferation of T lymphocytes was significantly enhanced by SMTG-d3, indicating that SMTG-d3 caused a large number of T lymphocytes to transition from a resting to an active state by regulating the cell proliferation cycle. Furthermore, compared with the same concentration of TG, SMTG-d3 had an obvious synergistic effect with ConA, and showed a significant positive result in promoting T-lymphocyte proliferation *in vitro*.

NK cells can directly destroy tumor cells after activation and secrete some cytokines, such as interleukins and interferon, which participate in immune killing and immune elimination (45, 48). Our data showed that TG and SMTG-d3 both increased the NK-cell activity in the 5–50 μg / mL range ($P < 0.01$); however, SMTG-d3 was more effective than TG at enhancing NK cell activity ($P < 0.01$) (Figure 2C). These findings indicate that SMTG-d3 may effectively kill YAC-1 cells or inhibit tumor cell development. Numerous crucial physiological processes, including immune response and regulation, and stimulation of cell proliferation and differentiation *in vivo*, are affected by cytokines. To further explore the cellular immune regulation mechanism of TG and SMTG-d3, and given that both TG and SMTG-d3 had good proliferative effects on T lymphocytes *in vitro*, the effects of TG and SMTG-d3 on the cytokines released by lymphocytes and macrophages were further investigated. IL-2 is produced by activated T lymphocytes and mainly promotes the activation and proliferation of T and B lymphocytes. After T lymphocytes are stimulated by ConA, they begin to secrete IL-2; meanwhile, they also express a high level of IL-2R. We demonstrated that both TG and SMTG-d3 had obvious inhibitory effects on the secretion of IL-2 by mouse T lymphocytes within the concentration range in this test ($P < 0.01$) (Figure 2D). Compared with the same concentration of TG, the effect of SMTG-d3 on inhibiting the secretion of IL-2 from T lymphocytes was significantly weaker ($P < 0.01$) (at the concentration of 10 μg/mL). The inhibitory effect of TG was in a negative dose–response relationship, and the secretion effect of SMTG-d3 on IL-2 was related to the dosage, showing a bell-jar type. The results above could be attributed to the antagonism of ConA by TG or SMTG-d3, as well as the decreased expression of IL-2R, and suppression of the proliferation signal. In earlier research (49–52), it was discovered that ginsenosides improved the reduction of IL-2 secretion in experimental animals under immunosuppression conditions, but had no appreciable impact on the immune function in healthy animals, which might indicate the immunomodulatory effect of ginsenosides. The difference between our findings and those of the previous studies may stem from the differences between *in vivo* and *in vitro* testing, and pathology and health, and further research is necessary to determine the action mechanism.

Many studies shown that activated T lymphocytes secrete IFN-γ, which can directly inhibit the proliferation of tumor cells and boost the expression of TNF-α; TNF-α can selectively destroy tumor cells or permit not-yet-diseased cells to move into an anti-tumor condition in a paracrine manner (3, 12, 53–55). Our data showed that both TG and SMTG-d3 significantly promoted the production of IFN-γ and TNF-α within the range of 5–50 μg/mL concentration ($P < 0.01$); and compared with TG at the same concentration, SMTG-d3 promoted the secretion of IFN-γ and TNF-α more considerably ($P < 0.01$) (Figures 2E, F). Many studies have suggested that the production of IFN-γ is inhibited in the whole organism and tumor region in cancer patients (5, 12, 38, 49). Recent discoveries have revealed that TG and their derivatives play an important role in assisting tumor cells in apoptosis and differentiation, as well as in increasing tumor cell sensitivity to chemotherapeutic treatments. TG in combination with chemotherapeutic medicines are currently utilized mostly to improve the efficacy of primary lung cancer and liver cancer treatment (5, 56, 57). Thus, promoting the production of IFN-γ by SMTG-d3 may be beneficial during radiotherapy and chemotherapy. Findinds above implying that SMTG-d3 could work as an antitumor immunomodulator, and serve as foundation for subsequent chemical modification, such as sulfation to change the pharmacological activity for TG and monomers contained therein, resulting in saponin derivatives with higher biological activity and reduced toxicity.

Conclusions

The capacity of TG and SMTG-d3 to improve immunity was discovered to be one of the primary mechanisms underlying their antitumor activity. For their immunoregulatory impact, TG and SMTG-d3 increased the activity of T lymphocytes, NK cells, and peritoneal macrophages, and aided in the production of IFN-γ and TNF-α throughout the antitumor process. Moreover, compared with TG, SMTG-d3 further enhanced antitumor immunity while lowering cytotoxicity. Hence, using ginseng's active ingredients as a starting point, and examining the relationship between structural modification and activity (toxicity), could be used as the basis for developing new drug target compounds; and the customized derivatives with desirable functional characteristics can be produced. SMTG-d3 appears to be a promising antitumor immunomodulator,

and it was expected to give greater value to the anticancer activity in traditional medicinal therapies.

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 strictly abided by the National Institutes of Health (NIH) Guidelines for Laboratory Animal Treatment and Use (58), and the study was approved by the Ethics Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences (Lanzhou, China).

Author contributions

ZG and LiW conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures, authored or reviewed drafts of the article, and approved the final draft. SH performed the experiments, analyzed the data, prepared figures, authored or reviewed drafts of the article, and approved the final draft. LuW conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures, and approved the final draft. WG and YL performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft. NI helped modify the final version manuscript and approved the final draft. 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.2023.1068315/full#supplementary-material

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Effects of thiram exposure on liver metabolism of chickens

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Pesticides are widely used to control crop diseases, which have made an important contribution to the increase of global crop production. However, a considerable part of pesticides may remain in plants, posing a huge threat to animal safety. Thiram is a common pesticide and has been proven that its residues in the feed can affect the growth performance, bone formation, and intestinal health of chickens. However, there are few studies on the liver metabolism of chickens exposed to thiram. Here, the present study was conducted to investigate the effect of thiram exposure on liver metabolism of chickens. Metabolomics analysis shows that 62 metabolites were down-regulated (ginsenoside F5, arbekacin, coproporphyrinogen III, 3-keto Fusidic acid, marmesin, isofumonisin B1, 3-Hydroxyquinine, melleolide B, naphazoline, marmesin, dibenzyl ether, etc.) and 35 metabolites were up-regulated (tetrabromodiphenyl ethers, deoxycholic acid glycine conjugate, L-Palmitoylcarnitine, austalide K, hericene B, pentadecanoylcarnitine, glyceryl palmitostearate, quinestrol, 7-Ketocholesterol, tetrabromodiphenyl ethers, etc.) in thiram-induced chickens, mainly involved in the metabolic pathways including glycosylphosphatidylinositol (GPI)-anchor biosynthesis, porphyrin and chlorophyll metabolism, glycerophospholipid metabolism, primary bile acid biosynthesis and steroid hormone biosynthesis. Taken together, this research showed that thiram exposure significantly altered hepatic metabolism in chickens. Moreover, this study also provided a basis for regulating the use and disposal of thiram to ensure environmental quality and poultry health.

KEYWORDS

thiram, liver, metabolism, chicken, pesticides

Introduction

Increasing evidence indicated that pesticides play a vital role in agricultural production. Statistical analysis indicated that China is the main consumer of pesticides, using 1.8 million tons per year, followed by the America (1–3). At present, pesticides have been listed as priority pollutants by the United Nations Environment Protection Agency (UNEP) (4, 5). Although, the use of pesticides has effectively increased crop yield and reduced disease. However, the extensive use of pesticides will also cause serious environmental pollution, posing a serious threat to food security and animal health (6). In addition, some pesticides may remain in plants and be introduced into nearby waters after rainfall, endangering the health of aquatic animals and causing drinking water safety problems (7–16). Moreover,

aerial spray of pesticides may cause the pollution of nearby or distant areas through transboundary movement (17). It is worth noting that humans and animals may also ingest plants containing pesticides through the food chain, seriously endangering public health and human security (18–21).

Poultry including chickens, ducks and geese are the largest livestock species. These species developed rapidly in the past few decades, effectively solving the problem of protein shortage. Among the above-mentioned poultry, chickens are widely farmed because of their fast growth and low price (22, 23). Consequently, any factors that endanger chickens should be given enough attention. However, chickens are likely to be exposed to feed containing pesticide residues (24–27). Previous studies have indicated that most pesticides could accumulate in multiple tissues and inhabit the exposed organisms from few months to several years, thus even very low concentration is also harmful to health (5, 28, 29). Liver is the vital metabolic and alexipharmic organ in the animal and humans, which is considered as one of the primary target organs for various hazardous substances such as pesticides and heavy metal (30–32). Therefore, the intake of feed containing pesticide residues will inevitably affect the liver of broilers.

Thiram is one of the common pesticides, mainly used to increase crop yield and reduce disease (33, 34). However, the abuse of thiram not only cause pesticide residues, but also pose a serious threat to the safety of humans and animals (35–37). Previous studies have shown that thiram exposure causes abnormal bone development and reduced growth performance in chickens (38–40). In addition, thiram exposure has been demonstrated to cause intestinal flora imbalance and liver histopathology injuries in chickens (18, 26). However, studies regarding the influences of thiram exposure on liver metabolism in chickens remain scarce. Taking advantage of this gap, we explored the effect of thiram exposure on liver metabolism in chickens.

Materials and methods

Animal experiments and sample acquisition

A group of 60 one-day-old healthy Arbor Acres chickens were purchased from a commercial hatchery and maintained under the standard ambient temperature, sanitary condition and illumination as previously described. Prior to the experiment, all the subjects were performed physical examinations to avoid deformity and other congenital diseases. After acclimatization for 3 days, an equal number of chickens (n = 30) regardless of sex were divided into control and thiram-treated groups. Throughout the trial, the control chickens were provided sufficient feed and water. Moreover, the chickens in thiram-treated group received same diet as controls but supplemented with thiram (50 mg/kg) purchased from Macklin Biochemical Co., Ltd. (Shanghai, China) in feed as suggested by previous research from days 3–7 (39). All chickens were euthanized and liver tissue was collected on days 18 of the experimental study. The achieved samples were snap-frozen utilizing liquid nitrogen and stored at −80°C for further study.

Sample preparation

The metabolomic procedure was conducted based on the previous protocols with minor improvements (41, 42). Briefly, the acquired liver samples (∼100 mg) were triturated in methanol and then centrifuged for 15 min at 14,000×*g*. The supernatant of mixture was collected and stored in Eppendorf tubes for 10 min. Subsequently, the deionized water (400 μl) was added to the obtained supernatant and kept at −80°C for further study. The extract (100 μl) of each sample was mixed for preparing quality control (QC) sample and QC samples were performed testing between every five samples. The 0.22 μm membranes were applied to filter the supernatant and then the filtered supernatant was performed UPLC-QTOF/MS (Waters, USA) analysis. The condition of UPLC was determined as described previously (42). Moreover, the reagents used in this study were HPLC grade.

Differential metabolite analysis

The original mass spectrometry was subjected to process using Marker View 1.1 (AB SCIEX, USA). Subsequently, PCA and PLS-DA were performed by importing metabolomics data into SIMCA (version 14.1, Umetrics, Sweden). The determination of differential metabolites was based on the variable weight value (VIP) and *p*-value obtained from the OPLS-DA model. To obtain pathways involved in differential metabolites, MetaboAnalyst and KEGG database (https://www.kegg.jp/kegg/pathway.html) was used to perform cluster analysis and metabolic pathway annotation of differential metabolites.

Results

Thiram exposure disrupts liver metabolism

The plots of PCA analysis showed that the samples in the thiram-exposed group were clustered closely and separated from the control group, indicating that thiram exposure significant changes in liver metabolome (Figures 1A, B). To further reveal the alterations of liver metabolome during thiram exposure, OPLS-DA score plots was applied for pattern discriminant analysis. Results indicated that there was a clear separation between both groups and no fitting occur (Figures 1C–F).

Identification of metabolites associated with thiram exposure

The differential metabolites were recognized based on the criterion of VIP > 1, $P < 0.05$. Results indicated that a total of 97 differential metabolites were detected between both groups (Table 1). Among significantly different metabolites, 62 metabolites (ginsenoside F5, arbekacin, coproporphyrinogen III, 3-keto Fusidic acid, marmesin, etc.) were down-regulated, whereas 35 metabolites (L-Palmitoylcarnitine, quinestrol, 7-Ketocholesterol,

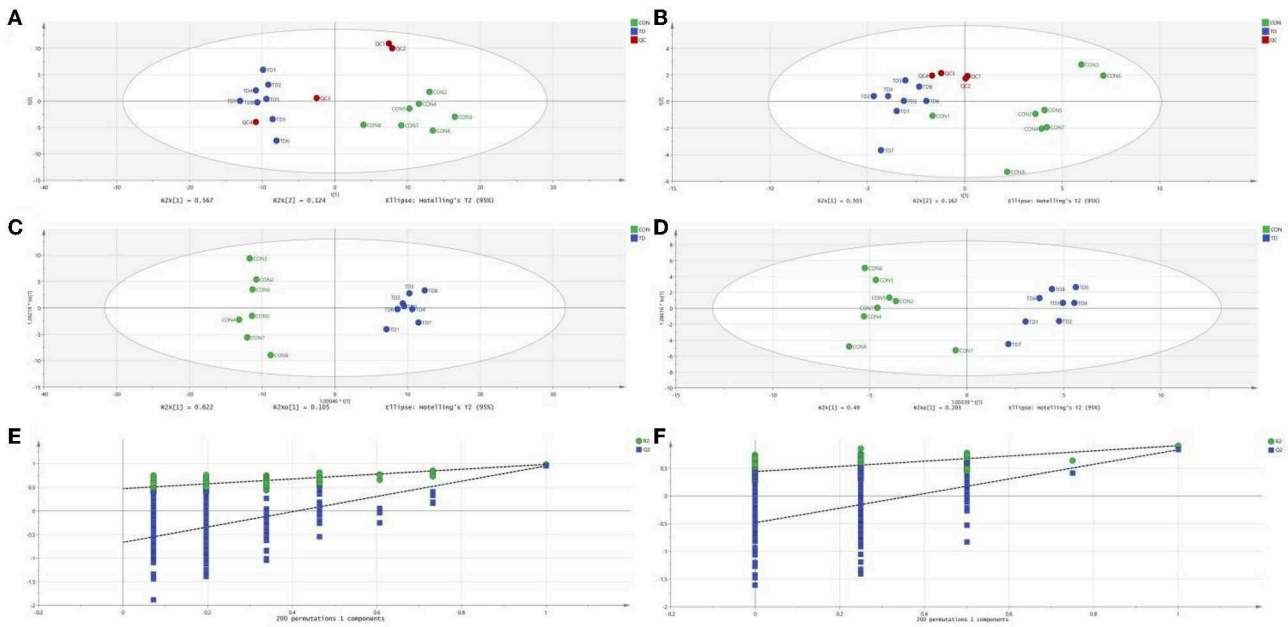

FIGURE 1
Thiram exposure altered liver metabolism. **(A, B)** PCA score plots based on positive-ion mode and negative-ion mode, respectively. **(C, D)** OPLS-DA plot based on positive-ion mode and negative-ion mode, respectively. **(E, F)** Permutation tests based on positive-ion mode and negative-ion mode, respectively.

tetrabromodiphenyl ethers, etc.) were up-regulated in thiram-induced chickens. Moreover, the alternations of metabolites also could be observed in the heatmap (Figure 2).

Metabolic pathway analysis

The differential metabolites were subjected to pathway analysis by utilizing MetaboAnalyst 4.0 and results indicated that 13 metabolic pathways (linoleic acid metabolism, glycerophospholipid metabolism, taurine and hypotaurine metabolism, vitamin B6 metabolism, alpha-Linolenic acid metabolism, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, sphingolipid metabolism, porphyrin and chlorophyll metabolism, arachidonic acid metabolism, fatty acid degradation, primary bile acid biosynthesis, purine metabolism and steroid hormone biosynthesis) involved in hepatotoxicity induced by thiram (Figure 3). Among above-mentioned differential pathways, 5 pathways with highest pathway impact value were the glycosylphosphatidylinositol (GPI)-anchor biosynthesis, porphyrin and chlorophyll metabolism, glycerophospholipid metabolism, primary bile acid biosynthesis and steroid hormone biosynthesis. The metabolic diagram in the intestine is shown in Figure 4.

Discussion

Thiram is widely used in agricultural production and is likely to accumulate in plants (43–45). Some plant-sourced feeds that accumulate pesticides are likely to enter poultry farming through the food chain, posing a serious threat to the health of poultry (18, 38, 39). At present, the harm of thiram exposure to various species such as mice, chickens and fish has been widely confirmed. For instance, thiram has been shown to dramatically affect the respiratory tract, central nervous system, stimulate skin and restrain the formation of white blood cells (34, 46, 47). Furthermore, some studies have also demonstrated the role of thiram exposure in the induction of lipid metabolism (18). The liver is an important metabolic and detoxifying organ in animals and humans, which is regarded as one of the main target organs for multiple stimulations including pesticides, heavy metals and various environmental pollutants (48–50). Therefore, pesticide residues in feed are likely to affect liver health, which will cause great damage to poultry production. However, study on thiram toxicities to liver of chicken is still lacking. In this study, we explored the effect of thiram exposure on liver metabolism in chickens.

In this study, 97 differential metabolites were totally recognized, which was closely related to multiple metabolic pathways including glycerophospholipid metabolism, porphyrin and chlorophyll metabolism, primary bile acid biosynthesis, steroid hormone biosynthesis and glycosylphosphatidylinositol (GPI)-anchor biosynthesis. These metabolic pathways may play an important role in the hepatotoxicity induced by thiram. Remarkably, some of the decreased metabolites including ginsenosides, arbekacin, coproporphyrinogen III, Fusidic acid, marmesin and fluorouracil play important roles in antioxidant capacity, anti-cancer and oxygen transport. Ginsenosides were widely recognized because

TABLE 1 Statistical analysis of differential metabolites between thiram-exposed and control groups.

Metabolites	Fold change	VIP	Trend
Isofumonisin B1	9.33	1.21	Down
3-Hydroxyquinine	5.86	1.20	Down
16b-Hydroxystanozolol	2.87	1.19	Down
9E,11E-Octadecadienoic acid	3.70	1.19	Down
Melleolide B	6.80	1.19	Down
Naphazoline	2.64	1.19	Down
Marmesin	3.32	1.19	Down
Dibenzyl ether	3.72	1.19	Down
Ginsenoside F5	11.28	1.19	Down
Imazamethabenz-methyl	4.93	1.18	Down
Darifenacin	30.79	1.18	Down
7-Aminonitrazepam	2.87	1.18	Down
Cer(d18:0/20:0)	7.18	1.17	Down
CPA(16:0/0:0)	2.67	1.17	Down
Momordicoside D	4.69	1.17	Down
Sintaxanthin	2.80	1.17	Down
Valero-1,5-lactam	2.13	1.17	Down
Tacrolimus	14.95	1.17	Down
Tetrabromodiphenyl ethers	3.69	1.17	Up
Pubescenol	3.28	1.16	Down
Sorbitan stearate	2.13	1.16	Down
[6]-Gingerdiol 4'-O-beta-D-glucopyranoside	2.68	1.16	Down
Allixin	2.19	1.16	Down
Fentanyl	3.74	1.16	Down
trans-2-Dodecenoylcarnitine	2.92	1.16	Down
PC(DiMe(11,3)/MonoMe(13,5))	3.65	1.16	Down
Pyrrhoxanthinol	3.14	1.16	Down
Bufotenine O-glucoside	3.60	1.15	Down
Hesperaline	4.72	1.15	Down
Momordin Ia	5.63	1.15	Down
Fluorouracil	3.66	1.15	Down
Deoxycholic acid glycine conjugate	2.07	1.15	Up
Coproporphyrinogen III	6.16	1.14	Down
Furcogenin 3-[2″-glucosyl-6″-arabinosylglucoside]	9.88	1.14	Down
Clionasterol	3.77	1.14	Down
L-Palmitoylcarnitine	2.70	1.14	Up
trans-Zeatin-O-glucoside riboside	2.40	1.14	Down

(Continued)

TABLE 1 (Continued)

Metabolites	Fold change	VIP	Trend
Myricetin 7-(6″-galloylglucoside)	2.80	1.14	Down
2-Methylbutyroylcarnitine	2.06	1.13	Down
Austalide K	4.74	1.13	Up
Glutamyl-Threonine	3.68	1.13	Down
25-Acetyl-6,7-didehydrofevicordin F 3-glucoside	5.65	1.12	Down
Adenosine diphosphate ribose	5.37	1.12	Up
N-Stearoyl GABA	2.04	1.12	Down
Lc3Cer	4.74	1.12	Up
(S)-Pterosin K	3.00	1.12	Down
Pentacosanoylglycine	3.96	1.11	Up
Ethanolamine Oleate	4.42	1.11	Up
Agavoside A	26.93	1.10	Down
Hericene B	3.19	1.10	Up
Pentadecanoylcarnitine	3.82	1.10	Up
Glyceryl palmitostearate	10.38	1.10	Up
Oseltamivir	4.65	1.10	Down
MG(18:0/0:0/0:0)	2.80	1.09	Up
Physapruin B	3.92	1.09	Down
N-Methoxyspirobrassinol	10.02	1.09	Up
Mactraxanthin	7.83	1.09	Down
Deoxycorticosterone	8.73	1.08	Down
2-Oxo-3-hydroxy-4-phosphobutanoic acid	2.23	1.08	Up
4,8 Dimethylnonanoyl carnitine	3.36	1.08	Down
Tridemorph	2.56	1.08	Up
PE([14:0/16:1(9Z))]	3.31	1.07	Up
1-Palmitoylglycerophosphoinositol	3.02	1.07	Down
45-Hydroxyyessotoxin	2.37	1.07	Up
PS([18:2(9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))]	3.55	1.07	Up
Rose bengal	3.62	1.06	Up
Remikiren	2.58	1.06	Up
Hydrocortamate	2.05	1.06	Down
DG[18:4(6Z,9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z)/0:0]	2.53	1.06	Down
Dehydrocarpaine I	2.28	1.06	Down
2-Stearoylglycerophosphoinositol	2.50	1.05	Up
Torvoside C	2.06	1.04	Down

(Continued)

TABLE 1 (Continued)

Metabolites	Fold change	VIP	Trend
Orange B	10.70	1.04	Down
7-Ketocholesterol	5.39	1.04	Up
MG(0:0/16:1(9Z)/0:0)	2.48	1.04	Up
Pergolide	8.78	1.03	Down
PGP[16:0/22:4(7Z,10Z, 13Z,16Z)]	3.33	1.03	Down
3-O-Protocatechuoylceanothic acid	3.37	1.02	Down
3'-N-Acetyl-4'-O-(9-octadecenoyl)fusarochromanone	2.12	1.01	Up
PC[18:0/22:6(4Z,7Z,10Z, 13Z,16Z,19Z)]	3.38	1.01	Up
Mifepristone	2.14	1.00	Down
Quinestrol	3.48	1.00	Up
(E)-2-Tridecene-4,6,8-triyn-1-ol	26.33	1.48	Down
PC[16:0/22:6(4Z,7Z,10Z,13Z, 16Z,19Z)]	6.05	1.28	Up
Arbekacin	8.07	1.25	Down
7-(4-Hydroxyphenyl)-1-phenyl-4-hepten-3-one	11.68	1.24	Down
Sulfolithocholylglycine	4.91	1.23	Up
Taurocholic acid	7.22	1.23	Up
12S-HHT	7.82	1.16	Up
SM[d18:1/24:1(15Z)]	18.06	1.16	Up
N-Stearoylsphingosine	2.96	1.07	Up
PC(18:3(9Z,12Z,15Z)/20:0)	4.09	1.07	Up
Mifepristone	14.97	1.05	Up
PC[15:0/20:3(5Z,8Z,11Z)]	4.33	1.04	Up
Mianserin	4.37	1.04	Down
3-keto Fusidic acid	4.16	1.01	Down
13-L-Hydroperoxylinoleic acid	4.59	1.00	Down

of multiple beneficial effects, such as inhibiting the growth of cancer cells, inducing tumor cell apoptosis, reversing the abnormal differentiation of tumor cells, and anti-tumor metastasis (51). Moreover, ginsenoside has been demonstrated to improve immunity and antioxidant capacity of host (52). Zhang et al. revealed that the concentrations of aspartate aminotransferase and alanine aminotransferase in thiram-induced chickens significantly increased, but antioxidant enzyme dramatically decreased, suggesting liver injury and antioxidant dysfunction (53). Therefore, we speculated that the decreased ginsenoside may be one of the important pathways for thiram exerts its toxic effects and cause antioxidant dysfunction. Previous research indicated that arbekacin have an inhibitory effect on multiple pathogens such as *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Acinetobacter baumannii* (54). Moreover, arbekacin can be used for treating multiple drug resistant pneumonia and septicemia as well as infections caused by resistant *Staphylococcus Aureus* (55, 56). Coproporphyrinogen III play a key role in the production of heme (57, 58). Heme is also an important component of hemoglobin, which plays a key role in the transport of oxygen. Oxygen has been demonstrated to play key roles in blood vessel development and bone formation (59). Previous studies indicated that the chickens exposed to thiram showed weight loss, accompanied by angiogenesis disorder and tibial dyschondroplasia (60, 61). Therefore, decreased coproporphyrinogen III may be one of the causes of angiogenesis disorder and abnormal bone development of chickens.

Fusidic acid can treat infections induced by methicillin-susceptible and methicillin-resistant Staphylococcus aureus (62). Marmesinpossess multiple pharmacological functions including anti-inflammatory, antihepatotoxic and antitumor activities (63, 64). Fluorouracil has anti-cancer effects (65). Moreover, we observed increased levels of L-palmitoylcarnitine, quinestrol, 7-ketocholesterol, and tetrabromodiphenyl ether during thiram exposure. L-palmitoylcarnitine is an ester derivative of carnitine, which participated in fatty acids metabolism and its abundance increased during hepatic lipid accumulation (66, 67). Consistent with this study, Sheng et al. indicated that the abundance of L-palmitoylcarnitine increased significantly in zebrafish exposed to organic pollutants (68). Moreover, increased L-palmitoylcarnitine was closely related to poorer prognosis in patients with chronic heart failure (69). Quinestrol can disrupt internal secretion and cause fertility disorders by inducing testicular damage (70). Moreover, quinestrol can increase the levels of serum MDA and aggravate the oxidative damage of cells (71). As a pro-oxidant and pro-inflammatory molecule, 7-ketocholesterol not only induces inflammation and nerve cell damage, but also affects membrane permeability and causes oxidative stress (72). Tetrabromodiphenyl ether is known to possess reproductive toxicity, which weaken sperm activity and increase the quantity of abnormal sperm (73). Moreover, tetrabromodiphenyl ether has also been demonstrated to induce liver inflammation and promote the expression of inflammatory genes including IL-6, TNF-α and IL-l β (74). Increasing evidence demonstrated that long-term pesticide exposure can result in cancer and reproductive disorders. In this study, we observed significant changes in metabolites associated with anti-cancer, oxidative stress and reproductive function, indicating that thiram may also be a potential cancer-inducing factor. Previous study indicated that thiram exposure could induce liver autophagy and apoptosis. Notably, some studies also showed that oxidative stress could cause the initiation and development of apoptosis and autophagy. Therefore, thiram induced liver apoptosis and autophagy may be mediated by differential metabolites related to oxidative stress.

In conclusion, this study investigated the effect of thiram exposure on liver metabolism in chickens. Results showed that thiram exposure can significantly alter liver metabolism, characterized by significant changes in some metabolites and metabolic pathways. These results filled in the blank of thiram exposure on liver metabolism characteristics of chickens, and conveyed an important message that hepatic metabolic disorder may be one of the important ways thiram affects broiler liver metabolism. Moreover, this study will help prevent and control

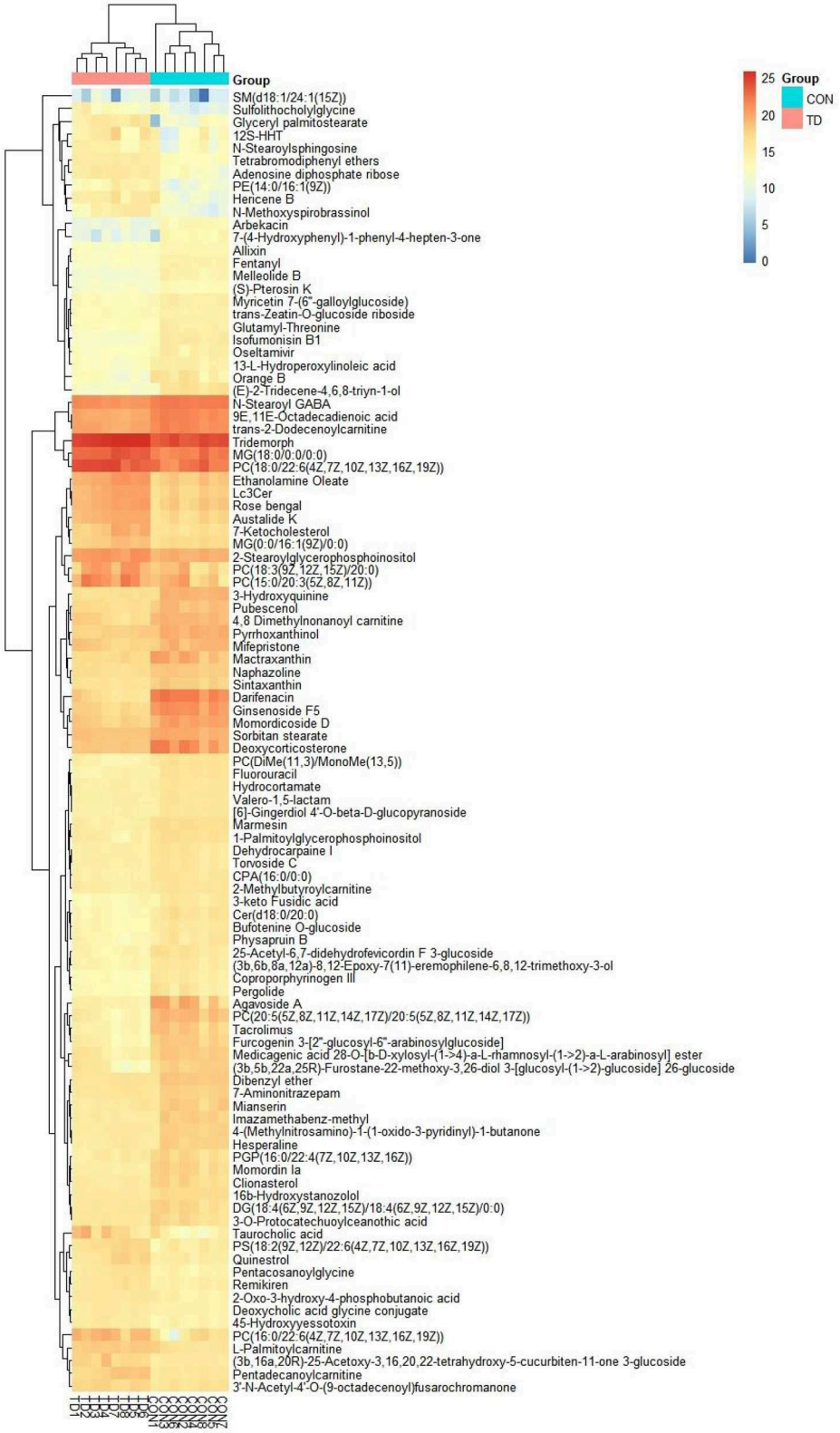

FIGURE 2
Heatmap revealed the differential metabolites in liver exposed to thiram. The color in the heatmap indicates the normalized relative abundance of each metabolite.

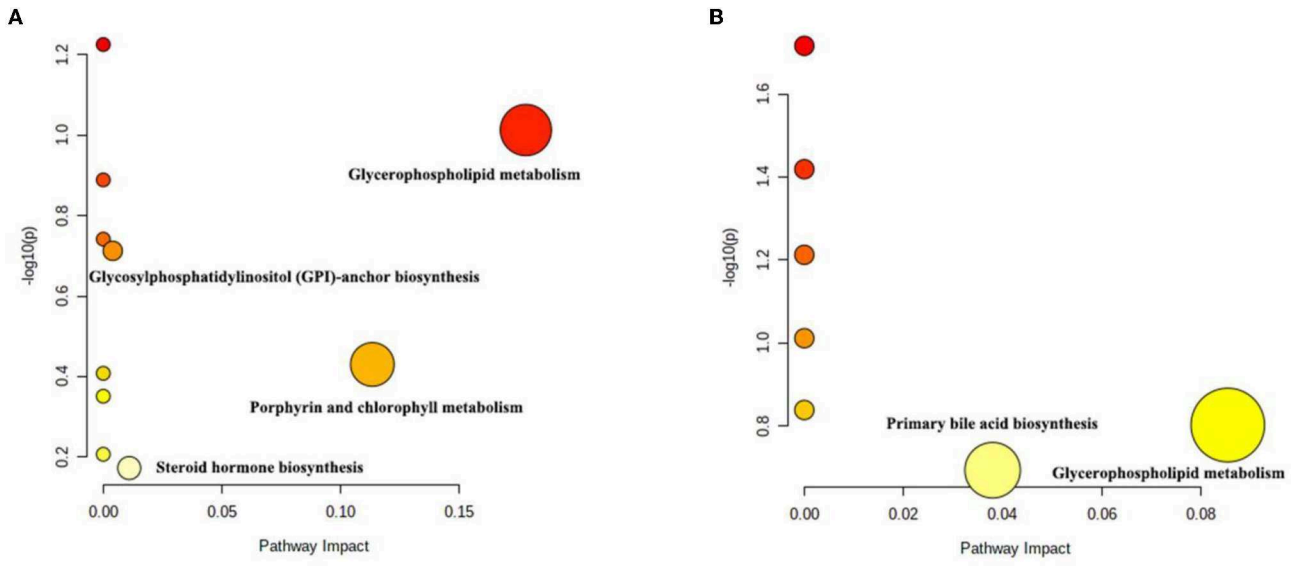

FIGURE 3
Differential metabolic pathway analysis based on the positive-ion mode **(A)** and the negative-ion mode **(B)**. Each circle represents a metabolic pathway.

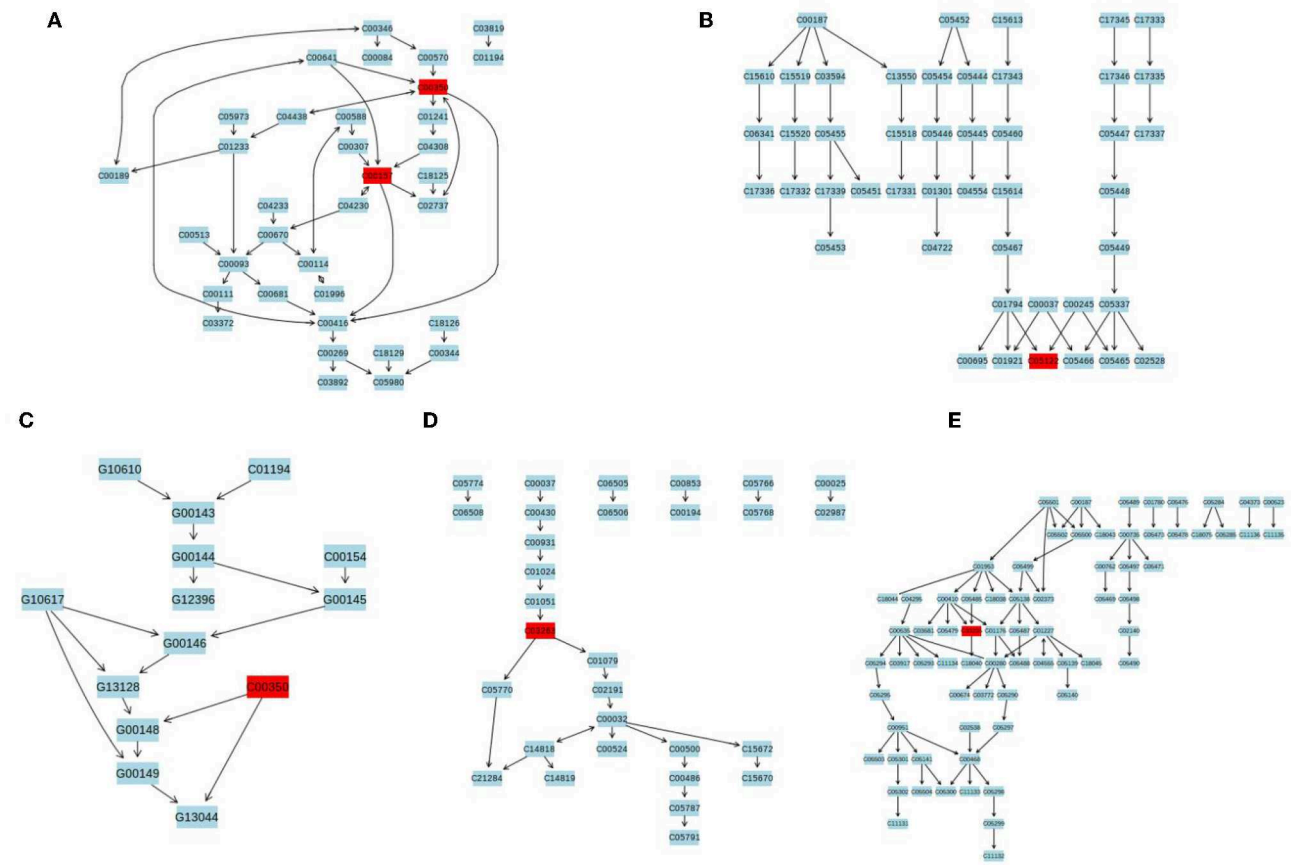

FIGURE 4
The representative schematic diagram of liver metabolic exposed to thiram. **(A)** Glycerophospholipid metabolism. **(B)** Primary bile acid biosynthesis. **(C)** Glycosylphosphatidylinositol (GPI)-anchor biosynthesis. **(D)** Porphyrin and chlorophyll metabolism. **(E)** Steroid hormone biosynthesis. The red boxes represent the differential metabolites associated with thiram exposure.

the effects of thiram on liver metabolism in chickens from the perspective of liver metabolism.

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 study was performed under the instructions and approval of Ethics Committee of the Wenzhou Vocational College of Science and Technology.

Author contributions

MW and LW conceived, designed the experiments, and wrote the manuscript. DZ, HLu, HLi, ZL, XS, CW, and YZ contributed sample collection and reagents preparation. MW analyzed the data. SS and MS revised the manuscript. All authors reviewed 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.

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Cell membrane-coated nanoparticles: An emerging antibacterial platform for pathogens of food animals

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Bacterial pathogens of animals impact food production and human health globally. Food animals act as the major host reservoirs for pathogenic bacteria and thus are highly prone to suffer from several endemic infections such as pneumonia, sepsis, mastitis, and diarrhea, imposing a major health and economical loss. Moreover, the consumption of food products of infected animals is the main route by which human beings are exposed to zoonotic bacteria. Thus, there is excessive and undue administration of antibiotics to fight these virulent causative agents of food-borne illness, leading to emergence of resistant strains. Thus, highprevalence antibiotic-resistant resistant food-borne bacterial infections motivated the researchers to discover new alternative therapeutic strategies to eradicate resistant bacterial strains. One of the successful therapeutic approach for the treatment of animal infections, is the application of cell membrane-coated nanoparticles. Cell membranes of several different types of cells including platelets, red blood cells, neutrophils, cancer cells, and bacteria are being wrapped over the nanoparticles to prepare biocompatible nanoformulations. This diversity of cell membrane selection and together with the possibility of combining with an extensive range of nanoparticles, has opened a new opportunistic window for the development of more potentially effective, safe, and immune evading nanoformulations, as compared to conventionally used bare nanoparticle. This article will elaborately discuss the discovery and development of novel bioinspired cell membrane-coated nanoformulations against several pathogenic bacteria of food animals such as *Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Salmonella enteritidis, Campylobacter jejuni, Helicobacter pylori*, and Group A Streptococcus and Group B Streptococcus.

KEYWORDS

bacteria, nanoparticles, cell, infection, animals, antibiotic, food

Introduction

Livestock and aquatic animals are the major sources of income, food, fertilizer, clothing, and building materials for a human being (1, 2). The continuous progression and growth of livestock sector offer significant opportunities for food security gains, poverty decline, better human nutrition, and agricultural development (3, 4). Farm animals constitute 40% of total agriculture output globally (5). These production animals support the livelihoods and the income of almost 1.3 billion people in the world (6). However, disease outbreak in livestock and aquaculture species may significantly reduce this quantum (7, 8). Highly contagious diseases especially the bacterial diseases of animals may have ripple effects on the revenue,

food supply, trade, and even human health as these may lead to reduced animal fertility, low yields (milk, eggs, etc.), animal death, and disease transfer in humans (9). There is a need to better understand the emerging animal diseases and their impact on the environment and society, as well as the development of innovative and potential therapeutic and preventive methodologies for their management.

Several therapeutic approaches have been considered to control livestock and fish bacterial diseases including the use of chemicals and antibiotic drugs (10–16). The use of antibiotics has become a vital input in food animal species to achieve the ultimate goal of successful farming with high production (17–19). Yet, the extensive use of antibiotics has raised the prevalence of antimicrobial resistant bacteria (20). In addition, the existence of drug residues in food animals further exaggerates the alarming situation for human beings (21–23). This lethal condition motivates scientists to search and develop new innovative nano-therapeutic agents which may successfully treat the resistant infections and avoid the undue excessive use of inaffective antibiotic drugs (24, 25). Several groups reported the development of nanoparticles which have been comprehensively analyzed in therapeutic context (26, 27). These tiny particles offer advantages of improved delivery of drug to target site (28) as well as controlled release of cargo (29). However, despite of these benefits, food and drug administration (FDA) approved only a few nanoparticles for clinical use. As our body system can easily recognize and rapidly remove the foreign substances *via* reticuloendothelial system, complex circulatory proteins and immune cells (30). Moreover, another strategy has been considered for surface modification of nanoparticles, that is the coating of Poly(ethylene glycol) (PEG) onto nanoparticles (31). Though pegylation was not a successful solution, as the studies showed that subsequent dosing of PEG-modified nanoparticles induce the rapid clearance by the hepatic phenomenon of accelerated blood clearance (32) *via* IgM antibodies production and complement system activation against PEG (33). Hence, a protective shield for cargo was needed in the form of an optimal delivery system to avoid rapid degradation and to offer a targeted and controlled drug delivery (34). Hence, the idea of development of biomimetic system start gaining attention to functionalize the nanoparticles with therapeutic potential. The cell membrane coating technology involves use of the cell membrane as drug-carrier, facilitating the controlled and targeted delivery of core nanoparticles irrespective to properties of nanomaterial. The cell membrane coated nano-moieties replicate the shape, surface composition, and movement of normal physiological cells (35).

A recent approach is the use of biomimetic nanoparticles in medical science for rapid diagnosis and biocontrol or treatment of bacterial diseases (36–39). Application of biomimetic nanoparticle can provide a sustainable and natural solution to control resistant bacterial infectionsin the livestock and fish farming industry (40, 41). The bioinspired nanotherapy may present a viable antibiotics alternative (40). The cell membrane coatings over nanoparticles give the appearance of surfaces of naturally occurring cells in the body, that when correctly selected and prepared do not have any risk of immune system activation and attack (38). The biomimetic therapeutic design demonstrates several therapeutic benefits like capability of biointerfacing (42, 43), biocompatibility (44), prolonged duration of circulation (45), immune evasion (46), and protection of the entrapped drug from active targeting and degradation (47).

A number of biomimetic nanoformulations have been designed using wide range of natural cell membranes coated over a variety of nanoparticles to ascertain antibacterial potential (48–52). Studies indicated that there is a significant medical importance of biomimetic and bioinspired nanoparticles to fight against the pathogenic bacteria of food animal host (45). However, our understanding of the design strategies for fabricating potentially effective and economically viable antibacterial nano-moieties remains limited. In this review, we will highlight the recent progress on the antibacterial activity of combo of different nanoparticles with natural cell-derived surfaces. Moreover, we will discuss recent research studies regarding successful development of novel biomimetic and biodegradable antibacterial nanomedicines against major bacterial pathogens responsible for veterinary bacterial diseases.

Concept of cell membrane coating over nanoparticles

The conception of coating several types of nanoparticles with plasma membranes of cells was raised in view of the troubleshooting of the functionalization of synthesized nanoparticles (53). Therefore, scientists developed new nature inspired nano-transporters, so that the nanoparticles may not be degraded by the internal environment of the organism and may easily reach their therapeutic targets (54). The synthesis of biomimetic nanoparticles combines the properties and specifications of cell membranes (external surface) and nanoparticles (core) (55, 56). This new emerging cell membranes coating technology allows the designing of biomimetic nanotransporters with covering surfaces which may directly imitate complicated functionalities of the cells, needed to have precise physiological interaction with other living cells and tissues (57, 58).

The plasma membrane coating concept was first reported in 2011, Hu and his coworkers (59) first employed red blood cell membranes for coating poly (lactic-co-glycolic acid) (PLGA) nanoparticles. The RBCs were first chosen for coating as they have extended circulation period of 120 days so the nanocarriers may stay longer in the blood circulation, the property which is highly needed for the nanocarriers. Since then, for this technology, wide variety of cell types have been considered including mesenchymal stem cells, bacterial cells, platelets, white blood cells, and cancer cells (60, 61).

Cell membrane coating techniques

To produce cell membrane-coated nanoparticles, the specific cell membrane must be coated over the nanoparticle core (62). The most frequently mentioned technique for cell membrane coating is membrane extrusion and ultra-sound to date (63). Membrane extrusion is one of the primary cell membrane-coating

techniques. Physical membrane extrusion is performed so that both the cell membranes and nanoparticles may move through membranes of various pore sizes concurrently, leading to the wrapping of the membranes over the nanoparticles (64). This method is beneficial as uniformly sized particles are obtained by using this methodology (65). The strategies employed for extrusion of membranes are membrane emulsification, vesicle extrusion and precipitation extrusion for emulsions, liposomes and nanoparticles/nanofibers respectively. The extrusion technique was first reported for uniform coating of PLGA nanoparticle core with red blood cell membrane (59). After that successful coating several research groups have stated this method for coating different types of cell membranes with varying pore size (66–68); however, mechanical forces may affect the structure of the membrane in this protocol (59).

Consistent and stable cell membrane-coated nanoparticles (CMCNPs) can be obtained by ultrasound technique. This technique also encourages the membrane and nanoparticles to naturally form a core-shell structure with a little loss of substantial material; however it may destroy the nanoparticles (69). Several researchers have reported to use this technique for assembling different types of cell membranes such as assembling or coating of membranes of platelet (54), stem cell (70), neutrophil (71) onto PLGA nanoparticles; Moreover, coating of cardiac stem cell membrane over microparticles of PLGA (72). Another example is assembling of a hybrid of platelet and RBC membranes over gold nanowires (73).

In another technique, nanoparticles and cell membrane vesicles are first mixed in the microfluidic system with the S and Y-shaped channels. Then, instantaneous pores are created on the vesicles by using electrical pulses at the exit of the channels and thus allowing nanoparticles to penetrate the vesicles, this technique is known as electroporation (74). An example of application of this technique is successful coating of RBC membranes onto ferric oxide (Fe_3O_4) nanoparticles resulting in uniform fabrication of RBC-Fe_3O_4 nanoparticles of good colloidal stability (75).

Another technique involves co-incubation of nanoparticles and live cells followed by the addition of serum-free media for secretion and production of exosomes (76). While wrapping the cell membrane over nanoparticles, it is vital to keep in mind that the coating process makes stable homogenous nanoparticles that do not interfere with either the function of the nanoparticles or the cellular membrane (77).

Concerning the membrane coating methods, the easiest strategy for coating among the above mentioned methods, is based on subsequent extrusion technique. Moreover, ultrasounds produce sonication forces to coat membrane surfaces over nanoparticles with better efficacy (69). Microfluidic technology is highly recommended for coating magnetic nanoparticles with good control as fine-tuning may be obtained *via* the flow speed (75).

Bioinspired nanotherapies against principal food borne pathogens

Biomimetic manufacturing of nanoparticles is a successful and rapidly developing field of nanotechnology (58). Depicting the concept of specific attachment sites and translocation protocols by pathogenic bacteria and host mammalian cells, the biomimetic nanoparticles showed several pharmacological functions like improved accumulation at site of infection/inflammation (78), efficient drug delivery to the target (79, 80), prolonged duration of circulation (81), and eliminate off-target adverse effects in the healthy host tissues (82). Thus, resulting in development of more effective and targeted nanoplatforms. All over the world, researchers have been working on bioengineered coated nanoparticles with special focus on antibacterial therapy (83, 84). The effectiveness, long-term safety, biopharmaceutical, and pharmacokinetic profiles of newly developed biomimetic nanotherapies are being confirmed by comprehensive anti-bacterial studies (85) (Table 1, Figure 1).

Major food animal pathogens treated through cell membrane-coated nanoparticles

Klebsiella pneumoniae

Klebsiella pneumoniae is commonly considered a responsible pathogen for nosocomial and community-acquired infections, including liver abscesses, infections of urinary tract and lower respiratory tract (102). The causative bacteria may be found in several niches like soil, meat, feaces, skin and intestine of livestock, and sea food animals (103). Antimicrobial-resistant species of *K. pneumoniae* have been found in various food products, including raw meat (bovine, chicken, and pork), sea food, vegetables, and ready-to-eat meals (104–108). World Health Organization (WHO) has listed cephalosporins and carbapenems resistant *K. pneumonia*e strain, as one of the top threats to public health globally. Thus emphasizing the urgent need for the discovery and development of new therapeutic agents (109, 110). Sepsis is a systemic inflammatory response syndrome caused by infection, with high incidence and mortality. Therefore, it is necessary to carry out an effective anti-infection treatment. In a recent research work, the scientists designed and developed a new biomimetic nanomedicine and named it γ3-RBCNPs. This bioinspired antibacterial agent involves the loading of ciprofloxacin into PLGA nanoparticles and then wrapping with RBCM. These functionalized nanoformulations specifically target intercellular adhesion molecule-1 (ICAM-1) *via* γ3 peptide on their surface and ultimately eradicate the *K. pneumoniae* with the help of loaded ciprofloxacin. The RBC membrane coating over the nanoparticles offers the properties of prolonged circulation time and immune escape (45, 46). Further investigations indicated that γ3-RBCNPs have good antibacterial efficacy, good bio-safety, and long half-life in mice mode (51).

Salmonella enteritidis

It is a foodborne pathogen, possess several zoonotic serotypes as well as a wide range of host organisms including poultry, ovine, bovine, and humans (111). It is the most common type of infectious

TABLE 1 Antibacterial cell membrane-coated nanoparticles against the food borne pathogens.

Food borne pathogens	Bioinspired therapeutic approach			Disease(s) of food animals	Host reservoir	References
	Cell membrane	Nano-core	Cargo			
Methicillin resistant *Staphylococcus aureus*	Platelet	PLGA	Docetaxel and Vancomycin	Soft tissue infection and mastitis	Poultry, pig, cow, sheep, goat, buffalo, sea food, human; NA	(54)
	RBC	PLGA	–	Sepsis, soft tissue infection and mastitis		(86)
	RBC	Fe_3O_4	–	Mastitis, septicemia arthritis, localized abscess formation		(40)
	Cholestrol enriched RBC	Cholestrol	Vancomycin	Skin infections, Mastitis		(87)
Campylobacter jejuni	*Campylobacter jejuni* membrane	PLGA	–	Gastrointestinal hemorrhage, pancreatitis cholecystitis, endocarditis, osteomyelitis meningitis	Chicken, swine, cattle, sea food, human	(88)
Group A Streptococcus	RBC	PLGA	–	Sepsis, pharyngitis, necrotizing fasciitis, impetigo and toxic shock syndrome	Fish, pig, cattle, human being	(89)
Group B Streptococcus	RBC	PLGA	–	Neonatal early-onset sepsis, intra mammary infection, respiratory infections, urinary tract infections, joint and bone infections, meningitis and bacteremia	Bovine and human	(90, 91)
Helicobacter pylori	Gastric epithelial cell membrane	PLGA	Clarithromycin	Gastric ulcer, lymphoma and gastric cancer in humans, Commensal in Sheep, cow pigs and camel sometimes cause digestive infection	Sheep, cow, pigs and camel milk, human	(92)
Endotoxin (LPS) producing bacteria including strains of *Escherichia coli*, *Staphylococcus*	Macrophage	$Ca_3(PO_4)_2$-Fe_3O_4@TiO_2	–	Osteomyelitis, sepsis, mastitis	Poultry, goat, sheep, broiler, pig, human	(39, 93)
	Endothelial cells	PLGA	Dexamethasone	Sepsis		(94)
	Macrophage	Fe_3O_4	–	Endotoxemia, sepsis, shock, and multiple organ dysfunction		(95)
	Macrophage	PLGA	–	Sepsis		(48)
Staphylococcus aureus	Macrophage	Gold-silver nanocage	–	Mastitis, septicemia arthritis, localized abscess formation	Cattle, poultry, chicken, sheep, goat, pigs, and humans	(96)
	Staphylococcus aureus	PEG	Rifampicin	Mastitis, septicemia, septic arthritis		(97)
		PLGA	Vancomycin			
	Staphylococcus aureus treated dendritic cells	CuFeSe2	–	Staphylococcus induced osteomyelitis		(98)

(Continued)

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TABLE 1 (Continued)

Food borne pathogens	Bioinspired therapeutic approach			Disease(s) of food animals	Host reservoir	References
	Cell membrane	Nano-core	Cargo			
Pseudomonas aeruginosa	Macrophage	Proteins secretions of Pseudomonas aeruginosa	–	Otitis and urinary tract infections, mastitis	Dairy cows, dogs; human	(99, 100)
Klebsiella pneumoniae	RBC	PLGA	Ciprofloxacin	Sepsis, urinary tract and pneumonic infections.	Chicken, pork, turkey and human	(51)
Salmonella Enteritidis	*Salmonella enteritidis* outer membrane protein	Chitosan	–	vomiting, high fever, diarrhea, and death in immunocompromised human patients	Poultry products (meat, eggs), layer, broiler chicken, and human	(49)
	Salmonella enteritidis outer membrane protein and flagellin protein (oral formulation)	Chitosan	–	Nausea, high temperature, diarrhea, vomiting and death in humans		(101)

PLGA, poly(lactic-co-glycolic acid); RBC, red blood cell; $CuFeSe_2$, Copper iron sulfide; PEG, polyethylene glycol; $Ca_3(PO_4)_2$, Calcium phosphate; Fe_3O_4, Iron (II, III) oxide; TiO_2, Titanium dioxide.

agent, causing animal and human Salmonellosis (112, 113). *S. enteritidis* is responsible for a reduction in egg production in poultry, leading economic losses to poultry industry (114, 115). Notably, *S. enteritidis*-infected meat and contaminated eggs are the primary cause of human Salmonellosis, so the control and treatment of Salmonellosis in the food animals would definitely lead to decline in cases of human Salmonellosis (116, 117). Moreover, the multi-drug resistant (MDR) strains of salmonella are emerging and their prevalence is also increasing day by day (118–120). In Egypt, the percentage of prevalence of multi drug resistant *S. enterica* was found to be 69.8% in the marketed meat samples in 2010, and 82.4 to 100% in 2020 (121–123).

Several therapeutic and preventive strategies have been considered to control Salmonella shedding in poultry and other livestock animals (124). It is expected that novel non-antibiotic therapeutic agents may reduce burden of bacterial colonization, environmental contamination and public health risk (125). Currently, Salmonella vaccines have been developed by coating of bacterial cell membranes over the chitosan nanoparticles. Scientists utilized *S. enterica* outer membrane proteins, flagellin proteins, and chitosan nanoparticles for the development of nano-vaccine against Salmonella (CNP-vaccine). Two experiments were performed, Experiment I was done to evaluate the optimal dose of CNP-vaccine to get a protective response against bacterial infection of *S. Enteritidis*. Whereas the Experiment II was performed to investigate the cross protection of nanoformulations against bacterial infection of *S. Heidelberg*. The results indicated that CNP-Salmonella vaccine was capable to raise the level of IgA and IgG in *S. Enteritidis* or *S. Heidelberg* infected broilers, thus showed cross-protection against both serovars of *S. enterica* (49).

Methicillin-resistant *Staphylococcus aureus* (MRSA)

This is a highly dangerous drug-resistant pathogen which is responsible for a wide range of infections such as endocarditis, sepsis and bacteremia leading to high rate of mortality (126–128). In food animals MRSA was first detected in the early 1970s in Belgium and induced bovine mastitis (129). After that, several cases have been reported on MRSA infection and colonization in other food-chain animals such as poultry, cattle, sheep, goat and pigs, intimating MRSA as an imperative veterinary and zoonotic pathogen (130–132).

Molecular typing of *S. aureus* indicated that certain animal lineages are specific to host while some are able to colonize or infect a wide variety of animals (133). The most notable case is ST398, which was primarily detected among pigs, and afterwards was found in various food-chain animals as well as in humans. The isolates stated to date and carrying mecC mainly belonged to lineages in cattle. However, mec C has also been indicated in other food animals such as sheep and rabbits as well as among various companion animals (cats, guinea pigs, horses, and dogs) (134, 135).

The pathogenesis of *S. aureus* pathogenesis is fueled by lethal secretion of toxins including α-toxin, which damages the membrane by forming pores and targets epithelium and endothelium of leukocytes and platelets (136). Most antibiotics are ineffective in treating MRSA infections as MRSA has a good

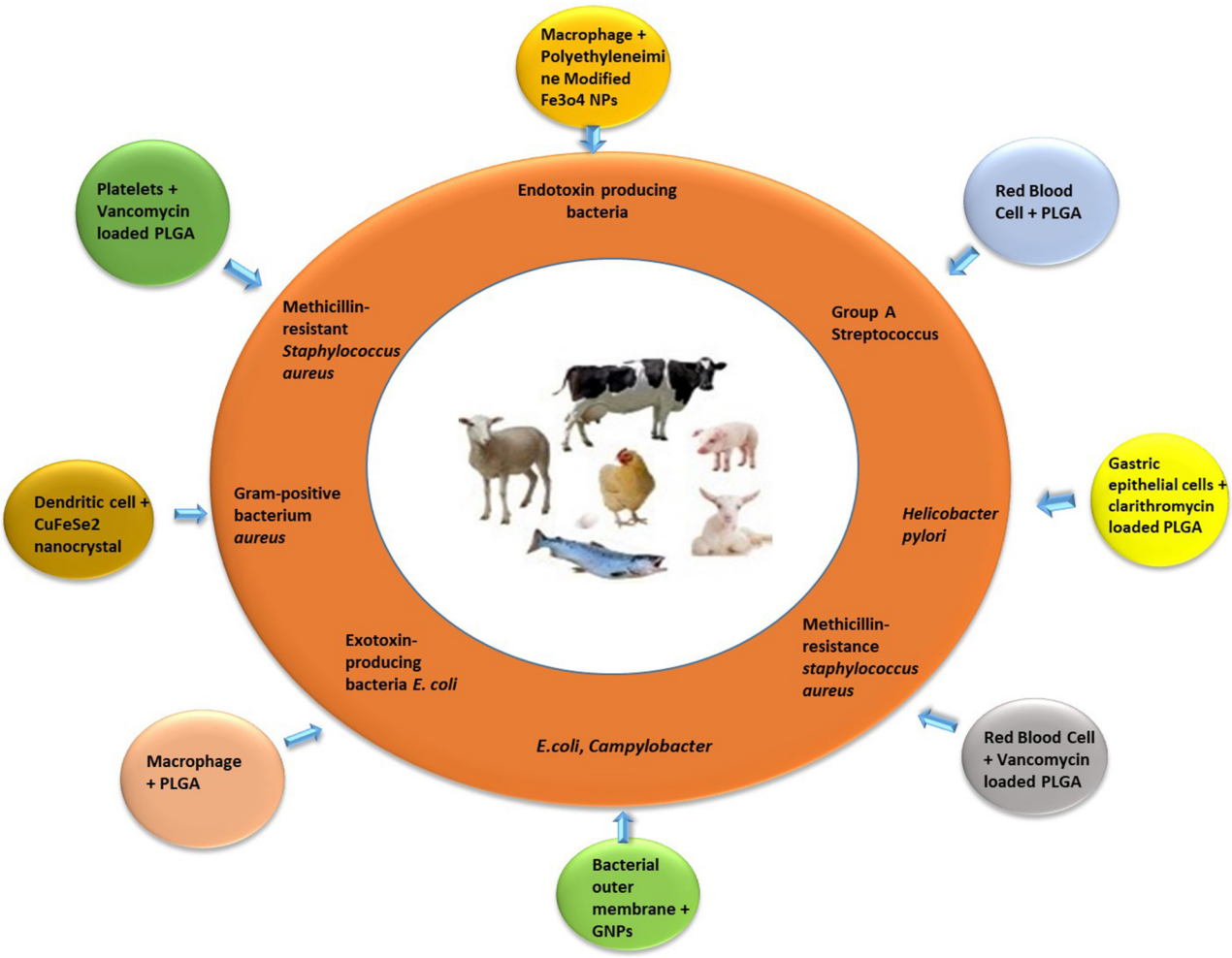

FIGURE 1
Bioinspired and biomimetic nanotherapies against specific bacterial pathogens of food animals.

survival strategy and self-preservation (137, 138). Nano-drug delivery systems have emerged as a new method to overcome this barrier (135). Recently, a study was conducted to synthesize an innovative nano-drug delivery system against MRSA infection. The researchers developed silver metal-organic frame structure (Ag-MOF) of methylimidazole and silver nitrate. Then the framework was loaded with Vancomycin (Vanc) to form Ag-MOF-Vanc complex and finally coated with platelet vesicles to get platelet membrane covered nanoparticles PLT@Ag-MOF-Vanc. The prepared nanoformulations killed MRSA through various strategies, such as interfering with bacterial metabolism, inhibiting production of reactive oxygen species, destructing the structure of cell membrane, and inhibiting formation of biofilm. The coating of the platelet membrane buttoned up to the surface of the pathogenic bacteria (MRSA) and the sites of MRSA infection. The results indicated good therapeutic effect in the mouse MRSA pneumonia model, and showed no toxic effect (139).

A biomimetic nanodecoy strategy was developed to capture and neutralize *S. aureus* toxins. The Platelets membranes were isolated and coated over PLGA nanoparticles and the resulting formulation of nanospongee inhibit the platelet and macrophage damage, induced by toxins of *S. aureus*, thereby supporting activation of platelets, nitric oxide production, macrophage oxidative burst, and bactericidal activity. Moreover, nanosponge also helped in release of neutrophils which are trapped in the extracellular fluid by a pathogenic organism. Thus, the prepared platelet membrane coated nanoparticles (PNPs) provided the therapeutic benefits of cytoprotection and enhanced host resistance to *S. aureus* infection (50).

The cell membrane-coated nanoparticles have great potential for treatment of bacterial infections. However, infection of bones involved inflammation and loss of bone mass, so the treatment must have anti-inflammatory and osteoconductive agents (39). The membrane coating through ultrasonication and extrusion strategies reduced the functionality of plasma membrane, so for coating the nanoparticles, another protocol was introduced in 2021, which involved electroporation procedure to help in retaining the functionality of cell membranes. A composite of nanoparticles with bactericidal TiO_2 and osteoconductive $Ca_3(PO_4)_2$ properties was assembled and coated by macrophage membrane. The

resulting macrophage membrane coated nanoparticles possess better functional membranous coating and showed significant bactericidal and anti-inflammatory potential against methicillin resistant *S. aureus* infection (39).

Escherichia coli

Escherichia coli (*E. coli*) is both commensal and pathogenic type of bacteria existing in humans and animals (140). Shiga-toxin producing *E. coli* (STEC), known as *E. coli* 0157 is responsible for causing resistant infectious disease (141–144). The symptoms of disease include fever, nausea, cramps, vomiting, bloody or watery diarrhea and sometimes kidney failure leading to death. Cows especially calves, sheep, goats, pig and deer may transfer the infection to humans. It is a food-borne pathogen and people got infection by either having contaminated food, including unpasteurized milk and undercooked beef or by having direct contact with *E. coli* O157 from stool of calves and cattle (145, 146). Animals carry *E. coli* O157 in the intestine and then shed in stool but still appear healthy and clean. The germs can quickly contaminate the animals' skin, fur, feathers, and the areas where they live and roam. Animals can lead healthy life but may potentially spread *E. coli* O157 infection to other animals and humans. *E. coli* may cause bovine mastitis in cattle (147, 148) and infection can be graded from being a subclinical illness of bovine mammary gland to a serious systemic infection. The age and lactation stage of the cows are the major factors to be considered for determining the severity of coliform mastitis.

The currently available antimicrobial agents are not effective enough for treatment of *E. coli* mastitis. However, cephalosporins and fluoroquinolones have shown partial therapeutic benefits for treatment of *E. coli* mastitis (149). The use of both drugs is restricted to a limit in food animals with special instructions (150). In *E. coli* mastitis, a potential therapeutic agent is needed, scientists are working to design and develop biomimetic nanoformulations for the successful treatment of the *E. coli* instigated disease. The disguised nanoparticles were prepared by using macrophage membranes to specifically neutralize and deactivate lipopolysaccharides secreted by *E. coli*, pathogenic bacteria. The *in vivo* experimentation in endotoxemia-induced mice model demonstrated that the coated nanoparticles reduced immune response and the inflammatory reaction. In addition, the nanoformulation improved the survival rate. These macrophage membranes coated nanoparticles are broadly considered for the treatment of LPS associated infectious diseases (95).

Helicobacter pylori

Helicobacter pylori is a spiral shaped gram negative bacterium which mainly colonize in the gastric environment (151). This highly virulent bacteria specie is responsible to cause ulcer, lymphoma and gastric cancer (152). The transmission route of the bacteria is still not known. However, studies were conducted in the past to evaluate the probability of zoonotic transmission. In a study, scientists isolated *H. pylori* from pigs, sheep, cow, and camel milk (153, 154) and the results showed that *H. pylori* may opt these animals as reservoirs. In another study, the prevalence and virulence of *H. pylori* was determined in the stomach of sheep, cow and goat. The zoonotic transmission of *H. pylori* was also examined. It was demonstrated in the results that cows, sheep, and human beings samples were found *H. pylori* positive; however the bacterium was not detected in goat samples. Moreover, the virulent *H. pylori* genotype (vacA s1a/m1a) linked to epithelial cell injury, was the predominant *H. pylori* genotype in cow, and goat population (155). *H. pylori* infection is the major cause of gastric and peptic ulcer, gastritis and gastric cancer, thus it is highly essential to treat the infection of food animals and humans with potential therapeutic agents. Currently, clarithromycin, amoxicillin and metronidazole are recommended for the treatment. However, mutations in *H. pylori* genes have led to the treatment failure (156).

Recently, a novel targeted nanotherapeutic agent was developed, inspired by the idea of natural interaction and adhesion of host and the bacteria. The plasma membranes of gastric epithelial cells are coated over chlarithromycin-loaded polymeric nanoparticles, the resulting biomimetic nanoparticles have the same surface properties as the epithelial cells and thus adhere to *H. pylori* bacteria. Thus, the nanoformulations provided the targeted drug delivery platform against colonized pathogenic bacteria. Thus, the bioinspired nanotherapeutic approach strategy reported here represented a novel drug delivery system to treat infectious disease of *H. pylori* (92).

Another versatile targeted therapeutic agent was synthesized by Zhang and his coworkers (157). The bioinspired moieties interfere with adhesion of bacteria to the host and thus evade the decorum of bacterial growth inhibition or killing, which may reduce the resistance development. The polymeric cores were wrapped with bacterial outer membranes resulting in synthesis of *H-pylori*-mimicking nanoparticles which compete with source bacteria for binding to gastric epithelial cells. Treatment of *H-pylori*-mimicking nanoparticles with gastric epithelial cells enhance the target adhesion and the efficacy is dependent on coated nanoparticles concentration and dosing (157).

Campylobacter jejuni

Campylobacter jejuni is highly responsible for foodborne zoonosis and is associated with handling and consumption of the poultry meat. The bacteria colonizes in the intestine of chicken and thus slaughtering process resulted in fecal contamination of the carcasses (158). One strategy to control *C. jejuni* infection is to halt the bacterial colonization of broilers (159).

The extent of contamination is directly linked to the number of bacteria in the cecal content of broiler chicken and the number of bacteria on the carcasses and cut meat pieces (160). Therefore, Campylobacter load is reduced at poultry production level before slaughtering (161). Several antibiotic drugs have been considered

for the treatment and control of the infection but still the infection prevails due to the existence of resistant strains of bacteria.

Several therapeutic and preventive strategies have been developed against *C. jejuni* colonization in poultry (159, 160, 162–164). In a study, biodegradable and biocompatible poly (lactide-co-glycolide) (PLGA) compound was used to prepare nanoparticle and then the synthesized nanoparticles were wrapped with outer membrane proteins of *C. jejuni*. The prepared coated nanoparticles were then administered through oral/subcutaneous routes. The results were interpreted based on intestinal colonization of *C. jejuni* in chicken. The *C. jejuni* colonization in cecal and cloacal contents was reduced post administration of prepared nano vaccine. It was concluded that nanoparticles encapsulated with outer membrane proteins of *C. jejuni* may be considered as a potential candidate vaccine for controlling the colonization of *C. jejuni* in chickens (88).

Considering the dynamic interaction of pathogen with host as well as understanding the versatile functionality of bacterial outer membrane proteins for intestinal adherence and invasion, bacterial outer-membrane vesicles have become a potential candidate for vaccine targeting against *C. jejuni* (165). The chitosan-nanoparticles were coated with outer-membrane vesicles and then evaluated in mouse model for induction of specific immune response against *C. jejuni*. The results indicated that intragastric delivery of chitosan-coated outer-membrane vesicles impart significant immune protection (166).

Further, immunization with the outer membrane vesicles resulted in potent cellular responses with an increased CD4+ and CD8+ T cell population. Moreover, significant upregulation of IFN-γ and IL-6 gene expression suggests that mucosal delivery of outer membrane vesicles promotes a Th1/Th2 mixed-type immune response. Together, as an acellular and nonreplicating canonical end product of bacterial secretion, mucosal delivery of outer membrane vesicles may represent a promising platform for developing an effective vaccine against *C. jejuni* (166).

Group A streptococcus and Group B streptococcus

The strains of Streptococcus cause mild to severe bacterial disease in animals as well as in human beings. The pathogenic organism is not host-specific as some strains which are associated with animals can be frequently found in humans (167, 168). Streptococcus infection may rapidly develop into a lethal disease despite administration of commercially available drugs (antibiotics and palliative medication). The streptococcus bacteria possess detrimental virulence factors, such as Group A streptococcus pore-forming streptolysin O and Group B streptococcus pore-forming toxin β-hemolysin/cytolysin. The toxins adversely effects the host tissues including failing of blood brain barrier, injury of lung cells, and apoptosis of immune cells (90). Many zoonotic cases of streptococci are sporadic, but some fish-linked strain of *S. agalactiae* are involved in outbreaks (169, 170). Pig associated strain of *S. suis*, has emerged as a major environmental pathogen causing deadly shock-like syndrome, septicemia, streptoccoccal meningitis, and other human diseases after contact with infected animals or derived food products, especially in the Asian countries (171–174). Moreover, in case of cattle, streptococci are declared to be the virulent bacteria causing bovine mastitis throughout the world (175, 176).

Several classes of antibiotic drugs are considered for the treatment of infected animals. First-line treatment for streptococci infection is penicillins either alone or in combo with tetracyclines, aminoglycosides, lincosamides, macrolides and fluoroquinolones (177). However, antibiotic-resistant phenotypes of streptococci have been reported in infected animals all over the world (178). New treatment strategies are being considered against streptococcal infection (179, 180), including a titratable detoxification therapy by a wraping of polymeric cores with donor red blood cell membranes to create biomimetic "nanosponges". The polymeric cores of biomimetic nanosponge retain the same repertoire of receptors over the cell membrane and proposed a non-specific toxin decoy stratagem to neutralize and sequester several bacterial toxins and pro-inflammatory chemokines of the host. The prepared nanosponge was evaluated to neutralize and sequester streptolysin O toxin of Group A Streptococcus (GAS) and inhibit the functioning of later virulent bacteria. Moreover, this therapeutic intervention inhibited toxin-induced apoptosis of macrophage and enhanced neutrophil trapping process leading to enhanced killing of pathogenic bacteria by phagocytic cells. *In vivo* studies of biomimetic nanosponge showed that the local administration of the prepared nanosponge reduced lesion size and bacterial colony-forming unit in the GAS-infected mouse model. Thus, the application of a toxin decoy assisted in the inactivation of secreted toxin and presented a novel approach to directly target virulence in severe GAS infections (89).

In the recent research work, red blood cell membrane vesicles were isolated and coated over polymeric nanoparticles to formulate biomimetic nanosponge. The prepared nanosponges proved to be successfully alleviate a sequence of toxic events like the hemolytic activity of living Goup B streptococcus bacteria, injury of lung epithelial and production of streptococcus -induced macrophage IL-1β. The results also indicated that red blood cell membrane-coated nanoparticles might be considered a first-in-class treatment option for streptococcal bacterial infection (90).

Conclusion

Traditional anti-infection therapeutic strategies mainly involved administration of antibiotics. However, emergence of drug-resistant bacteria and increasing endemic of lethal bacterial infections raised the importance of development of novel therapeutic agents for infectious diseases. Recent advances in nanotechnology, resulted in synthesis of a variety of nanoscale cell-membrane bagged nanoparticles. Several efforts have been directed toward nanoparticle functionalization by successful protection during the interaction of bioinspired nanoparticles with pathogens or exo/endotoxin. Food-borne zoonotic pathogens have been

targeted by biomimetic nanoparticles and showed potential antibacterial activity.

Author contributions

SA contributed to the design/plan of the work. KA and SA collected and interpreted the data and drafted, revised, and approved the final version of the article to be published.

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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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Oxidative stress and toxicity produced by arsenic and chromium in broiler chicks and application of vitamin E and bentonite as ameliorating agents

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The present study investigated the adverse effects of arsenic and chromium in broilers and ascertained the role of vitamin E and bentonite in alleviating their harmful effects. For this purpose, we experimented on 180 one-day-old broiler chickens. The feed was administered to broiler chicks of groups 2, 6, 7, 8, and 9 chromium @ (270 mg.kg^{-1} BW). Groups 3, 6, 7, 8, and 9 were administered arsenic @ (50 mg.kg^{-1} BW). Groups 4, 7, and 9 received vitamin E (150 mg.kg^{-1} BW), and groups 5, 8, and 9 received bentonite (5%), respectively. Group 1 was kept in control. All the broiler chicks treated with chromium and arsenic showed a significant ($p < 0.05$) decline in erythrocytic parameters on experimental days 21 and 42. Total proteins decreased significantly, while ALT, AST, urea, and creatinine increased significantly ($p < 0.05$). TAC and CAT decreased significantly ($p < 0.05$), while TOC and MDA concentrations increased significantly ($p < 0.05$) in chromium and arsenic-treated groups on experimental days 21 and 42. Pearson correlation analysis revealed a strong positive correlation between TAC and CAT (Pearson correlation value = 0.961; $p < 0.001$), similarly TOC and MDA positive correlation (Pearson correlation value = 0.920; $p < 0.001$). However, TAC and CAT showed a negative correlation between TOC and MDA. The intensity of gross and microscopic lesions was more in chromium (270 mg.kg^{-1}) and arsenic (50 mg.kg^{-1}) singly or in combination-treated groups. Thus, broiler chicks treated with chromium plus arsenic exhibited higher gross and microscopic lesion intensity than other treated groups. Fatty degeneration, severe cytoplasmic vacuolar degeneration, and expansion of sinusoidal spaces were the main lesions observed in the liver. Kidneys showed renal epithelial cells necrosis, glomerular shrinkage, and severe cytoplasmic vacuolar degeneration. Co-administration of bentonite along with chromium and arsenic resulted in partial amelioration (group 8) compared to groups 7 and 9, administered arsenic + chromium + vitamin E and arsenic + chromium + vitamin E + bentonite, respectively. It was concluded that arsenic and chromium cause damage not only to haemato-biochemical parameters but also lead to oxidation stress in broilers. Vitamin E and bentonite

administration can ameliorate toxicity and oxidative stress produced by arsenic and chromium.

KEYWORDS

broiler chicks, arsenic, chromium, toxicity, oxidative stress, vitamin E, bentonite

Introduction

The poultry industry of Pakistan has become an integral part of livestock, contributing 61.89% to agriculture and 14% to GDP. Intensive poultry farming has resulted in poverty alleviation by employing 1.5 million people. With investing > Rs. 750 billion, this industry has been expanding almost 7.5% per annum over the last decade, which has facilitated Pakistan to stand at 11th position among the largest poultry producer in the world and has more than enough room for additional expansion. The poultry industry contributes 38% of the total meat produced in the country (1).

Heavy metals are relentless in the environment and can render bioaccumulation in the food chain. Contamination of drinking water by heavy metals can risk poultry and human health (2). Water pollution due to heavy metals poses a potential risk to human and animal health (3). Wastewater from industrial and domestic sources is responsible for polluting the environment and has become a significant health issue in developing countries (4). Nowadays, heavy metal ions are among water's most toxic inorganic pollutants and have gained ecological significance (5). Heavy metals, including arsenic and chromium, are naturally found in the earth's crust and enter the environment due to natural or anthropogenic pursuits. They have become health hazards worldwide due to their non-biodegradability and bioaccumulation. Biological activities include rock weathering and volcanic eruptions, while anthropogenic activities include mining, ores smelting, and phosphate fertilizer (6).

As a global health issue, arsenic poisoning is influencing millions worldwide because of environmental and occupational disclosure. The fundamental source of arsenic toxicity to the general population is polluted soil, water, and food products (7). Arsenic name is derived from the Greek word "arsenikon" which means potent. Arsenic is the most common and toxic element among the most dangerous xenobiotics listed in the environment (8). Arsenic is found in the environment, e.g., organic and inorganic; their toxicity depends on the form and oxidation state. Arsenite is more toxic than arsenate due to its high affinity to thiol protein groups, while arsenate stops phosphorylation (7).

Chromium (Cr) is a heavy metal notorious as a toxic water pollutant. It is the 21st most abundant element. It naturally exists as mineral chromite and is metallic (9). Organic forms of chromium include chromium picolinate and chromium-enriched yeast. Among inorganic forms, metallic chromium is used to make alloys, i.e., stainless steel. Anti-corrosive property of stainless steel is due to chromium. Trivalent chromium found in water is used in the tannery and in making dyes and paints. Hexavalent chromium is the most treacherous form being utilized in chrome plating and is an important etiology of mutagenesis and cancer (10). Anthropogenic activities, i.e., metallurgy, smelting, electroplating, tannery effluents, and agriculture, has heightened chromium level ahead of the integration ability of the environment. Hence it has become the most toxic element in terrestrial and marine environments (11, 12).

Oxidative stress is a process triggered by inequity between the production and accretion of reactive oxygen species (ROS) in cells and tissues and the capacity of a biological system to cleanse these reactive manufactured items, such as free radicals (13, 14). Free radicals, including superoxide anion, hydroxyl radical, and nitric oxide, usually consist of partially filled orbital having unpaired electrons, which causes the reduction of a variety of organic macromolecules like lipid/protein/carbohydrate (15). Vitamin E renders free radicals inactive due to its ability to donate hydrogen. It is lipid-soluble vitamin located in the cell membrane. It is the best antioxidant available to combat the oxidative stress induced by heavy metals (16).

Among the natural adsorbents available, bentonite is the most widely used clay to eliminate heavy metals from the body. It is used in many ways in broiler feed, such as a binding agent of bacteria/viruses and the pelleting process (17). There are rare studies showing bentonite as an ameliorating agent in arsenic plus chromium intoxicated broiler chicks singly or amalgamation with vitamin E. Thus, this study was planned to know the arsenic plus chromium rendered oxidative stress and further amelioration with bentonite and vitamin E. This study shows how the co-administration of bentonite and vitamin E affects broiler health.

Materials and methods

Chemicals

Different chemicals, i.e., Potassium dichromate ($K_2Cr_2O_7$) and arsenic trioxide (As_2O_3), were procured from Merck KGaA, Darmstadt, Germany. We procured vitamin E (α-Tocopherol acetate) from Alpharma Inc., New Jersey, USA. Bentonite was obtained from the Potohar Plateau (Punjab Province, Pakistan), where it is abundantly available.

Abbreviations: ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; BW, Body Weight; CAT, Catalase; EDTA, Ethylenediaminetetraacetic Acid; ESR, Erythrocyte Sedimentation Rate; FCR, Feed Conversion Ratio; GFR, Glomerular Filtration Rate; Hb, Hemoglobin Concentration; MDA, Malondialdehyde; ROS, Reactive Oxygen Species; TAC, Total Antioxidant Capacity; Cr, Chromium; TBARS, Thiobarbituric Acid Reactive Species; TEC, Total Erythrocyte Counts; TLC, Total Leukocyte Counts; TOC, Total Oxidant Capacity; SOD, Superoxide Dismutase; GST, Glutathione S-transferases; GPx, Glutathione Peroxidase.

Experimental broiler chicks and management

The trial was conducted on 180 one-day-old broiler chicks bought from a regional hatchery and maintained these chicks in wire cages under standard housing and management conditions, i.e., humidity (60–65%) and temperature (24–35°C). We fed broiler chicks a basal diet (chick starter crumbs: 21% total proteins) and plenty of clean water. We administered the Newcastle disease (Nobilis® ND Lasota, Intervet SA (Pty) Ltd) vaccine to these chicks on days 2nd and 23rd. On days 8th and 21st, infectious bursal disease (IBD) [Nobilis Gumboro 228E, Intervet SA (Pty) Ltd], whereas administration of hydropericardium syndrome (BioAngara Plus, Sana Lab) vaccine was on day 19th.

Experimental design

After 2 days of acclimatization, we randomly divided broiler chicks into nine groups having twenty each. Arsenic, chromium, vitamin E, and bentonite medications began on the 3rd day and remained until 42 days. Potassium dichromate (17), arsenic trioxide (18), and vitamin E (17) were used @ 270, 50, and 150 $mg.kg^{-1}$ BW, respectively. All the treatments were administered daily in the feed. Group 1 served as control. We used 5 % bentonite in the feed (17). Groups 2 and 3 were given chromium and arsenic, respectively. Group 4 received vitamin E while group 5 bentonite. We administered chromium plus arsenic to chicks of Group 6, whereas group 7 received chromium plus arsenic and vitamin E. Group 8 administered chromium plus arsenic and bentonite. Group 9 received chromium + arsenic + vitamin E and bentonite. We assessed each bird in each group for weight weekly, weighed feed intake in each group daily, and then calculated the cumulative average of body weight and feed intake at the end of the experiment.

Hematobiochemical parameters

Broiler chicks ($n = 10$) were selected randomly from each group and killed humanely on experimental days 21 and 42 to collect blood with anticoagulant (EDTA; 1.0 mg/mL blood) for hematological studies, including total erythrocyte counts, hemoglobin concentration, hematocrit, and total leukocyte counts were determined. Briefly, total erythrocyte and leukocyte counts were figured out following the techniques already described (19) by diluting blood with Natt and Herrick solution and with the aid of a Neubauer counting chamber (Hemocytometer) were counted under a light microscope (20). Using Drabkin's solution, hemoglobin was determined spectrophotometrically (540 nm) through the cyanmethemoglobin method.

Biochemical studies

The collected blood without anticoagulant was centrifuged (3,000 rpm for 5 min) for serum separation and stored at −20°C. Serum biochemical parameters like total proteins (Cat # 997180), albumin (Cat # 997258), alanine aminotransferase (ALT; Cat # 30254), aspartate aminotransferase (AST; Cat # 30243), urea (Cat # 996060) and creatinine (Cat # 99108) were measured using commercial kits (M/S Canovelles; Barcelona, Spain) with the help of a chemistry analyzer. Globulin was determined by subtracting albumin from total proteins.

Antioxidant enzymes/parameters

As mentioned under biochemical studies, serum was procured for further studies. We measured total antioxidant capacity (TAC) in the serum samples following the method already mentioned (21). Briefly, by this method, antioxidants present in the sample lessen dark blue-green colored ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radial to colorless ABTS form determined through spectrophotometer at a wavelength between 660 and 670 nm.

The earlier method figured out the catalase (CAT) levels (22). Briefly, free radicals generated, like hydrogen peroxide, respond with molybdate founding a yellowish complex, the intensity of that is measured by spectrophotometer at a wavelength between 352 and 360 nm.

Total oxidant status

These included total oxidant capacity (TOC) and malondialdehyde (MDA). TOS was figured out using the method already described (23). By this method, ferrous-o-dianisidine is dissolved by oxidants present in the sample forming a ferric ion, which then oxidizes with glycerol by generating color. A spectrophotometer measures concentration at a wavelength of 560 nm.

The MDA was measured according to the earlier procedure (24). MDA oxidizes with deoxyadenosine and deoxyguanosine in DNA, forming complex DNA-MDA and other TBARS abridge with two equivalents of thiobarbituric acid to give a fluorescent red derivative that is assayed with a spectrophotometer at a wavelength of 532 nm.

Gross and histopathology techniques

The visceral organs, including the lungs, liver, kidneys, and heart, were examined for gross lesions on each killing. Morbid tissues were preserved in 10% buffered formalin and processed for histopathological studies using the routine method of dehydration and embedding in paraffin. For histopathological studies, sections 4–5 μm thick were cut and stained with hematoxylin and eosin (20). Prepared slides were examined under a light microscope. We made the scoring of microscopic lesions based on severity (mild, moderate, and severe).

Statistical analysis

The data thus gathered were evaluated statistically by utilizing two-factor factorials under a completely randomized design. Group means were equated by Duncan multiple range (DMR) test using Microcomputer Statistical Package (25). Pearson correlations were calculated using Minitab Statistical Software (Minitab Release 13.1). We considered the significance level at $p < 0.05$.

Results

Feed intake, body weight, and FCR

All physical parameters like feed consumed, body weight, and FCR decreased significantly ($p < 0.05$) in arsenic plus chromium-administered broiler chicks (group 6) on both experimental days, i.e., 21 and 42. Nevertheless, groups 7, 8, and 9 fed vitamin E and bentonite along with arsenic plus chromium exhibited increased ($p < 0.05$) feed consumption and body weight. In arsenic plus chromium-fed broiler chicks (group 6), there was increased FCR compared to control broiler chicks on investigational days 21 and 42. Groups 7, 8, and 9, treated with bentonite and vitamin E along with arsenic and chromium, exhibited significantly ($p < 0.05$) decreased/improved FCR (Table 1).

Hematological parameters

Displayed a significant ($p < 0.05$) decrease in TEC, hemoglobin, hematocrit, and ESR in groups treated with chromium (group 2), arsenic (group 3), and a combination of arsenic and chromium-treated broiler chicks (group 6) compared with control broiler chicks (group 1) on experimental days 21 and 42 (Tables 2, 3). Vitamin E and bentonite treated groups, along with chromium and arsenic (groups 7, 8, and 9), showed a non-significant ($p > 0.05$) difference compared with control (group 1).

Leukocyte counts significantly ($p < 0.05$) decreased in broiler chicks treated with chromium (group 2), arsenic (group 3), chromium + arsenic (group 6), and chromium + arsenic + vitamin E (group 7) at experimental days 21 and 42 (Figure 1). In broiler chicks treated with chromium + arsenic + bentonite (group 8), TLC was higher ($p < 0.05$) on both experimental days than in other 2, 3, 6, and 7 groups but lower ($p < 0.05$) than in control, 4, 5 and 9 groups (Figure 1).

Biochemical parameters

Significantly ($p < 0.05$) decreased plasma proteins, albumin, and globulin, and urea and creatinine significantly ($p < 0.05$) increased in chromium (group 2), arsenic (group 3), and chromium plus arsenic (group 6) treated broiler chicks compared with control group broiler chicks (Tables 4, 5) at experimental days 21 and 42. Whereas, total proteins, albumin, and globulin showed non-significantly ($p > 0.05$) on experimental days 21 and 42 in broiler chicks treated with chromium and arsenic plus vitamin E or bentonite (groups 7 and 8). Interestingly, broiler chicks in chromium + arsenic + vitamin E + bentonite (group 9) showed identical results in total proteins, albumin, and globulin concentration to control broiler chicks (group 1).

ALT concentrations increased significantly ($p < 0.05$) in chromium (group 2), arsenic (group 3), and chromium plus arsenic (group 6) treated broiler chicks compared to control (Figure 2) on experimental days 21 and 42 were observed. Whereas, a non-significant ($p > 0.05$) difference was seen in the concentration of ALT in chromium + arsenic + vitamin E (group 7), chromium + arsenic + bentonite (group 8), and chromium + arsenic + vitamin E + bentonite (group 9) treated broiler chicks with the control group on 21 and 42 trail days (Figure 2).

AST concentrations increased significantly ($p < 0.05$) in chromium (group 2), arsenic (group 3), chromium plus arsenic (group 6), chromium + arsenic + vitamin E (group 7), chromium + arsenic + bentonite (group 8) and chromium + arsenic + vitamin E + bentonite (group 9) treated broiler chicks compared with the control group at experimentation days 21 and 42 (Figure 3).

Antioxidant enzymes/parameters

Total antioxidant capacity

The TAC of broilers in groups given arsenic, chromium, bentonite, and vitamin E in various amalgamations has been presented in Tables 6, 7. At experimental days 21 and 42, the highest TAC (1.45 ± 0.09 and 0.85 ± 0.02 nmol/L, respectively) was recorded in control broiler chicks (group 1), while significantly ($p < 0.05$) the lowest values (0.82 ± 0.03 and 0.30 ± 0.01 nmol/L, respectively) were observed in broiler chicks treated with chromium and arsenic (group 6). Further analysis revealed that the TAC in broiler chicks of groups 2 and 3 were also significantly ($p < 0.05$) low compared to the control group. Groups administered bentonite and vitamin E along with arsenic and chromium (groups 7–9) showed non-significant ($p > 0.05$) TAC values compared with the control group.

Values for CAT of broilers in groups fed chromium, arsenic, bentonite, and vitamin E in various permutations have been presented in Tables 6, 7. A significantly ($p < 0.05$) highest CAT (63.7 ± 3.07 and 73.4 ± 3.01 Kilo U/L, respectively) was recorded in control broiler chicks (group 1). In comparison, the lowest ($p < 0.05$) values (35.2 ± 0.19 and 47.7 ± 2.07 Kilo U/L) were observed on chromium and arsenic-administered broiler chicks (group 6) on experimental days 21 and 42, respectively. Further analysis revealed that the CAT in groups 2 and 3 broiler chicks was also significantly ($p < 0.05$) lower than the control group. Groups treated with bentonite and vitamin E along with arsenic and chromium (groups 7 and 9) showed non-significant ($p > 0.05$) CAT values compared with the control group; however, group 8 showed significantly low ($p > 0.05$) CAT concentration than the control group on experimental days 21 (Table 6) and 42 (Table 7).

Total oxidant status

The total oxidant capacity (TOC) of broilers in various groups given chromium, arsenic, bentonite, and vitamin E in different

TABLE 1 Physical parameters like feed intake, body weight, and feed conversion ratio on experimental days 21 and 42 in broiler chicks fed chromium, arsenic, bentonite, and vitamin E singly or in blends.

Groups	Experimental day 21			Experimental day 42		
	Feed eaten (g/day)	Body weight (g)	FCR	Feed eaten (g/day)	Body weight (g)	FCR
G1	972 ± 14.2a	745 ± 27.5a	1.30	3,397 ± 26.9a	2,074 ± 29.2a	1.63
G2	820 ± 9.3b	515 ± 13.2c	1.59	3,259 ± 15.2b	1,629 ± 15.7c	2.00
G3	821 ± 9.7b	520 ± 12.5c	1.57	3,275 ± 19.9b	1,675 ± 15.9c	1.95
G4	974 ± 13.5a	752 ± 20.7a	1.29	3,392 ± 25.7a	2,079 ± 29.7a	1.63
G5	962 ± 12.9a	741 ± 21.3a	1.29	3,380 ± 23.2a	2,069 ± 29.3a	1.63
G6	795 ± 19.2b	490 ± 10.5c	1.62	3,201 ± 13.7b	1,572 ± 14.2d	2.03
G7	880 ± 11.3c	627 ± 18.5b	1.40	3,265 ± 21.5c	1,859 ± 17.5b	1.75
G8	865 ± 11.7c	617 ± 17.3b	1.40	3,247 ± 14.2c	1,855 ± 17.9b	1.75
G9	969 ± 14.3a	747 ± 23.5a	1.29	3,392 ± 25.3a	2„067 ± 28.3a	1.64

Mean ± SE values with dissimilar alphabets in a column vary significantly ($p < 0.05$). FCR, feed conversion ratio. Chromium, arsenic, and vitamin E were given @ 270, 50, and 150 $mg.kg^{-1}$, respectively, while bentonite was administered @ 5%. G1 = Group 1: Control (negative); G2 = Group 2: Chromium; G3 = Group 3: Arsenic; G4 = Group 4: Vitamin E; G5 = Group 5: Bentonite; G6 = Group 6: Chromium + Arsenic; G7 = Group 7: Chromium + Arsenic + Vitamin E; G8 = Group 8: Chromium + Arsenic + Bentonite; and G9 = Group 9: Chromium + Arsenic + Vitamin + Bentonite.

TABLE 2 Hematological parameters including total erythrocyte counts, hemoglobin concentration, hematocrit, and erythrocyte sedimentation rate on day 21 experiment in broiler chicks administered arsenic, chromium, vitamin E, and bentonite alone or in combination.

Groups	TEC ($\times 10^6/\mu L$)	Hb (g/dL)	Hematocrit (%)	ESR (mm/h)
G1	3.39 ± 0.01a	13.0 ± 0.19a	33.6 ± 2.03a	6.97 ± 0.95a
G2	2.75 ± 0.05b	6.35 ± 0.14d	19.5 ± 1.16b	2.97 ± 0.11b
G3	2.89 ± 0.07b	7.57 ± 0.13d	22.5 ± 1.14b	3.21 ± 0.19b
G4	3.37 ± 0.03a	11.3 ± 0.11b	33.1 ± 1.19a	6.92 ± 0.92a
G5	3.32 ± 0.06a	10.1 ± 0.12b	29.5 ± 1.16a	6.72 ± 0.97a
G6	2.65 ± 0.04b	5.15 ± 0.14d	17.3 ± 1.17b	2.85 ± 0.14b
G7	3.19 ± 0.02a	8.97 ± 0.15c	26.3 ± 1.16 b	5.67 ± 0.79a
G8	3.12 ± 0.04a	8.52 ± 0.16c	25.7 ± 1.13b	5.49 ± 0.70a
G9	3.36 ± 0.09a	10.5 ± 0.19d	30.5 ± 1.12a	6.35 ± 0.95a

Mean ± SE values with dissimilar alphabets in a column vary significantly ($p < 0.05$). Chromium, arsenic, and vitamin E were given @ 270, 50, and 150 $mg.kg^{-1}$, respectively, while bentonite was administered @ 5%. G1 = Group 1: Control (negative); G2 = Group 2: Chromium; G3 = Group 3: Arsenic; G4 = Group 4: Vitamin E; G5 = Group 5: Bentonite; G6 = Group 6: Chromium + Arsenic; G7 = Group 7: Chromium + Arsenic + Vitamin E; G8 = Group 8: Chromium + Arsenic + Bentonite; and G9 = Group 9: Chromium + Arsenic + Vitamin + Bentonite. TEC, total erythrocyte counts; Hb, hemoglobin concentration; ESR, erythrocyte sedimentation rate.

TABLE 3 Hematological parameters including total erythrocyte counts, hemoglobin concentration, hematocrit, and erythrocyte sedimentation rate on day 42 experiment in broiler chicks administered arsenic, chromium, vitamin E, and bentonite alone or in combination.

Groups	TEC ($\times 10^6/\mu L$)	Hb (g/dL)	Hematocrit (%)	ESR (mm/h)
G1	3.63 ± 0.07a	11.9 ± 0.12a	32.7 ± 2.04a	7.95 ± 0.92a
G2	2.82 ± 0.09c	6.49 ± 0.16c	20.1 ± 1.13b	3.51 ± 0.14b
G3	2.85 ± 0.02c	7.15 ± 0.11c	23.5 ± 1.12b	3.74 ± 0.17b
G4	3.63 ± 0.02a	9.57 ± 0.17a	33.0 ± 1.14a	7.96 ± 0.93a
G5	3.56 ± 0.03a	9.49 ± 0.15b	30.6 ± 1.16a	7.73 ± 0.99a
G6	2.77 ± 0.06c	5.62 ± 0.11c	19.1 ± 1.19b	3.42 ± 0.12b
G7	3.47 ± 0.04a	9.35 ± 0.12b	27.7 ± 1.16a	6.95 ± 0.74a
G8	3.39 ± 0.03a	9.31 ± 0.13b	25.4 ± 1.17a	6.73 ± 0.73a
G9	3.59 ± 0.01d	10.7 ± 0.12a	31.3 ± 1.11a	7.79 ± 0.95a

The footnote remains the same as that in Table 2.

TABLE 4 Biochemical parameters (g/dL) of broiler chicks on day 21 experiment administered arsenic, chromium, vitamin E, and bentonite alone or in combination.

Groups	Total proteins	Albumin	Globulin	Urea	Creatinine
G1	4.12 ± 0.07a	2.55 ± 0.01a	1.54 ± 0.05b	21.3 ± 1.02a	0.40 ± 0.01a
G2	3.45 ± 0.06c	2.14 ± 0.07b	1.31 ± 0.07c	43.5 ± 2.04b	1.17 ± 0.05d
G3	3.49 ± 0.04c	2.16 ± 0.07b	1.33 ± 0.03c	41.7 ± 2.07b	1.10 ± 0.04d
G4	4.14 ± 0.05a	2.54 ± 0.07a	1.36 ± 0.02c	21.0 ± 1.07a	0.40 ± 0.07a
G5	3.93 ± 0.02a	2.17 ± 0.06b	1.76 ± 0.09a	24.1 ± 1.03b	0.51 ± 0.01b
G6	3.37 ± 0.09c	2.10 ± 0.05b	1.27 ± 0.01d	44.5 ± 2.07b	1.23 ± 0.04d
G7	3.65 ± 0.03b	2.29 ± 0.07b	1.36 ± 0.06c	36.9 ± 2.06b	0.69 ± 0.03c
G8	3.61 ± 0.03b	2.22 ± 0.07b	1.39 ± 0.02c	37.7 ± 2.01b	0.75 ± 0.09c
G9	4.07 ± 0.02a	2.30 ± 0.09ab	1.77 ± 0.06a	21.8 ± 0.05a	0.54 ± 0.03b

Values (mean ± SE) with dissimilar letters in a column vary significantly ($p < 0.05$). Chromium, arsenic, and vitamin E were given @ 270, 50, and 150 mg.kg^{-1}, respectively, while bentonite was administered @ 5%. G1 = Group 1: Control (negative); G2 = Group 2: Chromium; G3 = Group 3: Arsenic; G4 = Group 4: Vitamin E; G5 = Group 5: Bentonite; G6 = Group 6: Chromium + Arsenic; G7 = Group 7: Chromium + Arsenic + Vitamin E; G8 = Group 8: Chromium + Arsenic + Bentonite; and G9 = Group 9: Chromium + Arsenic + Vitamin E + Bentonite.

TABLE 5 Biochemical parameters (g/dL) of broiler chicks on day 42 experiment administered arsenic, chromium, vitamin E, and bentonite alone or in combination.

Groups	Total proteins	Albumin	Globulin	Creatinine	Urea
G1	4.25 ± 0.09a	2.71 ± 0.07a	1.54 ± 0.02b	0.39 ± 0.09a	23.7 ± 1.07a
G2	3.25 ± 0.01c	1.87 ± 0.06c	1.38 ± 0.02d	1.49 ± 0.06c	38.2 ± 2.07b
G3	3.27 ± 0.07c	1.84 ± 0.02c	1.43 ± 0.07c	1.47 ± 0.09c	37.2 ± 2.07b
G4	4.25 ± 0.03a	2.72 ± 0.02a	1.53 ± 0.07b	0.39 ± 0.01b	23.0 ± 1.09a
G5	4.07 ± 0.09a	2.19 ± 0.07b	1.88 ± 0.01a	0.42 ± 0.06b	23.5 ± 1.05a
G6	3.23 ± 0.01c	1.83 ± 0.09c	1.40 ± 0.03c	1.51 ± 0.03c	39.6 ± 2.06b
G7	3.59 ± 0.05b	2.35 ± 0.07b	1.24 ± 0.09e	0.85 ± 0.07d	33.7 ± 2.06b
G8	3.52 ± 0.02b	1.97 ± 0.07b	1.56 ± 0.06b	0.89 ± 0.07d	35.2 ± 2.06b
G9	4.11 ± 0.07a	2.21 ± 0.03b	1.90 ± 0.05a	0.44 ± 0.02a	25.5 ± 1.01a

The footnote remains the same as that in Table 4.

combinations has been presented in Tables 6, 7. On experimental days 21 and 42, significantly ($p < 0.05$) high TAC (1.63 ± 0.05 and 1.79 ± 0.02 μMol/L, respectively) was recorded in chromium and arsenic administration broiler chicks (group 6), while the lowest ($p < 0.05$) values (1.15 ± 0.05 and 1.31 ± 0.01 μMol/L, respectively) was observed in control (group 1). Significantly ($p < 0.05$), high values of TOC were also recorded in chromium (group 2) and arsenic (group 3) treated broiler chicks compared control group. Though significantly ($p < 0.05$) high TOC values were observed in groups treated with bentonite and vitamin E along with arsenic and chromium (groups 7–8) but were significantly ($p < 0.05$) reduced TOC values than values in chromium + arsenic treated broiler chicks (group 6).

Malondialdehyde (MDA) of broilers in groups administered chromium, arsenic, bentonite, and vitamin E in different combinations have been presented in Tables 6, 7. At experimental days 21 and 42, the highest MDA (17.70 ± 0.12 and 19.92 ± 0.14 nmol/L, respectively) was recorded in administered broiler chicks (group 6), while the lowest values (9.47 ± 0.11 and 10.34 ± 0.15 nmol/L, respectively) was observed in control (group 1) with significant ($p < 0.05$) difference. Significantly ($p < 0.05$), higher values of MDA were also recorded in chromium-treated broiler chicks (group 2), followed by arsenic-treated broiler chicks (group 3) compared to the control group. However, higher MDA values were recorded in groups treated with bentonite and vitamin E plus arsenic and chromium (groups 7–9) than in the control group, but MDA values were lower than in chromium and arsenic-treated group (group 6).

Pearson correlation

Pearson correlation analysis revealed a strong positive and interconnected correlation between TAC with CAT (Figure 4). We also found a strong positive and significant Pearson correlation between TOC and MDA (Pearson correlation value = 0.920; $p < 0.001$), like that between TAC and CAT (Pearson correlation value = 0.961; $p < 0.001$). However, TAC showed a negative correlation between TOC (Pearson correlation value = −0.075; $p = 0.590$) and MDA (Pearson correlation value = −0.218; $p = 0.114$). Similarly, CAT showed a negative correlation

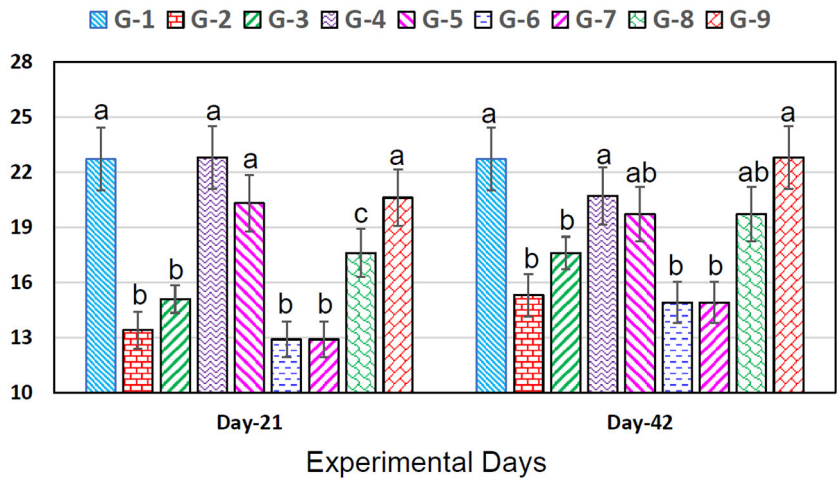

FIGURE 1
Leukocyte counts ($\times 10^3/\mu L$) in broiler chicks on 21 and 42 experiment days administered arsenic, chromium, vitamin E, and bentonite alone or in combinations. Bars (mean ± SE) having dissimilar letters under a specific experimental day vary significantly ($p < 0.05$). Chromium, arsenic, and vitamin E were given @ 270, 50, and 150 mg.kg^{-1}, respectively, while bentonite was administered @ 5%. G1 = Group 1: Control (negative); G2 = Group 2: Chromium; G3 = Group 3: Arsenic; G4 = Group 4: Vitamin E; G5 = Group 5: Bentonite; G6 = Group 6: Chromium + Arsenic; G7 = Group 7: Chromium + Arsenic + Vitamin E; G8 = Group 8: Chromium + Arsenic + Bentonite; and G9 = Group 9: Chromium + Arsenic + Vitamin E + Bentonite.

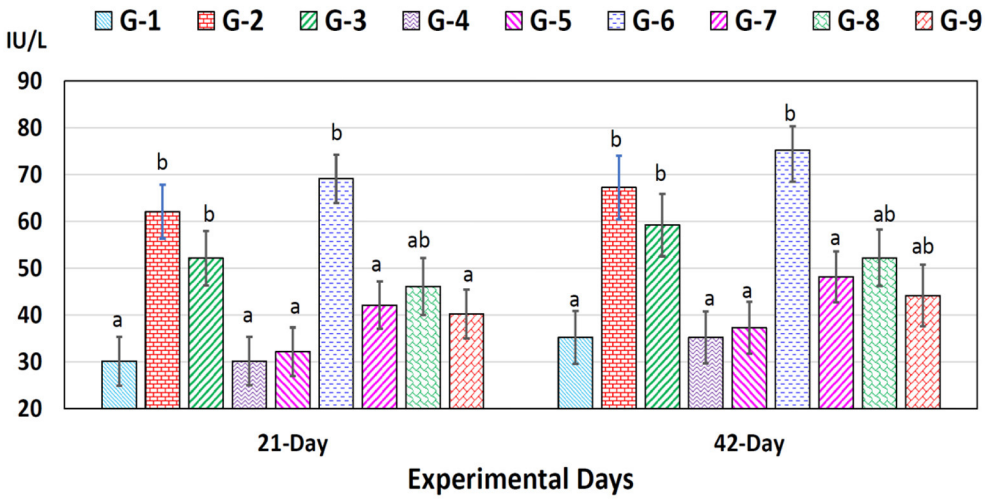

FIGURE 2
Alanine aminotransferase (ALT) in broiler chicks on 21 and 42 experiment days administered arsenic, chromium, vitamin E, and bentonite alone or in combinations. Bars (mean ± SE) having dissimilar letters under a specific experimental day vary significantly ($p < 0.05$). Chromium, arsenic, and vitamin E were given @ 270, 50, and 150 mg.kg^{-1}, respectively, while bentonite was administered @ 5%. G1 = Group 1: Control (negative); G2 = Group 2: Chromium; G3 = Group 3: Arsenic; G4 = Group 4: Vitamin E; G5 = Group 5: Bentonite; G6 = Group 6: Chromium + Arsenic; G7 = Group 7: Chromium + Arsenic + Vitamin E; G8 = Group 8: Chromium + Arsenic + Bentonite; and G9 = Group 9: Chromium + Arsenic + Vitamin E + Bentonite.

between TOC (Pearson correlation value = −0.021; $p = 0.882$) and MDA (Pearson correlation value = −0.101; $p = 0.446$). Interestingly, with the increase of antioxidant enzymes (TAC and CAT), TOC and MDA decrease and have a negative correlation (Figure 5). TAC1 to TAC6 (group 1: control, group 2: chromium; group 4: vitamin E, group 5: bentonite, and group 6: chromium + arsenic) except TAC3 (group 3: arsenic) showed weak positive Pearson correlation with total oxidant capacity and malondialdehyde (Figure 5).

Gross and histopathology

The intensity of gross and microscopic lesions was more in arsenic and chromium singly or in combination-treated groups. It is worth mentioning that broiler chicks of group 6 were treated with chromium (270 mg.kg^{-1}) plus arsenic (50 mg.kg^{-1}) showed higher intensity of gross and microscopic lesions as compared with other treated groups (Table 8).

Grossly, lungs were normal in size and shape in all treated and control groups except the lungs of groups 2, 3, & 6 (++),

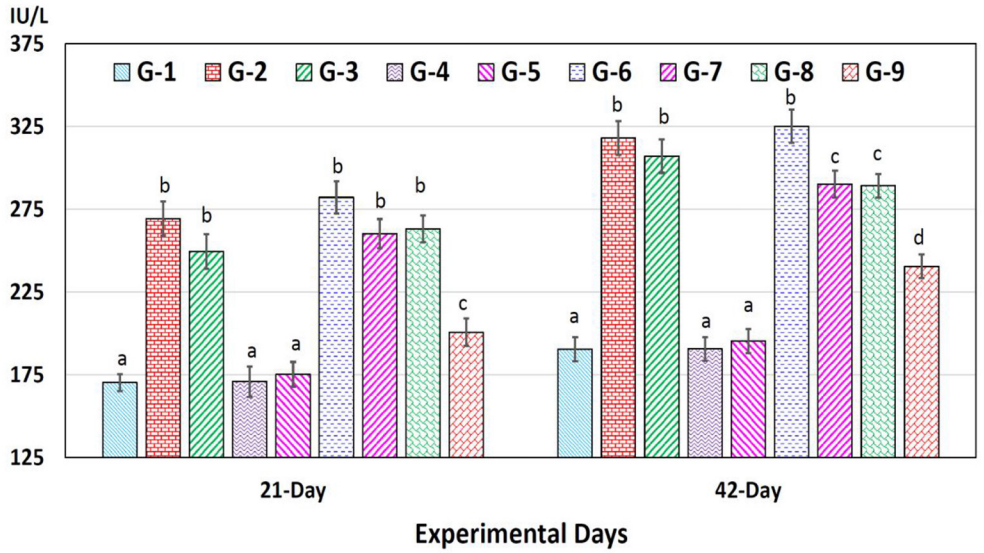

FIGURE 3
Aspartate aminotransferase (AST) in broiler chicks on 21 and 42 experiment days administered arsenic, chromium, vitamin E, and bentonite alone or in combinations. Bars (mean ± SE) having dissimilar letters under a specific experimental day vary significantly ($p < 0.05$). Chromium, arsenic, and vitamin E were given @ 270, 50, and 150 mg.kg^{-1}, respectively, while bentonite was administered @ 5%. G1 = Group 1: Control (negative); G2 = Group 2: Chromium; G3 = Group 3: Arsenic; G4 = Group 4: Vitamin E; G5 = Group 5: Bentonite; G6 = Group 6: Chromium + Arsenic; G7 = Group 7: Chromium + Arsenic + Vitamin E; G8 = Group 8: Chromium + Arsenic + Bentonite; and G9 = Group 9: Chromium + Arsenic + Vitamin E + Bentonite.

TABLE 6 Antioxidant enzymes/parameters (TAC and CAT) and oxidant enzymes (TOC and MDA) in broiler chicks on day 21 experiment administered arsenic, chromium, vitamin E, and bentonite alone or in combinations.

Groups	TAC (mmol/L)	CAT (Kilo U/L)	TOP (μMol/L)	MDA (nmol/L)
G1	1.45 ± 0.09a	63.7 ± 3.07a	1.15 ± 0.05a	9.47 ± 0.11f
G2	0.87 ± 0.01b	37.1 ± 1.07c	1.55 ± 0.05b	17.65 ± 0.13a
G3	0.91 ± 0.09b	39.2 ± 1.09c	1.61 ± 0.05b	16.54 ± 0.12b
G4	1.46 ± 0.04a	62.2 ± 3.03a	1.16 ± 0.04a	9.67 ± 0.19f
G5	1.41 ± 0.02a	57.5 ± 2.09a	1.19 ± 0.09a	11.57 ± 0.12d
G6	0.82 ± 0.03b	35.2 ± 0.19c	1.63 ± 0.05b	17.70 ± 0.12a
G7	1.26 ± 0.07a	52.3 ± 2.02a	1.42 ± 0.02c	12.72 ± 0.11c
G8	1.23 ± 0.03a	44.1 ± 1.05b	1.47 ± 0.03c	13.00 ± 0.11c
G9	1.42 ± 0.04a	61.5 ± 3.01a	1.16 ± 0.02a	10.43 ± 0.12e

Mean ± SE values with dissimilar alphabets in a column vary significantly ($p < 0.05$). Chromium, arsenic, and vitamin E were given @ 270, 50, and 150 mg.kg^{-1}, respectively, while bentonite was administered @ 5%. G1 = Group 1: Control (negative); G2 = Group 2: Chromium; G3 = Group 3: Arsenic; G4 = Group 4: Vitamin E; G5 = Group 5: Bentonite; G6 = Group 6: Chromium + Arsenic; G7 = Group 7: Chromium + Arsenic + Vitamin E; G8 = Group 8: Chromium + Arsenic + Bentonite; and G9 = Group 9: Chromium + Arsenic + Vitamin + Bentonite. Antioxidant parameters (TAC, total antioxidant capacity; CAT, catalase); oxidant enzymes (TOC, total oxidant capacity; MDA, malondialdehyde).

and 7, 8 & 9 (+) were hemorrhagic and frothy exudate was seen in the trachea. Microscopically, the major lesions of the lungs, like edema, congestion, and emphysema, were noted in groups 7 & 8 (+), 2 & 3 (++), and 6 (+ + +). However, mononuclear cell infiltration, hemorrhages, congestion, thickening of alveolar and bronchial septae, and alveolar edema along with emphysema and necrosis were also observed but with intensity. While the lungs of group 1 did not show any microscopic lesions and had well-arranged normal-sized bronchioles and alveoli.

Grossly, the liver was normal in size, shape, and consistency in all groups. Microscopically, pyknosis, condensation of nuclei, along with advanced fatty change in hepatocytes (Figure 6) and mononuclear cells infiltration and separation of cells from the basement membrane in hepatic lobule were observed in groups 7 (+), 2, 3 & 8 (++), and 6 (+ + +). Advanced fatty degeneration, detachment of cells from the basement membrane, and expansion of sinusoidal spaces were also observed in some treated groups like groups 6 (+ + +) (Table 8). Nuclear degenerative changes such as karyorrhexis, and karyolysis in broiler chicks treated with

TABLE 7 Antioxidant enzymes/parameters (TAC and CAT) and oxidant enzymes (TOC and MDA) in broiler chicks on day 42 experiment administered arsenic, chromium, vitamin E, and bentonite alone or in combinations.

Groups	TAC (mmol/L)	CAT (Kilo U/L)	TOP (μMol/L)	MDA (nmol/L)
G1	0.85 ± 0.02a	73.4 ± 3.01a	1.31 ± 0.01a	10.34 ± 0.15a
G2	0.32 ± 0.02b	49.8 ± 2.09b	1.75 ± 0.02b	18.85 ± 0.19c
G3	0.35 ± 0.09b	50.9 ± 2.02b	1.72 ± 0.02b	17.50 ± 0.19d
G4	0.84 ± 0.03a	73.3 ± 3.01a	1.32 ± 0.01a	10.77 ± 0.12a
G5	0.74 ± 0.09a	70.7 ± 3.09a	1.32 ± 0.02a	11.05 ± 0.11a
G6	0.30 ± 0.01b	47.7 ± 2.07b	1.79 ± 0.02b	19.92 ± 0.14b
G7	0.55 ± 0.05a	61.6 ± 3.04a	1.64 ± 0.01a	15.56 ± 0.19e
G8	0.51 ± 0.07a	59.5 ± 2.03b	1.60 ± 0.09ab	14.50 ± 0.17f
G9	0.82 ± 0.02a	72.6 ± 3.05a	1.32 ± 0.03a	11.67 ± 0.19a

The footnote remains the same as that in Table 6.

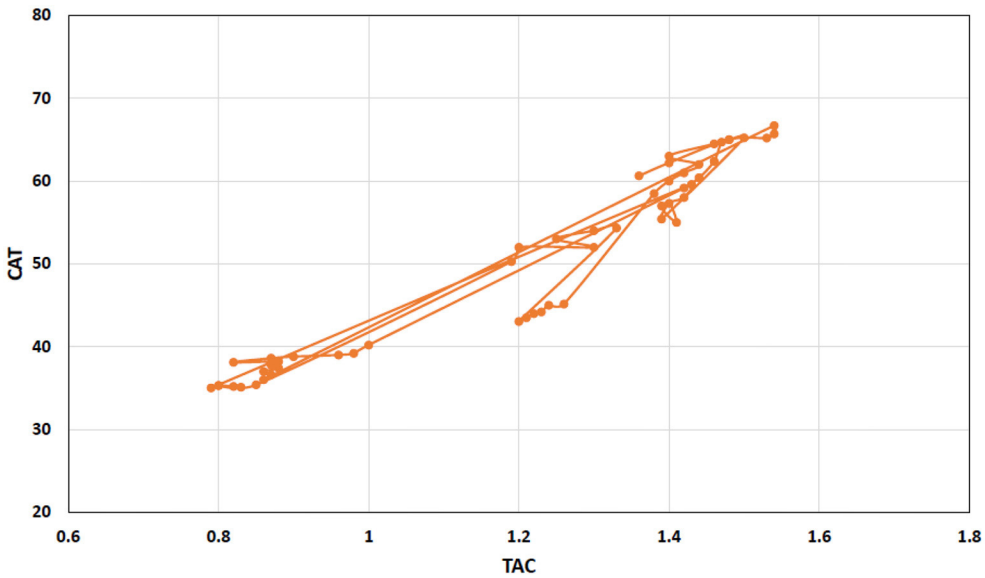

FIGURE 4
Scattered and point connected graph showing total antioxidant capacity (TAC) positive Pearson correlation with catalase in broiler chicks treated with chromium, arsenic, and their combination along and amelioration with vitamin E and bentonite clay.

chromium + arsenic (group 6) was observed. The liver of group 1 showed no microscopic lesion, and hepatocytes had a well-preserved lobular pattern.

Grossly, kidneys were swollen in groups 2 & 7 (+), 3 & 8 (++), and 6 (+ + +). Microscopically, renal epithelial cells necrosis characterized by pyknotic nuclei, glomerular shrinkage, increased urinary spaces, and cytoplasmic vacuolar degeneration was observed in a mild form (+) in group 7, moderate (++) in groups 2, 3 & 8, and severe form (+ + +) in group 6 (Table 8). Degeneration, congestion, disintegration of cells from the basement membrane (Figure 7), karyorrhexis, and karyolysis were also seen in group 6 (+ + +). There was no microscopic lesion in group 1.

Grossly, the heart was normal in size and shape in all treated groups, i.e., groups 2–9, and did not show microscopic lesions and had fairly well-arranged smooth muscles.

Discussion

The data of the present study suggested that arsenic and chromium provoke adverse effects not only on hematobiochemical parameters but also on TAC is severely damaged, leading to oxidative stress. The current study showed a significant ($p < 0.05$) decrease in feed intake and body weight in broiler chicks administered with arsenic plus chromium compared with the control group. Earlier reports have also shown a significant reduction in feed intake and body weight in broiler chicks (26) and rats/mice (13) treated with chromium and arsenic, respectively. Decreased feed intake and body weight could be due to the treatment of broiler chicks with heavy metals that could have led changes in liver glycogen and triglyceride along with a disturbance in metabolic enzymes leading to weight loss (27). Decreased feed intake and body weight could also result from metabolic

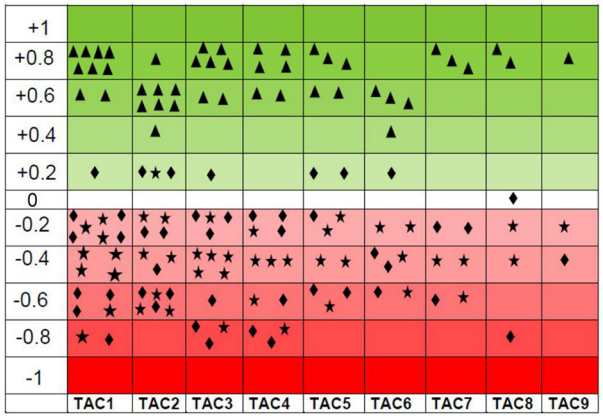

FIGURE 5
Scattered graph showing total antioxidant capacity (TAC) positive Pearson correlation (green shaded) with catalase (▲) and negative Pearson correlation (red shaded) with oxidant enzymes, i.e., total oxidant capacity (♦) and malondialdehyde (★) at experimental day 21. There were nine experimental groups. Broiler chicks were treated with chromium and arsenic, and their combination and amelioration of toxic effects with vitamin E and bentonite clay. A positive Pearson correlation has been shown in the green-shaded area from 0 to +1, whereas a negative Pearson correlation has been shown in the red-shaded area from −1 to 0. TAC also has shown a weak positive Pearson correlation with total oxidant capacity and malondialdehyde. A similar trend was observed on experimental day 42. Thus, only the results of experimental day 21 are presented here.

deregulations due to chromium's toxic effects on the liver (11). Another reason could be due to the inhibitory effect of chromium on the specific area of the hypothalamus in the brain regulating feed intake leading to restricted feed intake and ultimately decreased body weight (28).

Erythrocyte indices decreased significantly ($p < 0.05$) in the present study in broiler chicks treated with arsenic and chromium. At the same time, ESR increased significantly in arsenic plus chromium given to broiler chicks compared to the control group in the current experiment. Earlier studies reported decreased hematological parameters because of chromium and arsenic treatment in rats (29) and broilers (30), respectively. A decrease in erythrocytes numbers, hemoglobin concentration, and hematocrit levels indicates an anemic condition, which could be a decrease in the availability of iron for hemoglobin synthesis as a result of heavy metals exposure leading to the development of anemia (31). Another possible reason could be the ability of chromium to cross the red blood cell membrane where it forms DNA protein crosslinks leading to anemia. Anemia could also occur due to the binding of chromium to the β-chain of hemoglobin; thus, hemoglobin would not be available for heme synthesis ultimately anemia develops (28). Chromium taken up by erythrocytes undergoes reduction to the trivalent form with the help of reduced glutathione (32), chromium-hemoglobin complexes, and other intracellular proteins are sufficiently stable to retain chromium for a substantial fraction of the RBC lifetime (33). In this mechanism, arsenic triggers eryptosis either by increasing cytosolic calcium and ceramide concentration or depleting energy (34). The cytopathic effect of arsenic is attributed to its interference with the erythrocytes' energy production pathway leading to interference in ATP production. Arsenic also interferes with mitochondrial enzymes, so there could be no ATP production; ultimately cell lysis occurs (35).

Significantly ($p < 0.05$) decreased leukocyte counts were recorded in arsenic plus chromium-administered broiler chicks compared to control broiler chicks in the present experiment. Earlier studies reported decreased leukocytes following arsenic/chromium administration in rats (11, 36). A decrease in leukocytes could be due to the inhibitory effect of heavy metals on the immune system leading to leukopenia (29). Chromium affects the cortisol level and may be partially liable for its immunostimulatory effects (37). Cortisol influences antibody production and the functions of lymphocytes and other leukocyte populations (38), thus leading to leukopenia. A decrease in leukocytes could result from chromium contact with biological compounds, leading to the peroxidation of these biological complexes present in the cell (11). In effect, some negative changes, such as cell membrane impairment due to the peroxidation of unsaturated fatty acids or inhibition of both mitochondrial trans-membrane potential occur in lymphocytes (39). Still, another possible reason could be due to the capability of chromium to enter the leukocytes and reside there till its life. In this way, it becomes lethal for the leukocytes, thus leading to leukopenia development.

In the current study, ALT and AST increased significantly ($p < 0.05$), while plasma proteins decreased significantly ($p < 0.05$) in arsenic plus chromium-administered broiler chicks compared to the control group. Earlier studies reported increased ALT and AST concentrations in rats (37) and mice (40) for chromium and arsenic, respectively. An increase in the level of ALT and AST could be due to leakage of these enzymes indicating damage to hepatocytes due to heavy metals (chromium/arsenic). Another reason for their increased levels could be a result of the biotransformation of chromium in hepatocytes rendering injury to hepatocytes (41). Heavy metals are known to produce ROS in the body (42). Hepatocytes develop various defensive mechanisms to block ROS consequences. Among the antioxidative enzymes, CAT concentration has been reported to be very high in liver tissue (43). Thus, CAT provides the first line of antioxidative defense enzymatic system, leading to elevated ALT and AST. In the case of arsenic, high serum hepatic enzymes could be due to its binding to the thiol groups of enzymes and proteins of liver cells while arsenic being bio-converted from its toxic (monomethyl arsenic) to less toxic (dimethyl arsenic) metabolites (44). This hepato-cellular membrane damage leads to elevated ALT levels and loss of functions (45).

For the decreased proteins in the present study, there is a possibility that arsenite and trivalent organic (methylated) arsenicals respond with thiols (-SH) in proteins and impede their action (46). A decrease in plasma proteins could also be due to the impairment of podocytes (visceral epithelial cells in Bowman's capsule). The mechanism of podocyte injury could be due to the production of ROS, which is deleterious for podocyte contractile, modulating, and linkage proteins. ROS induces unrepairable damage to podocytes leading to changes in cell integrity, thus affecting the glomerular filtration rate (GFR) and leakage of proteins (47).

TABLE 8 Gross and microscopic lesions recorded in broiler chicks administered arsenic, chromium, bentonite, and vitamin E, alone or in combinations.

Organ	Gross/microscopic	Lesions	G2 (Cr)	G3 (As)	G6 (As + Cr)	G7 (As + Cr + Vit E)	G8 (As + Cr + BN)	G9 (As + Cr + Vit E + BN)
Lungs	Gross	Hemorrhages	++	++	++	+	+	+
		Frothy exudate	++	++	++	+	+	+
	Microscopic	Edema	++	++	+++	+	+	–
		Congestion	++	++	+++	+	+	–
		Emphysema	++	++	+++	+	+	–
Liver	Microscopic	Pyknotic nuclei	++	++	+++	+	++	–
		Expended sinusoidal spaces	+	++	+++	+	+	–
		Cytoplasmic vacuolar degeneration,	++	++	+++	+	++	–
		Leucocytic infiltration	++	++	+++	+	++	–
Kidneys	Gross	Swollen	+	++	+++	+	++	–
	Microscopic	Tubular necrosis	++	++	+++	+	++	–
		Pyknotic nuclei	++	++	+++	+	++	–
		Vacuolation	++	++	+++	+	++	–
		Congestion	++	++	+++	+	++	–
		Glomerular shrinkage	++	++	+++	+	++	–

No lesion was recorded in G1 (Control), G4 (Vit E), and G5 (Bentonite); therefore, they were excluded from this table.

Lesion categorization: No lesion (–), mild (+), moderate (++), and severe (+++). Cr, Chromium; As, Arsenic, Vit E: Vitamin E, BN: Bentonite. Chromium, arsenic, and vitamin E were given @ 270, 50, and 150 $mg.kg^{-1}$, respectively, while bentonite was administered @ 5%. G1 = Group 1: Control (negative); G2 = Group 2: Chromium; G3 = Group 3: Arsenic; G4 = Group 4: Vitamin E; G5 = Group 5: Bentonite; G6 = Group 6: Chromium + Arsenic; G7 = Group 7: Chromium + Arsenic + Vitamin E; G8 = Group 8: Chromium + Arsenic + Bentonite; and G9 = Group 9: Chromium + Arsenic + Vitamin + Bentonite.

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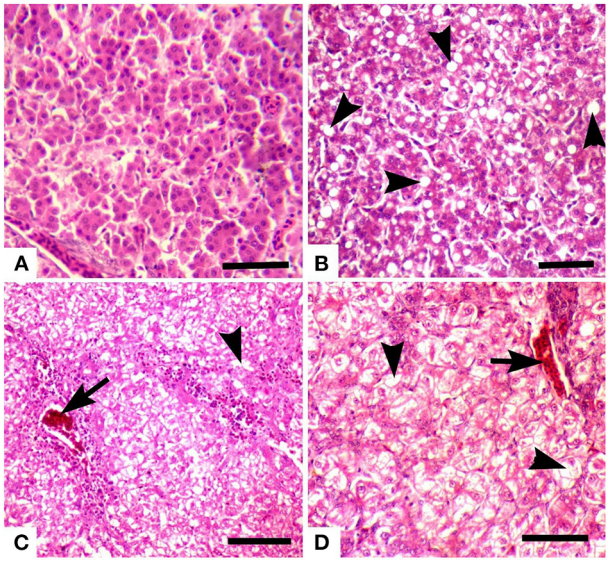

FIGURE 6
Photomicrograph of the liver of broiler chicks stained with H&E (scale bar = 50): **(A)** control group showing no microscopic lesions, and hepatocytes have well-preserved lobular pattern, **(B)** group 6 (chromium + arsenic treated) showing advanced fatty change in hepatocytes (arrow heads), **(C)** group 2 (chromium treated) showing vacuolar degeneration (arrowheads) and congestion (arrow), and **(D)** group 3 (arsenic treated) showing vacuolar degeneration (arrowheads) and congestion (arrow).

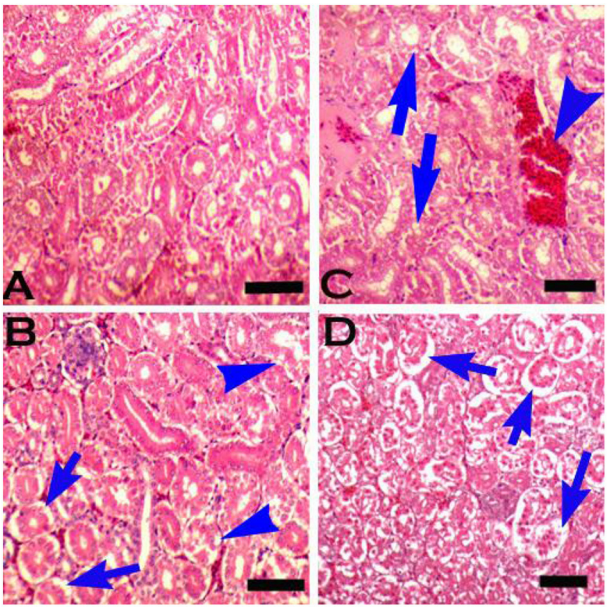

FIGURE 7
Photomicrograph of the kidneys of broiler chicks stained with H&E (scale bar = 50): **(A)** control group showing normal histology, **(B)** group 2 (chromium treated) showing pyknosis and disintegration of cells from the basement membrane (arrows), and degeneration in the form of vacuolation (arrow heads), **(C)** group 3 (arsenic treated) showing congestion (arrowhead) and degenerative changes in the form of vacuolation (arrows), and **(D)** group 6 (chromium + arsenic treated) showing pyknosis and disintegration of cells from the basement membrane (arrows).

Urea and creatinine increased significantly ($p < 0.05$) in arsenic plus chromium-treated broiler chicks compared to the control group in the current study. Earlier reports showed a rise in urea and creatinine in rats (48) for chromium, ducks (49), and goats (50) for arsenic. Urea and creatinine levels could be due to ROS generation, which then causes lipid peroxidation. These lipid droplets sediment in the endothelium of glomeruli, thereby affecting GFR, ultimately damaging the membrane components and leading to necrosis. Therefore, elevated urea and creatinine occur (51). Arsenic has a great affinity to the sulfhydryl group of glomerular filtration membrane; thus, the renal injury could be due to defective GFR (52). After protein metabolism, ammonia is produced. The liver converts it into a less dangerous form as urea which is water soluble and only accumulates in the plasma if the renal system fails to eliminate it from the body (53).

The inequality between the production of ROS and the antioxidant defense system is oxidative stress (13, 14, 54). ROS production is a peculiar feature of heavy metals like arsenic and chromium (46). In this process, mitochondria are the primary organelle affected as the center of cell metabolism. Oxidative stress considerably injures proteins, lipids, and nucleic acids within the mitochondria, resulting in substantial mitochondrial changes in structures and functions (55). Arsenic, a heavy metal, can ruin the anatomy and physiology of mitochondria and yield excess electrons that can convert oxygen (O_2) into superoxide anion (O_2^-). Superoxide anions persuade oxidative stress and produce ROS, resulting in lipid peroxidation and MDA formation (56). ROS also persuades DNA breakage, thus generating many molecules of 8-hydroxy-2 deoxyguanosine (8-OHdG) (57). In the meantime, arsenic can trigger the antioxidant defense system and boost the countenance of molecules such as CAT, SOD, GST, and GPx which remove excessive free radicals and peroxides (56). However, if the degree of oxidation exceeds the ability of these antioxidant molecules, then it will reduce the levels of CAT, SOD, GST, and GPx; this is what we have observed in our study in arsenic and chromium-treated broiler chicks.

Total antioxidant capacity is the primary measurement to evaluate the state and potential of oxidative stress. The imbalance between antioxidants and oxidants generates the condition of oxidative stress (56). In the current study, TAC and CAT decreased significantly ($p < 0.05$), while TOC and MDA increased significantly ($p < 0.05$) in chromium and arsenic administration broiler chicks. Heavy metals like chromium and arsenic cause oxidative stress by lessening antioxidant enzymes (TAC, CAT, SOD, GPx, and glutathione reductase) and elevating lipid peroxidation in both target and non-target animals (58). Oxidative stress mediated by ROS is a common denominator in arsenic toxicity (46).

Arsenic and chromium, as individual metals and in combination, affect animals'/birds' health more terribly. The acquaintance with these metals results in the upsurge of oxidative stress that leads to the creation of an uneven number of electrons, triggering the deterioration of proteins, RNA, and DNA and even leading to cell death (56). However, due to the cleansing systems of bare birds/animals, exposure to different toxicants yields rapid

and increased formation of ROS. ROS production triggers the lipid peroxidation process, leading to cell membrane damage and the development of TBARS (19). As a result, the increased concentration of oxidative stress indices (TOC and MDA) in the present investigation might be connected to antioxidant enzyme diminution and misbalancing (58).

Depression in CAT concentration after feeding rats (59) chromium and goats (60) for arsenic has been reported. A decrease in CAT levels could be due to the involvement of free radicals. In this situation, SOD is slowed down due to the overproduction of free radicals leading to hyperaccumulation of superoxide because of decreased dismutation of superoxide to hydrogen peroxide; thus, decreased TAC and CAT activity is evident (13).

Levels of TOC and MDA increased significantly ($p < 0.05$) in chromium and arsenic-treated broiler chicks compared to the control group in the current study. Earlier studies reported increased MDA in rats (36) for chromium and cattle (61) for arsenic. MDA is a good marker of lipid peroxidation (62). MDA is well-known toxic metabolite formed by lipid peroxidation due to oxidative stress (63). An increase in MDA level could be due to oxygen free radicals, which further target polyunsaturated fatty acids leading to the production of lipid peroxides that then change membrane fluidity and permeability, ultimately rendering cellular damage (64–66).

Histopathological biomarkers use target organs of toxicity in heavy metal studies, mostly the liver and kidneys (67). The liver and kidneys perform several important functions related to the metabolism and excretion of substances. Thus, lesions in such organs caused by chemical pollutants/heavy metals may negatively affect detoxification and homeostasis (68–70). The present study showed gross and microscopic lesions in chromium (270 $mg.kg^{-1}$) and arsenic (50 $mg.kg^{-1}$) singly or in combination-treated groups. In the liver, the main lesions were fatty degeneration, disintegration of cells from the basement membrane, expended sinusoidal spaces, and cytoplasmic vacuolar degeneration.

In contrast, renal epithelial cell necrosis, glomerular shrinkage, and cytoplasmic vacuolar degeneration were kidney lesions. In White Pekin ducks, inorganic arsenic toxicity has been reported (71). Skin lesions due to chronic arsenic toxicity have been reported (70), as the present study was not chronic, thus, we did not observe these lesions. Chromium toxicity also produces severe lesions in the liver and kidneys (72), as observed in the present study (Table 8).

Several synthetic, as well as natural mixtures have been experienced for the amelioration of arsenic and chromium toxicity (40, 69, 73, 74). Vitamin E, an integral part of the plasma membrane, is an effective antioxidant as it is present at the site of free-radical production; it might counteract the toxic effects of ROS (73). The oral vitamin E and $K_2Cr_2O_7$ ameliorate all these vicissitudes and ensue in normal hepatic cellular structure and contents (17, 74). Vitamin E is the most effective fat-soluble non-enzymatic antioxidant, which safeguards the cell membrane from radical-induced peroxidation, rouses the initiation of antioxidant enzymes, and lessens the concentration of oxidative stress produced by heavy metal-induced toxicity (75, 76).

It has been explained earlier that vitamin E allows free radicals to non-concrete a hydrogen atom from the antioxidant molecule rather than from polyunsaturated fatty acids, thus breaking the chain of free radical regeneration, thus resultant antioxidant radical being a comparatively unreactive species (40). Vitamin E, due to its hepatoprotective properties, has got wide attention, which is principally due to its capability to lessen the tempted oxidative stress in various tissues by reducing MDA levels, restoring the levels of CAT, SOD, GSH, and the recovery of impaired hepatocytes (74).

Vitamin E administration improves various hemato-biochemical and oxidative stress parameters in arsenic plus chromium-administered broiler chicks compared to the control group in the present study. Oxidative stress results from a disproportion in free radical generation and antioxidant production. A possible mechanism of vitamin E-induced restoration of antioxidant enzyme levels is breaking the chain reaction initiated by free radicals (77). It repairs the oxidizing radicals responsible for the chain elongation by autoxidation. Its antioxidant activity could also be due to its ability to restore the cell membrane to normal by interacting with the unsaturated fatty acid chain (77). Moreover, vitamin E is an important component of the cytoplasm and cell membrane (75). Vitamin E is efficient only in protecting the outer cellular layer of cells from oxidation stress, although very low concentration still prevents lipids and proteins from oxidation (13, 36). Mechanism regarding chain-breaking antioxidants states that SOD, GPx, and CAT are responsible for removing super oxides and peroxides before metal catalyzes them to generate free radicals (78). However, some free radicals escape the protective mechanism of the enzymes, and a peroxidative chain reaction occurs. Here chain breaker antioxidants, i.e., vitamin E plays an important role by donating electrons, thus limiting this cascade of deterioration (14).

Bentonite is a clay mineral collection of fine particles with high opening volume and particular active sites (17, 79). Bentonite is a widely used porous adsorbent aluminum phyllosilicate clay with high adsorption capability, chemical and mechanical solidity, and unique inter-lamellar structural properties (80, 81). The metal ion belongings, preliminary concentration, adsorbent quantity, and operational circumstances (contacting time, pH, temperature, etc.) are the most important factors for the successful application of raw and altered bentonite (79). Metals like arsenic, chromium etc., interfere with various body processes and are toxic to many organs and tissues (82), thus leading to the generation of ROS, consequently creating oxidative stress in the body (46). Bentonite absorbs these toxic substances and acts as an ameliorating agent (80, 83). In the present study, bentonite administration improved arsenic and chromium-treated broiler chicks compared to the control group. Partial amelioration by bentonite could be due to its ability to prevent heavy metal absorption by forming inert, stable, and insoluble complexes with toxic substances (84, 85). Another proposed mechanism of partial amelioration of bentonite could be due to its adhesive ability and absorptive nature by which it attaches to toxic substances occurs, rendering their absorption lessened (17, 86, 87).

Conclusion

The data suggested that arsenic and chromium aggravate adverse effects not only on hematobiochemical parameters but

also lowering total antioxidants, thus enhancing oxidative stress. All the broiler chicks treated with chromium and arsenic showed a significant ($p < 0.05$) decline in erythrocytic parameters. Total proteins decreased significantly, while ALT, AST, urea, and creatinine increased significantly ($p < 0.05$). TAC and CAT decreased significantly ($p < 0.05$), while TOC and MDA concentrations increased significantly ($p < 0.05$) in chromium and arsenic-treated groups. There was a strong positive correlation between TAC and CAT (Pearson correlation value = 0.961; $p < 0.001$), with similar TOC and MDA positive correlation (Pearson correlation value = 0.920; $p < 0.001$). However, TAC and CAT showed a negative correlation between TOC and MDA. The intensity of gross and microscopic lesions was more in chromium (270 $mg.kg^{-1}$) and arsenic (50 $mg.kg^{-1}$) singly or in combination-treated groups. Lungs of arsenic and chromium treated broiler chicks were hemorrhagic and had frothy exudate in the trachea and microscopically, lungs edema, congestion, thickening of alveolar and bronchial septae, and necrosis were observed. Heart was also normal in size and texture in treated broiler chicks. In the liver, fatty degeneration, detachment of cells from the basement membrane, severe cytoplasmic vacuolar degeneration, and expansion of sinusoidal spaces, while in kidneys, renal epithelial cells necrosis, glomerular shrinkage, and severe cytoplasmic vacuolar degeneration were the main lesions. Co-administered bentonite along with chromium and arsenic resulted in partial amelioration compared to groups administered arsenic plus chromium plus vitamin E and arsenic plus chromium plus vitamin E plus bentonite, respectively. Vitamin E and bentonite administration can ameliorate toxicity and oxidative stress produced by arsenic and chromium.

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 Bioethics Committee, University of Agriculture, Faisalabad, Pakistan.

Author contributions

AK tailored the research project and managed the experiment. JM executed the project with the help of STG, RH, LA, RM, and UF. JM, STG, and RH carried out laboratory work and data analysis. AK, RH, FAAS, and AFA interpreted the data. The manuscript was written by AK and checked by ZG. All authors approved 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.

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Copper sulfate induces clinico-hematological, oxidative stress, serum biochemical and histopathological changes in freshwater fish rohu (*Labeo rohita*)

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Despite being an essential trace element for numerous metabolic processes and micronutrients, copper (Cu) has induced adverse effects on the environment and public health due to its continuous and widespread use for the last several decades. The current study assessed the hematological and histopathological alterations in the freshwater fish (*Labeo rohita*) exposed to graded concentrations of copper sulfate. For this purpose, *L. rohita* fish ($n = 72$), weighing ~200–215 g, were randomly divided into four experimental groups and then exposed to acute doses of $CuSO_4$, i.e., control, 0.28, 0.42, and 0.56 μgL^{-1}. For comparative analysis of hematological and biochemical changes, blood/serum samples were obtained on 12, 24, and 36 days. Overall, the body weight of fish decreased with the time and dose of $CuSO_4$; as the dose increases, body weight decreases. Dose and time-dependent results were observed in other parameters also. Results showed a significant increase in leukocytes, whereas red blood cells count, Hb, and Hct were significantly reduced in treated groups compared to the control. The mean corpuscular hemoglobin (MHC) and mean corpuscular hemoglobin concentration (MCHC) showed a non-significant decrease in treated groups compared to the control group. Serum biochemical parameters, including total proteins, albumin, and globulin, decreased significantly ($p < 0.05$). At the same time, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), glucose, and cholesterol were significantly ($p < 0.05$) increased in the treated groups compared to the control group. Significantly ($p < 0.05$) increased levels of lipid peroxidation while decreased values of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (RGSH) in the blood of fish were recorded. Histopathological examination of fish gills, liver, and kidneys showed inflammation and degenerative changes due to $CuSO_4$ exposure. In the brain tissue, degenerative changes like neuron necrosis, intracellular edema, cytoplasmic vacuolization, and congestion

were observed. In conclusion, the study indicates that exposure to copper sulfate, even in smaller concentrations, can cause adverse hematological and histopathological changes in *L. rohita* fish.

KEYWORDS

copper sulfate, *Labeo rohita*, blood-biochemistry, oxidative stress, histopathology

Introduction

Increased agricultural activities and extensive industrialization ultimately enhance the levels of various metals in the aquatic environment. Copper (Cu), an essential micronutrient for humans and animals, is an important and vital trace element needed for the multiple normal metabolic functions in various living organisms (1, 2). Copper also has an essential role in the regular activity of connective tissue, iron metabolism, and several functions in the central nervous system (3, 4). Plants and animals need copper to perform everyday tasks in different organs, e.g., hemoglobin synthesis. It is also a cofactor for various enzymes (5–7).

Widespread application of copper sulfate ($CuSO_4$) in various sectors like building materials, agricultural sector, electronic industry, water pipes, transportation sector, intra-uterine contraceptive devices, and as an antifungal agent and growth promoter in poultry has also induced toxicological impacts in the exposed species (8). Copper sulfate is used worldwide as a fungicide in agriculture and as an algaecide in aquaculture (9). Unlike other synthetic and organic pesticides, Cu cannot be degraded and can easily be deposited in various tissues of the treated animals (8, 10, 11). Due to frequent application, Cu can damage the cells due to its potential to catalyze the generation of reactive oxygen species (12). Copper sulfate ($CuSO_4$) negatively affected male Nile tilapia's reproductive function (13). Studies have reported that persistent dietary and environmental Cu exposure may cause different neurological and reproductive disorders and hepatic diseases in various animals (8, 14, 15). Cu also causes toxic impacts in terms of poor feed intake, increased oxidative stress, reduced body mass, and biochemical and hematological changes (5, 8, 16).

Over the last years, aquatic contamination has become a great concern for marine life and public health (17–19). Anthropogenic activities and accidental industrial waste discharge have become major causes of aquatic pollution in freshwater (20). Pollution due to heavy metals occur in the aquatic ecosystem due to agricultural discharge, geological weathering, municipal, direct air deposition, and other industrial waste products (21–24). Due to anthropogenic activities, several toxicants, including $CuSO_4$, enter the aquatic ecosystem and cause detrimental effects on the exposed aquatic organisms (13). Fishes are considered an important component of the aquatic food chain among different aquatic organisms and reflect almost all the adverse effects upon exposure to toxicants (25–28). High pollution due to various heavy metals in water has become a significant problem because of the threat to aquatic life and public health concerns related to fish consumption (23, 29, 30). In the natural environment and laboratory specimens, various metals have been linked with different fish abnormalities (31). Constant subjection to several metals, like Zn, Cu, Pb, Cd, Ni, and As, causes harmful effects in organisms (32).

Biochemical parameters are often considered indicators when a clinical diagnosis of aquatic organisms' physiology is applied to determine the effects of external stressors and toxic substances (33). For the contaminants, cellular enzymes (33) and biochemical and oxidative stress parameters (34–40) serve as a biomarker for assessing the risk of environmental hazards (41). The microscopic evaluation of target tissues using histopathological examination is the endpoint in determining the risks of contaminants in the environment (42). Histopathological alterations can be employed to analyze the effects of numerous environmental contaminants on organisms as well as the general health of the aquatic population (43, 44). Investigation of biochemical parameters is recommended to give early indicators regarding modifications in the organisms exposed to toxicants and is especially effective in identifying target organs with toxicity and animal health (26, 45). Thus, the objectives of this study were to ascertain the toxicological impact of $CuSO_4$ in *Labeo rohita* along with dose and time-dependent manner so that a suitable safe, and eco-friendly dose of $CuSO_4$ could be determined for the removal of various contaminants from fish pounds.

Materials and methods

Experimental species, management, and treatments

A total of 72 freshwater fish *L. rohita*, F. Hamilton, 1822, healthy and free from any clinical ailments, with approximately the same age and weight (200–215 g), were collected from a local fish hatchery and breeding center. All the fish were immediately transported to the laboratory and reared in glass aquaria having 100 L of water at 26 ± 1.0 °C and photo-regime (12/12 h) light/dark for 2 weeks under laboratory conditions. Before the start and during

Abbreviations: ALT, Alanine aminotransferase; ALP, Alkaline phosphatase; ANOVA, Analysis of variance; AST, Aspartate aminotransferase; Cd, Cadmium; CAT, Catalase; Cu-NPs, Copper nanoparticles; CuO-NPs, Copper oxide nanoparticles; CuSO4-NPs, Copper Sulfate nanoparticles; CuSO4, Copper Sulfate; Cu, Copper; Cr, Cromiuim; Hct, Hematocrit; Hb, Hemoglobin; HDL, High-density lipoprotein; IL-1, Interleukon-1; LDH, Lactate dehydrogenase; Pb, Lead; LDL, Low-density lipoprotein; MCHC, Mean corpuscular hemoglobin concentration; MHC, mean corpuscular hemoglobin; Ni, Nickle; RBCs, Red blood cells; RGSH, Reduced glutathione; SOD, Superoxide dismutase; TBARS, Thio-barbituric acid reactive substances; TNF-α, Tissue necrosis factor alpha; WBCs, White blood cells; Zn, Zinc.

the trial, all the fish were offered Optimum Aquarium Fish Food having 28% crude proteins (Perfect Companion, Thailand). The feed was provided to all the fish at 2–3% of body mass twice daily. After 2 weeks, the fish were divided randomly into equal four groups (A-D) having 18 fish in each group. Copper sulfate ($CuSO_4$) of analytical grade was procured from the local market. The concentrations of $CuSO_4$ were selected following the previous studies (46, 47). Briefly, $CuSO_4$ was dissolved in water @ 0.28, 0.42, and 0.56 $\mu g.L^{-1}$, respectively and fish in groups B-D were treated with the prepared solution for 36 days.

Physical parameters

Clinical and behavioral signs include loss of equilibrium, mucus secretion from mouth and gills, air gulping, increased swimming, rapid operculum movement, bulging eyes, loss of coordination, erratic swimming, and swimming in isolation in fish treated with various concentrations of copper sulfate were estimated numerically roughly. Each sign was given score – to +++ (– means no sign while + to + + + denoted mild, moderate, and severe, respectively (48). Data thus collected were averaged and presented.

Blood sampling and estimation of hemato-biochemical parameters

On days 12th, 24th, and 36th post-treatment, ∼2.0 mL of blood from the caudal vein of six randomly selected fish was collected using a 26-gauge hypodermic sterile needle after the fish was anesthetized with clove oil (4 $mg.L^{-1}$) (27). A blood sample was placed in anticoagulant-coated glass test tubes on each collection day. Various hematological parameters, including hemoglobin (Hb) quantity, hematocrit (Hct), red blood cells (RBCs) counts, total and differential white blood cells (WBCs) counts, lymphocytes, and neutrophils were determined as described previously (28). Total proteins (Cat # 997180) were assessed according to the earlier method (27). After centrifugation, serum was separated and stored at −20°C. Serum biochemical parameters like aspartate aminotransferase (AST; Cat # 30243), alanine aminotransferase (ALT; Cat # 30254), creatinine (Cat # 99108), cholesterol (Cat # 30183), alkaline phosphatase (ALP; Cat # 30134) and triglycerides (Cat # 30364) were measured using commercial kits (Canovelles; Barcelona, Spain). The quantity of lactate dehydrogenase (LDH) was measured by using a commercial kit (Cat # 21213; Analytical, Germany), while the quantity of urea (Cat # 996060) and albumin (Cat # 997258) was measured with the help of commercially available kits (Quimica Clinica Aplicada, S.A. Spain) using chemistry analyzer (Model: Rx Monza # 328-15-1001; UK) at days 12th, 24th and 36th of the trial (27).

Body mass, absolute and relative organ weight, and histopathology

Randomly selected, six fish were weighed on the 12th, 24th, and 36th day of the trial, then an autopsy was conducted. Visceral organs like the kidneys, brain, gills, and liver were dissected and weighed immediately. The relative weight was measured as a percent of the body weight. Similarly, the liver, kidneys, brain, and gills were preserved in 10% paraformaldehyde for microscopic studies. After fixation, all the visceral tissues were processed for histopathological changes using eosin and hematoxylin staining procedures (27). Tissue slides were examined for the presence of lesions by two pathologists, and if discrepancy come across, then a third pathologist was asked for judgment. Thus, collected histopathological lesions were graded by earmarking numerics. All the slides in each group were studied; median of the numerics was calculated and an cumulative picture of each lesion in each group was drawn. The severity/degree of different histopathological lesions were indicated on the basis of scoring of lesions as mild (25%), moderate (50%), severe (75%) and very severe (100%).

Status of oxidative stress parameters and antioxidant enzymes in erythrocytes

TBARS values were determined according to the procedure already described (49), while reduced glutathione (RGSH), superoxide dismutase (SOD), and catalase (CAT) were determined according to modified methods of Beutler et al. (50), Marklund and Marklund (51) and Chance and Maehly (52), respectively.

Data analysis

The data thus collected from hemato-biochemical, oxidative stress, and antioxidant enzymic parameters were presented as means ± standard error (SE). The data were subjected to ANOVA (one-way analysis of variance) using statistical software (IBM SPSS Statistics, v.22.00) followed by *post hoc* Tukey's test to determine the significant variation at $p < 0.05$.

Results

Physical observations

The present investigation observed no mortality in the treatment groups (B, C, D) during the trial. Clinical and behavioral signs include loss of equilibrium, mucus secretion from mouth and gills, air gulping, increased swimming, rapid operculum movement, bulging eyes, loss of coordination, erratic swimming, and swimming in isolation detected in the treated fish. The severity of various clinical and behavioral signs increases parallelly with increased concentration and exposure time to $CuSO_4$ (Table 1).

The body weight and absolute weight of various visceral tissues of *Labeo rohita*

Results showed that the body weight of fish decreased significantly ($p < 0.05$) in group D ($CuSO_4$: 0.56 μgL^{-1}) compared to the control group on the 36th experimental day (Table 2). The absolute weight of the brain, kidneys, and liver increased gradually

TABLE 1 Intensity of various clinical signs exhibited by the *Labeo rohita* treated with various concentrations of copper sulfate.

Clinical ailments	Groups/treatments		
	B (0.28 $\mu g.L^{-1}$)	C (0.42 $\mu g.L^{-1}$)	D (0.56 $\mu g.L^{-1}$)
Loss of equilibrium	− − +	− + +	+ + +
Mucus secretion from mouth and gills	− − +	− + +	+ + +
Air gulping	− − +	− + +	+ + + +
Increased swimming	− +	− + +	+ + +
Rapid operculum movement	− +	− + + +	+ + + +
Bulging eyes	− +	+ + +	+ + +
Coordination loss	− − +	− + +	+ + +
Erratic swimming	− +	+ + +	+ + + +
Swimming in isolation	− +	+ + +	+ + +
Average	Mild	Moderate	Severe

Fish kept in group A were kept as control and showed no clinical ailments. Copper sulfate was dissolved in sterile water and the fish in groups B to D were treated to 0.28, 0.42, and 0.56 $\mu g.L^{-1}$, respectively for 36 days.

at $CuSO_4$ doses of 0.28 μgL^{-1} and 0.48 μgL^{-1} on experimental days 12 and 24. In contrast, at the $CuSO_4$ dose of 0.56 μgL^{-1}, the absolute weight of these organs increased significantly ($p < 0.05$) than the control group on experimental days 12, 24, and 36 (Table 2). Moreover, the absolute weight of the kidneys and liver also increased significantly ($p < 0.05$) in the control group at $CuSO_4$ dose 0.48 μgL^{-1} on experimental day 36. The absolute weight of gills increased significantly ($p < 0.05$) at $CuSO_4$ dose 0.56 μgL^{-1} than the control group on the 36th experimental day (Table 2).

The relative weight of visceral organs of *Labeo rohita*

Results showed that the relative liver weight of fish increased significantly ($p < 0.05$) at $CuSO_4$ doses of 0.48 μgL^{-1} and 0.56 μgL^{-1} on experimental day 36th than the control group (Table 2). In contrast, the relative gills weight increased significantly ($p < 0.05$) on experimental days 24th and 36th in groups C ($CuSO_4$: 0.48 μgL^{-1}) and D ($CuSO_4$: 0.56 μgL^{-1}) compared to the control group. As far as the relative weight of the kidneys is concerned, that showed a significant ($p < 0.05$) increase on experimental days 12th, 24th, and 36th in group D ($CuSO_4$: 0.56 μgL^{-1}) and also in group C ($CuSO_4$: 0.48 μgL^{-1}) on experimental day 24 compared to control group (Table 3). Significantly ($p < 0.05$) relative weight of the brain increased on experimental days 12th, 24th, and 36th in groups C ($CuSO_4$: 0.48 μgL^{-1}) and D ($CuSO_4$: 0.56 μgL^{-1}) compared to the control group (Table 3).

Hematological parameters

Different hematological parameters of fish exposed to several concentrations of $CuSO_4$ are presented in Table 4. Results showed that RBC counts, hemoglobin, hematocrit, and lymphocytes decreased significantly ($p < 0.05$) while WBC counts and neutrophils increased significantly ($p < 0.05$) on experimental days 12, 24, and 36 in high dose group ($CuSO_4$: 0.56 $\mu g.L^{-1}$) compared to control group. In addition, hemoglobin and hematocrit decreased significantly ($p < 0.05$) at $CuSO_4$ dose 0.42 $\mu g.L^{-1}$ on experimental day 36 compared to the control group. WBCs counts also increased significantly ($p < 0.05$) on experimental day 24 and 36 at $CuSO_4$ dose 0.42 $\mu g.L^{-1}$ compared to control group (Table 4). MHC and MCHC in all treatment groups varied non-significant ($p > 0.05$) throughout the experiment as compared to the control group.

Serum biochemical parameters

Serum biochemical parameters of fish exposed to several concentrations of $CuSO_4$ are presented in Table 5. The concentration of urea, creatinine, AST, LDH, cholesterol, glucose, and triglycerides increased significantly ($p < 0.05$) in the blood serum of fish treated with $CuSO_4$ dose 0.56 $\mu g.L^{-1}$ on experimental days 24 and 36 and also with $CuSO_4$ dose 0.42 $\mu g.L^{-1}$ on experimental day 36 compared to control group. In contrast, ALP and ALT increased significantly ($p < 0.05$) in fish treated with $CuSO_4$ dose 0.56 $\mu g.L^{-1}$ on experimental days 12, 24, and 36 and also with $CuSO_4$ dose 0.42 $\mu g.L^{-1}$ on experimental day 36 compared to control group. Total proteins and albumin concentration decreased significantly ($p < 0.05$) with the treatment of $CuSO_4$ dose 0.56 $\mu g.L^{-1}$ on experimental days 24 and 36, and total proteins also decreased significantly ($p < 0.05$) with the treatment of $CuSO_4$ dose 0.42 $\mu g.L^{-1}$ on experimental day 36 (Table 4).

Oxidative stress and antioxidant enzymes

The results of oxidative stress (Thio-barbituric acid reactive substances) and different antioxidant enzymes (reduced

TABLE 2 Body weight and absolute weight of various visceral organs of *Labeo rohita* treated to copper sulfate with different doses.

Parameters/day	Groups/treatments			
	A (0.0)	B (0.28 $\mu g.L^{-1}$)	C (0.42 $\mu g.L^{-1}$)	D (0.56 $\mu g.L^{-1}$)
Body weight (g)				
12	166.0 ± 2.04	164.25 ± 3.81	160.75 ± 3.42	156.5 ± 1.84
24	168.5 ± 4.36	162.75 ± 3.03	162.5 ± 3.27	158.75 ± 2.28
36	169.25 ± 4.21	162.25 ± 1.95	158.0 ± 1.65	155.75 ± 4.21*
Absolute weight of brain (g)				
12	0.71 ± 0.27	0.72 ± 0.26	0.75 ± 0.21	0.98 ± 0.20*
24	0.74 ± 0.26	0.73 ± 0.01	0.76 ± 0.24	0.99 ± 0.29*
36	0.71 ± 0.25	0.74 ± 0.23	0.77 ± 0.25	0.99 ± 0.30*
Absolute weight of gills (g)				
12	4.65 ± 0.46	4.82 ± 0.51	5.05 ± 0.45	5.15 ± 0.39
24	5.05 ± 0.42	5.01 ± 0.49	5.06 ± 0.90	5.59 ± 0.87
36	5.06 ± 0.42	5.29 ± 0.38	5.57 ± 0.48	7.82 ± 0.49*
Absolute weight of kidneys (g)				
12	0.53 ± 0.07	0.52 ± 0.04	0.56 ± 0.08	0.70 ± 0.18*
24	0.55 ± 0.03	0.62 ± 0.12	0.57 ± 0.16	0.89 ± 0.05*
36	0.58 ± 0.06	0.65 ± 0.05	0.76 ± 0.15*	0.98 ± 0.18*
Absolute weight of liver (g)				
12	0.73 ± 0.16	0.73 ± 0.84	0.75 ± 0.04	0.87 ± 0.03*
24	0.74 ± 0.09	0.79 ± 0.078	0.78 ± 0.23	0.88 ± 0.08*
36	0.76 ± 0.18	0.80 ± 0.05	0.89 ± 0.11*	0.98 ± 0.22*

Values (means ± SE) bearing asterisk in a row significantly ($p < 0.05$) different compared with control group.

glutathione, superoxide dismutase, and catalase) are presented in Table 6. Results recorded a significantly ($p < 0.05$) increased quantity of TBARS in fish treated with $CuSO_4$ dose 0.56 $\mu g.L^{-1}$ on experimental days 24 and 36 and with $CuSO_4$ dose 0.42 $\mu g.L^{-1}$ on experimental day 36 compared to the control group. The TBARS also increased significantly ($p < 0.05$) with the treatment of $CuSO_4$ dose 0.42 $\mu g.L^{-1}$ on experimental day 36 (Table 5). There were significantly ($p < 0.05$) decreased concentrations of different antioxidant enzymes, including reduced glutathione, superoxide dismutase, and catalase in erythrocytes of fish treated with $CuSO_4$ dose 0.56 $\mu g.L^{-1}$ on experimental days 24 and 36 and also with $CuSO_4$ dose 0.42 $\mu g.L^{-1}$ on experimental day 36 compared to control group (Table 6).

Histological alterations in various tissues

Histological investigation of the liver, gill, kidneys, and brain tissues on experimental days 12, 24, and 36 of the control and copper sulfate treated fish was carried out by the use of H and E staining, and various alterations in the tissue morphology were observed and presented below in Tables 7–9 and Figures 1, 2. Dose and time-dependent histopathological alterations in kidneys, gill, liver, and brain tissues occurred; those were fewer at $CuSO_4$ dose 0.28 $\mu g.L^{-1}$ and experimental day 12 and increased significantly ($p < 0.05$) with increasing the dose of $CuSO_4$ from 0.42 $\mu g.L^{-1}$ to 0.56 $\mu g.L^{-1}$ and experimental day from 24 to 36. The degree of histopathological lesions was moderate to severe (Figure 1) at experimental day 36 with $CuSO_4$ dose 0.42 $\mu g.L^{-1}$ whereas these were severe to very severe (Figure 2) at $CuSO_4$ dose 0.56 $\mu g.L^{-1}$ at day 36. The Control group did not exhibit any histopathological alterations.

Discussion

Copper sulfate has been reported to cause deleterious effects at higher levels (53–55). Sometimes the water quality, like pH, dissolved organic carbon, and hardness, also affect the toxicity of metal (e.g., Cu, Zn, Ni) in aquatic environments (22). Among all the environmental issues, metal pollution is the most serious and major hazard to the safety of humans and worldwide ecosystems (21, 44, 56–58).

The current study showed that various concentrations of $CuSO_4$ affect fish health in several ways. In our trial, we observed no mortality in both control and $CuSO_4$-treated experimental groups. However, the treated groups showed different clinical and behavioral signs like rapid operculum movement, tremors of fins, hypersecretion of mucus, erratic swimming, increased swimming

TABLE 3 Relative weight of visceral organs of *Labeo rohita* treated with various concentrations of copper sulfate.

Parameters/day	Groups/treatments			
	A (0.0)	B (0.28 μg.L^{-1})	C (0.42 μg.L^{-1})	D (0.56 μg.L^{-1})
Liver (g)				
12	0.27 ± 0.03	0.27 ± 0.02	0.30 ± 0.03	0.31 ± 0.11
24	0.30 ± 0.05	0.28 ± 0.05	0.32 ± 0.04	0.35 ± 0.13
36	0.30 ± 0.03	0.31 ± 0.07	0.38 ± 0.11*	0.41 ± 0.12*
Gills (g)				
12	2.76 ± 0.26	3.14 ± 0.23	3.15 ± 0.28	3.23 ± 0.30
24	3.24 ± 0.25	3.07 ± 0.34	3.47 ± 0.53	3.81 ± 0.51*
36	3.19 ± 0.23	3.08 ± 0.27	3.93 ± 0.34*	3.97 ± 0.21*
Kidneys (g)				
12	0.34 ± 0.05	0.33 ± 0.03	0.33 ± 0.05	0.44 ± 0.12*
24	0.36 ± 0.03	0.38 ± 0.07	0.34 ± 0.12	0.45 ± 0.04*
36	0.35 ± 0.04	0.39 ± 0.03	0.48 ± 0.08*	0.49 ± 0.13*
Brain (g)				
12	0.32 ± 0.16	0.34 ± 0.16	0.46 ± 0.17*	0.51 ± 0.18*
24	0.36 ± 0.16	0.35 ± 0.01	0.55 ± 0.15*	0.58 ± 0.12*
36	0.34 ± 0.15	0.36 ± 0.13	0.58 ± 0.14*	0.59 ± 0.18*

Values (means ± SE) bearing asterisk in a row significantly ($p < 0.05$) different compared with control group.

area, loss of equilibrium, loss of coordination, increased surface breathing, and air gulping. Though no mortality was recorded in the present study, however high survival rate in the Japanese Medaka (*Oryzias latipes*) fish has been reported with exposure to low (10 BBP) compared with high (100 BBP) dose of $CuSO_4$ (59). In the present study, the relative and absolute weight of fish exposed to $CuSO_4$ showed a significant ($p < 0.05$) reduction in the treated groups compared to the control group. Similarly, it has been reported that *Epinephelus coioides* supplemented with $CuSO_4$ and copper nanoparticles (Cu-NPs) rearing water exhibited lower weight gain than the control group (60). A decrease in growth performance could be due to two factors: in homeostasis and detoxification, metabolic expenditure increases, and higher exposure to Cu reduces feed intake, which ultimately leads to growth reduction (61).

In the present trial, various hematological and histopathological parameters were studied. Hematological signs are important for environmental monitoring, toxicological research, and indicators for many diseases. These parameters are critical for determining the physiological condition of fish under stress caused by metals or contaminants. In the current study, hematological parameters such as WBCs, and neutrophils were increased with the increase in concentration and duration. However, erythrocytes (4.83 ± 0.18 vs. 3.09 ± 0.05 $\times 10^6$/mm^3), hematocrit (34.14 ± 2.39 vs. 24.70 ± 0.58 %), and hemoglobin (8.96 ± 0.23 vs. 6.91 ± 0.18 g.dL$^{-1)}$, and lymphocytes (18.61 ± 0.54 vs. 14.85 ± 0.16 %) were decreased significantly ($p < 0.05$) after the exposure to $CuSO_4$ (0.28 μg.L^{-1}) on all experimental days. Similarly, $CuSO_4$ (0.05 mg/L) exposure to fish *Oreochromis niloticus* had been reported to significantly ($p < 0.05$) decrease erythrocytes (1.78 ± 0.03 vs. 1.41 ± 0.02 $10^6/\mu$L), hematocrit (31.95 ± 0.74 vs. 24.83 ± 0.37 %), and hemoglobin (8.38 ± 0.09 vs. 5.94 ± 0.12 g/dL) values (17). It is also on record that $CuSO_4$-NPs render more reduction in these parameters than $CuSO_4$ itself (17). The lower hematological values in this study might be due to hemoglobin oxidation, erythrocyte destruction, and hemolysis (23, 47, 53, 56). Previous studies also described a decline in RBCs, Hb, and Hct contents in freshwater fishes exposed to Cd and Ni (58). In the current study, the hematological abnormalities might be due to the destruction or decreased production of RBCs in hematopoietic tissues, increased production of free radicals, and insufficient supply of oxygen to blood-forming tissues through gills. Moreover, several other studies have reported similar lower hematological values in different organisms like albino rats (62), yellow catfish (61), freshwater fish (47), and *Catla Catla* (23) exposed to various toxicants or pollutants. Additionally, the increased WBCs values and neutrophil percentage could be related to the stress conditions induced by the inflammatory response.

In the present study, except total proteins (3.75 ± 0.04 vs. 2.08 ± 0.04 mg.dL^{-1}), all biochemical parameters including serum urea (8.04 ± 0.05 vs.11.57 ± 0.05 mg.L^{-1}), creatinine (1.28 ± 0.02 vs. 1.94 ± 0.02 mg.L^{-1}), cholesterol (158.9 ± 1.50 vs. 188.3 ± 3.50 mg.L^{-1}), glucose (30.7 ± 1.19 vs. 37.4 ± 1.19 mg.L^{-1}), triglycerides (156.3 ± 3.41 vs.183.7 ± 2.41 mg.L^{-1}) along with serum enzymes i.e., ALT (23.10 ± 0.13 vs. 32.60 ± 0.13 UL^{-1}), AST (13.80 ± 0.13 vs.18.10 ± 0.13 UL^{-1}), ALP (24.40 ± 0.13 vs. 33.90 ± 0.13 UL^{-1}) and LDH (253.3 ± 2.61 vs. 274.4 ± 5.61 UL^{-1}) increased significantly ($p < 0.05$) in *L. rohita* with

TABLE 4 Hematology of fish treated with various concentrations of copper sulfate.

Parameters/day	Groups/treatments			
	A (0.0)	B (0.28 μg.L^{-1})	C (0.42 μg.L^{-1})	D (0.56 μg.L^{-1})
Red blood cell count ($\times 10^6$/mm^3)				
12	4.83 ± 0.18	4.06 ± 0.19	3.96 ± 0.16	3.09 ± 0.05*
24	5.05 ± 0.29	4.03 ± 0.27	3.85 ± 0.03	3.33 ± 0.11*
36	5.02 ± 0.07	4.01 ± 0.11	3.83 ± 0.17	3.29 ± 0.18*
Hemoglobin concentration (g.dL^{-1})				
12	8.96 ± 0.23	8.73 ± 0.31	7.72 ± 0.28	6.91 ± 0.18*
24	9.03 ± 0.30	8.38 ± 0.47	7.69 ± 0.16	6.81 ± 0.26*
36	9.38 ± 0.20	8.68 ± 0.28	6.45 ± 0.13*	6.30 ± 0.21*
White blood cell counts ($\times 10^3$/mm^3)				
12	12.55 ± 0.21	15.09 ± 0.42	15.44 ± 0.51	16.67 ± 0.50*
24	13.01 ± 0.43	14.94 ± 0.22	16.28 ± 0.44*	18.47 ± 0.35*
36	12.56 ± 0.61	14.28 ± 0.42	17.22 ± 0.28*	18.96 ± 0.31*
Hematocrit				
12	34.14 ± 2.39	35.78 ± 0.94	29.53 ± 0.96	24.70 ± 0.58*
24	35.18 ± 0.41	34.73 ± 1.01	30.52 ± 0.95	23.48 ± 0.25*
36	36.50 ± 0.32	32.78 ± 0.36	26.35 ± 0.32*	23.30 ± 2.29*
MCH (pg)				
12	38.25 ± 0.28	35.88 ± 0.35	32.92 ± 1.05	14.48 ± 1.05
24	36.48 ± 0.96	36.55 ± 0.30	19.20 ± 1.72	15.94 ± 1.15
36	38.08 ± 0.89	37.43 ± 0.68	34.13 ± 1.64	17.95 ± 0.95
MCHC (g.dL^{-1})				
12	34.70 ± 0.67	33.25 ± 0.18	31 ± 0.86	26.02 ± 1.41
24	34.70 ± 0.82	32.85 ± 0.14	31.55 ± 0.44	25.53 ± 2.68
36	35.48 ± 0.50	33.93 ± 0.05	32.20 ± 0.88	24.98 ± 1.91
Lymphocytes (%)				
12	18.61 ± 0.54	17.50 ± 0.34	15.85 ± 0.18	14.85 ± 0.16*
24	20.34 ± 0.18	19.20 ± 0.37	16.79 ± 0.41	13.67 ± 0.18*
36	19.97 ± 0.36	18.35 ± 0.35	16.31 ± 0.29	12.75 ± 0.17*
Neutrophils (%)				
12	15.78 ± 0.22	17.37 ± 0.26	19.67 ± 0.65	22.50 ± 0.36*
24	15.97 ± 0.26	17.29 ± 0.29	19.78 ± 0.79	22.75 ± 0.43*
36	15.89 ± 0.23	17.35 ± 0.26	19.79 ± 0.69	22.62 ± 0.33*

Values (means ± SE) bearing asterisk in a row significantly ($p < 0.05$) different compared with control group.

the exposure of $CuSO_4$ (Table 5). However, along with other biochemical parameters such as glucose (105 ± 1.1 vs. 115 ± 0.5 mg/dL), AST (55.8 ± 0.3 vs. 57.4 ± 0.2 µg/L), ALT (28.6 ± 0.4 vs. 30.8 ± 0.5 µg/L) and uric acid (12.7 ± 0.04a 13 ± 0.1 mg/dL), total proteins (5.6 ± 0.1 vs. 6.4 ± 0.1 g/dL) also increased significantly ($p < 0.05$) in Nile tilapia (*Oreochromis niloticus*) with the treatment of both $CuSO_4$ (15 mg/L) and CuO-NP (24). Increased total proteins in Nile tilapia with the treatment of $CuSO_4$ (24) could be the reason that during exposure to Cu, fish may require higher protein concentration to meet the increased demand for repairing tissue and enhance the immunological response (20). Similarly, Atta et al. (63) reported that birds supplemented with excessive $CuSO_4$ revealed an increase in ALP, ALT, and AST activities while reducing albumin, globulin levels, and total serum protein are consistent with our results. The hypoproteinemia in this study could be due to impairment of protein synthesis or

TABLE 5 Serum biochemistry of fish treated to various concentrations of copper sulfate.

Parameters/day	Groups/treatments			
	A (0.0)	B (0.28 μg.L^{-1})	C (0.42 μg.L^{-1})	D (0.56 μg.L^{-1})
Albumin quantity (mg.dL^{-1})				
12	2.85 ± 0.01	2.81 ± 0.01	2.77 ± 0.01	2.73 ± 0.01
24	2.81 ± 0.01	2.77 ± 0.01	2.72 ± 0.01	2.18 ± 0.01*
36	2.79 ± 0.01	2.74 ± 0.01	2.70 ± 0.01	2.15 ± 0.01*
Total proteins (mg.dL^{-1})				
12	3.89 ± 0.03	3.72 ± 0.03	3.55 ± 0.03	3.38 ± 0.03
24	3.83 ± 0.03	3.63 ± 0.03	3.44 ± 0.03	3.14 ± 0.03*
36	3.75 ± 0.04	3.49 ± 0.04	3.14 ± 0.04*	2.08 ± 0.04*
Aspartate aminotransferase (UL^{-1})				
12	13.65 ± 0.06	15.05 ± 1.06	15.35 ± 0.06	15.75 ± 0.06
24	13.07 ± 0.09	15.55 ± 1.06	16.05 ± 0.06	17.52 ± 0.09*
36	13.80 ± 0.13	16.60 ± 0.13	17.30 ± 0.13*	18.10 ± 0.13*
Alkaline phosphatase (UL^{-1})				
12	24.55 ± 0.10	26.17 ± 0.11	26.82 ± 0.11	30.45 ± 0.10*
24	25.15 ± 0.10	26.82 ± 0.11	27.47 ± 0.11	32.15 ± 0.10*
36	24.40 ± 0.13	27.20 ± 0.13	29.10 ± 0.13*	33.90 ± 0.13*
Alanine aminotransferase (UL^{-1})				
12	22.05 ± 0.06	22.45 ± 0.06	22.75 ± 0.06	28.15 ± 0.06*
24	22.45 ± 0.10	23.07 ± 0.11	23.72 ± 0.11	29.35 ± 0.10*
36	23.10 ± 0.13	23.9 ± 0.13	29.80 ± 0.13*	32.60 ± 0.13*
Lactate dehydrogenase (UL^{-1})				
12	248.3 ± 2.19	249.5 ± 3.19	250.7 ± 1.19	251.9 ± 5.19
24	251.7 ± 1.48	254.6 ± 2.47	257.6 ± 2.47	269.6 ± 3.48*
36	253.3 ± 2.61	257.0 ± 3.61	268.7 ± 3.61*	274.4 ± 5.61*
Urea (mg.L^{-1})				
12	8.02 ± 0.04	8.57 ± 0.04	8.81 ± 0.04	9.06 ± 0.04
24	8.08 ± 0.05	8.87 ± 0.05	9.16 ± 0.05	10.45 ± 0.05*
36	8.04 ± 0.05	9.02 ± 0.05	10.29 ± 0.05*	11.57 ± 0.05*
Creatinine (mg.L^{-1})				
12	1.17 ± 0.01	1.18 ± 0.02	1.21 ± 0.02	1.23 ± 0.04
24	1.20 ± 0.04	1.22 ± 0.04	1.25 ± 0.04	1.87 ± 0.04*
36	1.28 ± 0.02	1.29 ± 0.02	1.72 ± 0.02*	1.94 ± 0.02*
Cholesterol (mg.L^{-1})				
12	154.6 ± 2.17	155.7 ± 3.17	156.8 ± 3.17	157.9 ± 2.17
24	156.5 ± 3.39	158.9 ± 3.39	161.2 ± 3.39	173.6 ± 2.39*
36	158.9 ± 1.50	162.1 ± 2.50	175.2 ± 2.50*	188.3 ± 3.50*
Glucose (mg.L^{-1})				
12	29.3 ± 1.16	30.2 ± 1.15	31.1 ± 1.15	32.1 ± 2.16
24	29.6 ± 1.19	30.8 ± 1.19	32.2 ± 1.19	35.2 ± 1.19*
36	30.7 ± 1.19	31.9 ± 1.19	35.6 ± 1.19*	37.4 ± 1.19*

(Continued)

TABLE 5 (Continued)

Parameters/day	Groups/treatments			
	A (0.0)	B (0.28 μg.L^{-1})	C (0.42 μg.L^{-1})	D (0.56 μg.L^{-1})
Triglycerides (mg.L^{-1})				
12	151.6 ± 1.34	157.7 ± 2.32	159.8 ± 1.32	160.9 ± 2.34
24	153.2 ± 2.35	158.4 ± 2.35	161.6 ± 1.35	179.8 ± 3.35*
36	156.3 ± 3.41	159.7 ± 2.41	181.2 ± 2.41*	183.7 ± 2.41*

Values (means ± SE) bearing asterisk in a row significantly ($p < 0.05$) different compared with control group.

TABLE 6 Oxidative stress and antioxidant enzymes in erythrocytes of fish (*Labeo rohita)* treated to copper sulfate.

Parameters/day	Groups/treatments			
	A (0.0)	B (0.28 μg.L^{-1})	C (0.42 μg.L^{-1})	D (0.56 μg.L^{-1})
Thio-barbituric acid reactive substances (TBARS) contents (nmol TBARS/h/mg protein)				
12	37.21 ± 1.11	38.41 ± 1.13	40.13 ± 1.04	41.25 ± 1.17
24	38.71 ± 1.21	39.21 ± 1.11	40.89 ± 1.37	46.35 ± 1.33*
36	39.31 ± 2.01	41.71 ± 1.28	47.31 ± 1.55*	53.19 ± 166*
Reduced glutathione (μmol GSH/mg protein)				
12	0.12 ± 0.04	0.11 ± 0.03	0.11 ± 0.01	0.19 ± 0.03
24	0.13 ± 0.01	0.12 ± 0.08	0.10 ± 0.07	0.08 ± 0.01*
36	0.13 ± 0.02	0.11 ± 0.06	0.07 ± 0.01*	0.06 ± 0.02*
Superoxide dismutase (units/mg protein)				
12	2.33 ± 0.19	2.19 ± 0.13	1.99 ± 0.17	1.88 ± 0.11
24	2.37 ± 0.15	2.11 ± 0.15	1.88 ± 0.11	1.64 ± 0.05*
36	2.38 ± 0.15	2.03 ± 0.16	1.45 ± 0.21*	1.39 ± 0.11*
Catalase (units/mg protein)				
12	2.45 ± 0.07	2.43 ± 0.09	2.39 ± 0.13	2.33 ± 0.15
24	2.47 ± 0.14	2.35 ± 0.23	2.22 ± 0.21	1.68 ± 0.05*
36	2.46 ± 0.12	2.25 ± 0.16	1.71 ± 0.02*	1.37 ± 0.22*

Values (means ± SE) bearing asterisk in a row significantly ($p < 0.05$) different compared with control group.

the functional damage to the liver (31), and excessive loss of protein caused by degenerated glomerulus or could be explained due to the oxidative stress of copper on the liver and kidney tissue (64). There is also the possibility that the release of intracellular enzymes as a result of hepatic degeneration and necrosis caused by $CuSO_4$ could lead to lipid peroxidation, which resultantly ends in hypoproteinemia (65). In the present research, the cholesterol levels increased significantly in *L. rohita* exposed to $CuSO_4$ in a dose-dependent manner. Similarly, the findings of Almansour (66) are consistent with our results, who observed an increase in the cholesterol, total lipid, and LDL with no variation in HDL level in quail that were exposed to copper. Contrarily, no alteration was seen in total plasma cholesterol levels in broilers exposed to $CuSO_4$ (67).

In this experimental trial, TBARS (39.31 ± 2.01 vs. 53.19 ± 166 nmol TBARS/h/mg protein) increased significantly ($p < 0.05$) whereas RGSH (0.13 ± 0.02 vs. 0.06 ± 0.02 μmol GSH/mg protein), SOD (2.38 ± 0.15 vs. 1.39 ± 0.11 units/mg protein) and CAT (2.46 ± 0.12 vs. 1.37 ± 0.22 units/mg protein) decreased significantly ($p < 0.05$) in red blood cells of *L. rohita* with the exposure of $CuSO_4$ (Table 6). It has been reported that antioxidant enzymes such as SOD (11.6 ± 0.1 vs. 12.1 ± 0.13 IU/L), TAC (1.1 ± 0.01 vs. 0.9 ± 0.02 μM/L) and CAT (10.7 ± 0.1 vs. 11.5 ± 0.1 IU/L) increased significantly ($p < 0.05$) in Nile tilapia (*Oreochromis niloticus*) with the treatment of both $CuSO_4$ (15 mg/L) and CuO-NP (24). The reduced quantity of different oxidant enzymes in erythrocytes of $CuSO_4$ treated fish could be due to higher turnover of free radicals leading to depletion of antioxidants enzymes and induction of oxidative stress (40, 55, 68–70). Reduction in different antioxidant enzymes, including SOD, CAT, POD, and RGSH in erythrocytes of fish can possibly be due to different physiological disorders in erythrocytes and poor integrity of erythrocytic membranes in association with induction of low-grade inflammatory mechanisms in treated fish leading to inhibition of release of TNF-α and IL-1 (71). Studies have recorded that monitoring and evaluation of oxidative stress are of vital importance, which plays a vital role in

TABLE 7 Severity/degree of different histopathological lesions in various organs/tissues of *Labeo rohita* treated to various concentrations of copper sulfate at 12th day.

Histopathological lesions	Groups		
	B (0.28 $\mu g.L^{-1}$)	C (0.42 $\mu g.L^{-1}$)	D (0.56 $\mu g.L^{-1}$)
Brain			
Congestion	++	+++	++++
Neuron's necrosis	++	+++	++++
Intracellular edema	+	++	+++
Cytoplasmic vacuolization	+	++	++++
Gills			
Congestion	++	+++	++++
Lamellar pillar cell's necrosis	++	+++	++++
Cartilaginous core congestion	++	+++	++++
Cartilaginous core degeneration	+	++	+++
Lamellar fusion	+	++	++++
Lamellar atrophy	+	+++	++++
Lamellar disorganization	+	+++	++++
Telangiectasia	++	+++	++++
Lamellae uplifting	+	+++	++++
Primary lamellae disruption	+	++	++++
Secondary lamellae curling	+	++	+++
Liver			
Congestion	++	+++	++++
Vacuolar degeneration	+	++	+++
Hemorrhages	++	+++	++++
Hepatocytes with eccentric nuclei	+	+++	++++
Hepatocyte degeneration	++	++	+++
Pyknosis	+	+++	++++
Karyorrhexis	+	++++	++++
Karyolysis	+	+++	++++
Nuclear hypertrophy	++	++	++++
Ceroid formation	++	+++	++++
Kidneys			
Ceroid formation	++	+++	++++
Congestion	++	++	+++
Edema	++	++	+++
Bowman's space increased	+	++	+++
Tubular cells necrosis	+	++	+++
Glomerulus deterioration	+	++	+++
Renal tubule degeneration and abolition	++	+++	++++
Nuclear hypertrophy	++	+++	++++
Melanomacrophage aggregates	++	+++	++++
Thyroidization	++	+++	++++

The control group did not show any lesion; thus was not mentioned in the Table. The degree of histopathological lesions was based on severity as mild (+, 25%), moderate (++, 50%), severe (+++, 75%) and very severe (++++, 100%).

TABLE 8 Severity/degree of different histopathological changes in various tissues of *Labeo rohita* exposed to various concentrations of copper sulfate at day 24th.

Histopathological lesions	Groups		
	B (0.28 $\mu g.L^{-1}$)	C (0.42 $\mu g.L^{-1}$)	D (0.56 $\mu g.L^{-1}$)
Brain			
Congestion	++	+++	++++
Neuron's necrosis	++	+++	++++
Intracellular edema	++	+++	++++
Cytoplasmic vacuolization	+	++	++++
Gills			
Congestion	++	+++	++++
Lamellar pillar cell's necrosis	++	+++	++++
Cartilaginous core congestion	++	+++	++++
Cartilaginous core degeneration	+	++++	++++
Lamellar fusion	+	++++	++++
Lamellar atrophy	+	++++	++++
Lamellar disorganization	++	+++	++++
Telangiectasia	++	+++	++++
Lamellae uplifting	++	++++	++++
Primary lamellae disruption	++	++++	++++
Secondary lamellae curling	++	+++	++++
Liver			
Congestion	++	++	++++
Vacuolar degeneration	+	+++	++++
Hemorrhages	++	+++	++++
Hepatocytes with eccentric nuclei	++	++++	++++
Hepatocyte degeneration			
Pyknosis	+	+++	++++
Karyorrhexis	+	++++	++++
Karyolysis	++	++	++++
Nuclear hypertrophy	++	+++	++++
Ceroid formation	+	++	++++
Kidneys			
Ceroid formation	++	++	++++
Congestion	++	+++	++++
Edema	++	+	++++
Bowman's space increased	++	+++	++++
Tubular cells necrosis	++	+++	++++
Glomerulus deterioration	++	+++	++++
Renal tubule degeneration and abolition	+	++	++++
Nuclear hypertrophy	++	++	++++
Melanomacrophage aggregates	+	++	++++
Thyroidization	++	++	++++

The control group did not show any lesion; thus was not mentioned in the Table. The degree of histopathological lesions was based on severity as mild (+, 25%), moderate (++, 50%), severe (+++, 75%) and very severe (++++, 100%).

TABLE 9 Severity/degree of different histopathological changes in various tissues of *Labeo rohita* exposed to different concentrations of copper sulfate at day 36.

Histopathological lesions	Groups		
	B (0.28 $\mu g.L^{-1}$)	C (0.42 $\mu g.L^{-1}$)	D (0.56 $\mu g.L^{-1}$)
Brain			
Congestion	++	+++	++++
Neuron's necrosis	++	+++	++++
Intracellular edema	++	+++	++++
Cytoplasmic vacuolization	+	++	++++
Gills			
Congestion	++	+++	++++
Lamellar pillar cell's necrosis	++	+++	++++
Cartilaginous core congestion	++	+++	++++
Cartilaginous core degeneration	+	++++	++++
Lamellar fusion	+	++++	++++
Lamellar atrophy	+	++++	++++
Lamellar disorganization	++	+++	++++
Telangiectasia	++	+++	++++
Lamellae uplifting	++	++++	++++
Primary lamellae disruption	++	++++	++++
Secondary lamellae curling	++	+++	++++
Liver			
Congestion	++	+++	++++
Vacuolar degeneration	++	+++	++++
Hemorrhages	++	+++	++++
Hepatocytes with eccentric nuclei	++	+++	++++
Hepatocyte degeneration	++	+++	++++
Pyknosis	+	+++	++++
Karyorrhexis	++	++++	++++
Karyolysis	++	+++	++++
Nuclear hypertrophy	++	+++	++++
Ceroid formation	++	+++	++++
Kidneys			
Ceroid formation	++	+++	++++
Congestion	++	+++	++++
Edema	++	+++	++++
Bowman's space increased	++	+++	++++
Tubular cells necrosis	++	+++	++++
Glomerulus deterioration	++	+++	++++
Renal tubule degeneration and abolition	++	++++	++++
Nuclear hypertrophy	++	+++	++++
Melanomacrophage aggregates	+	+++	++++
Thyroidization	++	++	++++

The control group did not show any lesion; thus was not mentioned in the Table. The degree of histopathological lesions was based on severity as mild (+, 25%), moderate (++, 50%), severe (+++, 75%) and very severe (++++, 100%).

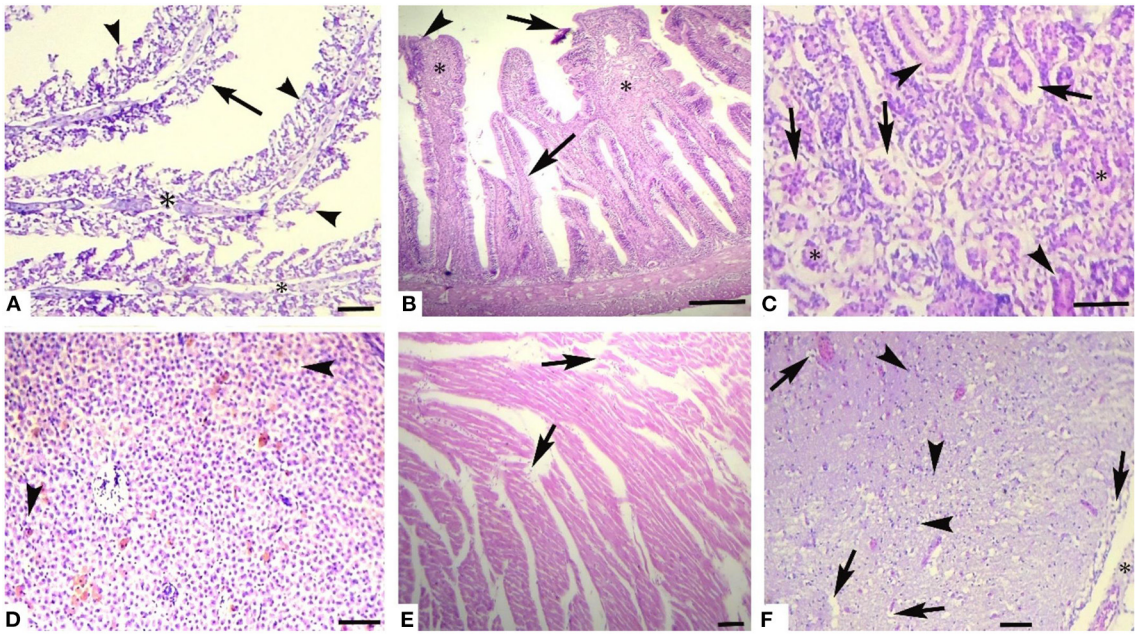

FIGURE 1
Photomicrograph of different tissues of freshwater fish treated with $CuSO_4$ (0.42 $\mu g.L^{-1}$) at experimental day 36: **(A)** Gills showing aneurysm (arrowheads), disorganization of primary lamellae (double asterisk) and necrosis of epithelium (arrows), **(B)** Intestine showing sloughing of epithelium from villi (arrows), necrosed epithelium (arrowheads) and necrosis of middle portio of villi (asterisk), **(C)** kidneys showing detachment of epithelium from the basement membrane (arrows), necrosis of renal tubules epithelial cells (arrowheads), protenecious material in tubulat lumen (asterisk) **(D)** Liver showing vacuolar degeneration (arrowheads) and necrosis of hepatocytes, **(E)** Heart showing disorganization of cardiac muscles (arrow), and **(F)** Brain showing necrosis of neurons (arrowheads), vacuolar degeneration of neurons (arrows) and microgliosis (asterisk). H and E. Bar is equal to **(A)** and **(E)** = 50 μm, and **(B–D)** and **(F)** = 100 μm.

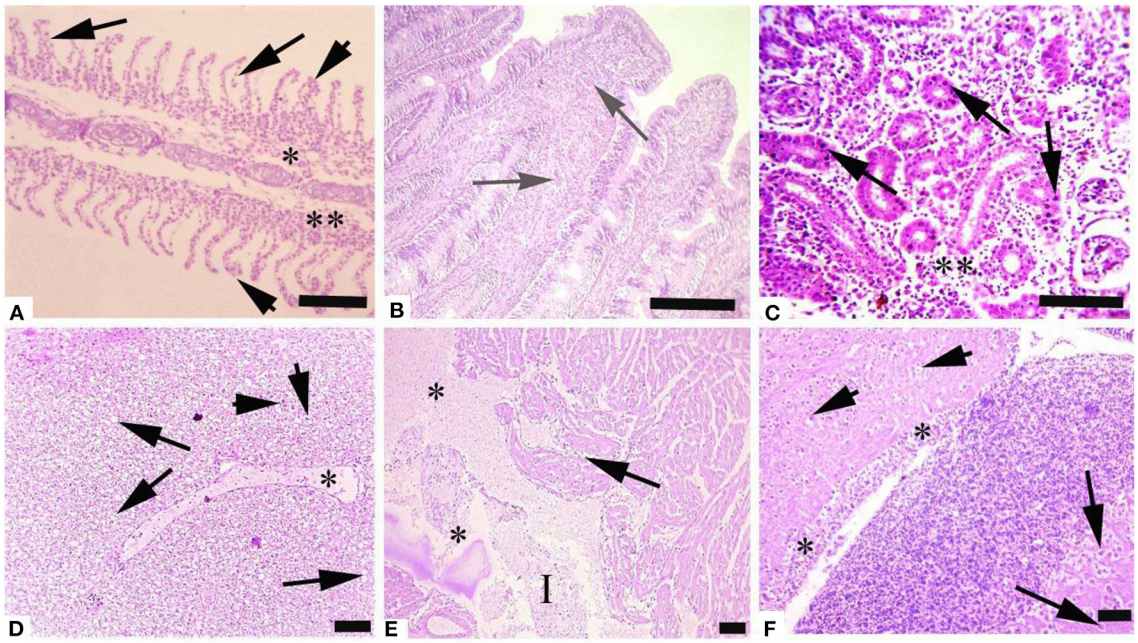

FIGURE 2
Photomicrograph of different tissues of freshwater fish treated with $CuSO_4$ (0.56 $\mu g.L^{-1}$) at experimental day 36: **(A)** Gills showing aneurysm (arrowheads), disorganization of primary lamellae (double asterisk) and necrosis of epithelium of secondary lamellae (arrows), **(B)** Intestine showing hemorrhages (arrows), **(C)** kidneys showing necrosis of renal tubules (asterisk) and necrosis of tubular epithelial cells (arrows), **(D)** Liver showing edema (asterisk), vacuolar degeneration (arrows) and necrosis (arrowheads), **(E)** Heart showing disorganization of cardiac muscles (arrow), edema (asterisks) and inflammatory exudate (I) and **(F)** Brain showing necrosis of neurons (arrowheads), degeneration of neurons (arrows) and microgliosis (asterisk). H and E. Bar is equal to **(A)** and **(E)** = 50 μm, and **(B–D)** and **(F)** = 100 μm.

the development of different physiological disorders in animals and also leads to lipid peroxidation, DNA damage, apoptosis, enzymic deactivation, and cell necrosis (71–75).

Histopathological changes have been extensively utilized as biomarkers while assessing the health of exposed fish to toxins/pollutants (76). Moreover, histopathology is the microscopic assessment of alteration in morphology that indicates disease in an organism (77, 78). We observed histopathological alterations in liver tissues, like ceroid formation, congestion, vacuolar degeneration, hemorrhages, pyknosis, karyorrhexis, degenerated hepatocytes, hepatocytes with eccentric nuclei, and karyolysis. While in kidney tissues, histopathological changes like widened Bowman's space, congestion, necrosis of tubular cells, edema, ceroid formation, melano-macrophage aggregates, glomerulus deterioration, thyroidization, and degeneration of kidney tubules were observed in *L. rohita* exposed to copper sulfate. These changes were dose-dependent.

The liver is considered as cutting edge immune organ (79). $CuSO_4$ may possibly accrue in the liver. Liver is involved in the detoxification of metals at cellular level (80). Continual exposure to copper sulfate could have many detrimental effects on living beings (81). Copper have a tendency to bioaccumulate, disturbing human/animals food chain and eventually life menacing. Overexposure to copper generates inflammatory response, and oxidative stress, thus leading to liver injury (82–84).

Copper sulfate produces multiorgan (lungs, liver, gastrointestinal tract, and kidneys) injury/damage leading to disfunction *via* ROS production rendering oxidative stress (85). In hepatotoxicity, a vicious circle between oxidative stress and inflammatory response has been reported (86). Direct effect of copper usually leads to DNA and cell damage which then results in oxidative stress or enhanced discharge of ROS due to stimulation of phagocytic cells and increased tissue damage (6, 40, 70, 72, 87) as have been observed at high doses of $CuSO_4$ in the present study. Moreover, endoplasmic reticulum and oxidative stress leads to nephrotoxicity as a result of $CuSO_4$-exposure (5). Injury to various organs may result in fish mortality (88). Copper sulfate toxicity usually causes degeneration in renal tubules and glomerulus, and hepatocytes necrosis. These types of lesions hamper kidney and liver functions (89–91) which result in elevated enzymes such as LDH, ALT, and AST that has been seen in the present study.

Similarly, Abdel-Latif et al. (92) conducted a study on Nile tilapia. They found that exposure to sub-lethal concentration of CuO-NPs resulted in necrosis, hepatocytes degeneration, and severe congestion of blood sinusoids corresponding to the exposure dose, as well as increased Bowman's spaces, pyknotic nuclei, necrosis of kidney tubules, multi-focal inter-tubular hemorrhages and renal edema.

The gills are the first organ affected by exposure to CuSO4 in water (81). In the current study, histopathological alterations of gill tissues such as congestion of the cartilaginous core, congestion, telangiectasia, necrosis of lamellar pillar cells, lamellar fusion, lamellar atrophy, cartilaginous core degeneration, primary lamellae disruption, uplifting of lamellae, and disorganization of lamellar were observed, while in brain tissue degenerative changes like neurons necrosis, intracellular edema, cytoplasmic vacuolization, and congestion were observed in the studied fish at different exposure intervals during the experiment, which increases with an increase in concentration. Previously it has been indicated edematous changes in the gills could possibly due increased capillary permeability (88). Copper sulfate causes hyperplasia of gills epithelial cells, and it is dose dependent (93, 94) and it also leads to primary and secondary lamellar epithelial cells degeneration (76). Hyperplasia of gills epithelium along with degeneration and complete to partial lamellar fusion could be responses of the gills to pollutants (95, 96). Hyperplasia with thickening of gill filament epithelium may lead to the lamellar fusion (97). Augmented absorbency of the gill capillary walls subsequent vessel distension at the site of toxicant injury might be liable for the noted lamellar edema (98). There is another possibility that hyperplasia of gills could be a defensive aspect against irritants/heavy metals by reducing the mucosal membrane of respiratory tract and increasing the distance between toxicant–blood dissemination and its escalation which could result in the thickness of epithelial layers that could support by the intensifications of lamellar width and epithermus. Hence, lesions found in the current study could possibly impede the secretory, excretory, and respiratory functions of fish gills (81, 88, 99).

In the present study, $CuSO_4$ dose and time-dependent histopathological alterations in brain tissues including congestion, cytoplasmic vacuolization, intracellular edema, and neuron necrosis were recorded. In the published literature, $CuSO_4$ or CuO-NPs post disclosure, neuropil degeneration was obvious in Nile tilapia exposed to copper (24). These lesions could have developed as copper can cross blood-brain barrier (100–102). Several other studies (103–105) have reported similar lesions in brain of in response to various toxic chemicals and pollutants. Histopathological lesions in brain of *L. rohita* (106), *O. punctatus* (107), *C. gariepinus* (103), *C. catla* (108), *C. Carpio* (104), and *O. mossambicus* (97, 105) have been reported due to several toxicants exposure.

Conclusion

Many changes in the hematological and serum biochemical parameters and histopathological status in *Labeo rohita* were observed in the current study. These changes directly affect the health status of fish and all other aquatic organisms exposed to these pollutants/chemicals/compounds. This study observed that exposure to even a low concentration of $CuSO_4$ (0.28 $\mu g.L^{-1}$) might result in physiological and histopathological alterations in multiple fish tissues. Based on the results derived from this study, further studies are proposed to find out the more suitable dose of $CuSO_4$ for regular use to decontaminate fish ponds. It is also suggested that due to possible effects on the ecosystem, aquatic organisms, and animal and human health, we should regularly monitor exposure to CuSO4 in waters and continuously observe the environment/ecosystem. Hence, we must prioritize studies about environmental monitoring of pollutants and their possible effects on different organisms.

Novelty of the study

While studying copper sulfate toxicity in fish in the past, people have reported mostly hematological or biochemical parameters singly or in combination. Whereas, in this study, we have reported comprehensively and simultaneously, physical, hematological, or biochemical, oxidative stress parameters along with gross and histopathology of fish exposed to various doses and at various time intervals.

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 Bioethics Committee, The Islamia University Bahawalpur, Pakistan.

Author contributions

SN and RH had the initial idea for the study. They were involved in the experiment, laboratory, data analysis, writing and development of the article, and the final proof stage. RH, SJ, and AC were involved in experimental studies, laboratory studies, and the article's writing. ML was involved in experimental studies, laboratory studies, and data analysis. ZG and AK performed and interpreted the statistical analysis. ZR arranged the facilities (enzymes, reagents). AK valuable suggestions regarding histopathological studies. All authors have read and approved the final version of the manuscript.

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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Impact of epidermal growth factor and/or β-mercaptoethanol supplementations on the *in vitro* produced buffaloes' embryos

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The present study investigated the effects of epidermal growth factors (EGF) and/or β-Mercaptoethanol (βME) supplementations to oocyte maturation, fertilization, and culture media on the buffalo *in vitro* embryo production. The ovaries were collected and transferred within 2 h to the laboratory. The cumulus oocytes complexes were aspirated from 3 to 8 mm diameter follicles. Firstly, EGF; 0, 10, 20, or 50 ng/mL or βME; 0, 25, 50, 100, or 200 μM were supplemented to the *in vitro* maturation (TCM-199), fertilization (IVF-TALP), or culture (IVC: SOF) media. Our results revealed that supplementing EGF (20 ng/mL) to the TCM-199, IVF-TALP, or SOF media could efficiently improve the growth rates and development of buffalos' embryos, while EGF (50 ng/mL) could stimulate the embryo production only after treatment of the IVF-TALP /or SOF media, but not the IVM medium. However, βME was less efficient than EGF; it stimulated the growth rates of buffalo embryos when supplemented with the maturation and fertilization (IVF-TALP) media in a 50 μM concentration. Secondly, combined EGF (20 ng/mL) and βME (50 μM) were supplemented to the maturation media as effective concentration. The combined treatment of EGF (20 ng/mL) and βME (50 μM) showed no significant enhancing effect on the buffalo embryos compared to each alone. For future perspectives, further study is required to examine the effects of combined EGF and βME on the maturation and fertilization of buffalo oocytes at different categories of age and seasonal localities.

KEYWORDS

buffalo, IVF, EGF, βME, fertilization, embryo production, oocyte

1. Introduction

Buffaloes (*Bubalus bubalis*) are important milk and meat producers and efficiently resist adverse environmental conditions. However, buffaloes have lower productivity and reproductive performance than cows due to; anoestrus, delayed puberty, or lower conception rates (1, 2). Compared to cows, the donner buffaloes were known to give lower ovarian follicles numbers, a low embryo recovery rate, and less effective superovulation (3), which may be attributed to the genetic-merits advancements through reproduction-biotechnologies of the cows compared to buffaloes (4, 5). *In-vitro* embryo production (IVEP) is an effective technique for producing high-quality bovine embryos (6).

However, IVEP in buffaloes is limited due to several factors, including low-quality oocytes obtained by ovarian stimulation. Oocyte maturation is essential for fertilization and implantation and completion of meiosis in response to substances naturally originating from follicular fluid and cells (7, 8).

Cavalieri et al. (9), and Marin et al. (10) reported *in vitro* embryo production (IVEP) efficacy was poor as 30–40% of the total oocytes were developed into the blastocyst stage in cattle. However, the blastocyst production rates in buffaloes were usually lower than those achieved in cattle (around 22 vs. 40%, respectively). Thus, different laboratories with scientific and commercial characteristics have worked in the search to improve embryo production rates.

On the other side, Epidermal growth factor (EGF) promotes oocyte maturation, including cellular growth, proliferation, and differentiation and so mitosis stimulation (11) and meiosis induction (12, 13). Also, EGF regulates various ovarian functions, including granulosa and theca cells, follicle-stimulating hormone action, and enhancement of oocyte maturation. Also, Adding EGF alone to IVM media increases the cumulus cell expansion and enhances the nuclear maturation of bovine oocytes. For oocyte maturation and embryo development, antioxidants play a pivotal role in many mammalian species (14) because excessive production of reactive oxygen species negatively affects oocyte maturation and embryo development (15).

β-Mercaptoethanol (βME) is a critical sulphuric amino acid component of oocyte glutathione (GSH) that plays an important role in protection against the toxic effect of oxidative stress. βME is essential to enhance the oocyte GSH synthesis and improve the quality of bovine embryos, and βME can effectively improve oocyte maturation, likely due to the decrease in the oocytes' intracellular free radicals (16). Furthermore, previous studies showed that adding βME solely to the IVM media enhances the expansion of the cumulus cells, the oocyte maturation, and the fertilization rates (17). Also, Sidi et al. (18) reported that βME supplementation could protect the bovine embryos against oxidative stress and enhance the development of embryos with increased blastocysts viability.

EGF and antioxidants could increase the cumulus expansion, helping cellular maturation, but not nuclear (19). Combined supplementation of EGF (20 ng/mL) plus βME (100 μM) in buffalo was proposed to increase the proportion of oocytes with the extruded polar body (20). Similar findings reported significant increases in cleavage and blastocyst rates in response to a combined treatment of EGF and cysteamine, an amino acid component essential for the synthesis of GSH (21). Despite previous studies on supplementing EGF and/or antioxidants to *in-vitro* embryo production media, it is still unclear what is the most effective development stage (IVM, IVF, or IVC) they were involved in buffaloes' embryos. Consequently, the optimum effective concentrations of the EGF and/or βME should be clarified.

The hypothesis of the current study depended on the supplementation of the EGF, βME, or their combination of the IVM (TCM-199), IVF (IVF-TALP), or IVC (SOF) media. The specific aim of this study was to unveil the effects of EGF as a growth promotor and/or βME, as an antioxidant, on the production of buffalo embryos throughout the different developmental stages *in vitro*; maturation, fertilization, and culture.

2. Materials and methods

2.1. Biological materials

2.1.1. Ovaries

Ovaries were obtained from apparently normal reproductive organs of the slaughterhouse of adult buffaloes (Hide for reviewers). Instantly, the ovaries were placed in a thermos containing warm saline (35°C) with penicillin; 100 IU/mL, and streptomycin; 100 μg/mL, till they reached the laboratory for further processing.

2.1.2. Semen

Frozen 0.25 mL-straws containing 30×10^6 spermatozoa/straw of superior buffalo bulls; obtained from the Animal Reproduction Research Institute (ARRI), El-Haram, Giza, Egypt, were thawed in a water bath (Laboratory Circulating Water Bath HWS-28, China) at 37°C for 30 s. One straw was randomly examined, and at least 50% sperm progressive motility and not exceeding 25% abnormality were approved in the experiment.

2.1.3. Chemicals and media

In vitro used media, including; EGF (E-5036), βME (M-6250), and TCM-199 (M-7528), and chemicals used for the preparation of IVF-TALP and SOF media, all were purchased from Sigma-Aldrich (Co. St. Louis County, MO, USA). Other chemicals were obtained from Oxford Laboratory, Maharashtra, India, including; sodium bicarbonate, sodium pyruvate, EDTA, and calcium lactate with reference numbers; S-07990, S-08302, E-03819, and C-02186, respectively. D-glucose (S-2837) was purchased from Alpha Chemika, Mumbai, Maharashtra, India.

2.2. Preparation of media

All media were filtered using a 0.2 μm (Millipore, USA) syringe filter and incubated for at least 2 h in a humidified atmosphere (95%) under 5% CO_2 at 38°C before use.

2.2.1. Aspiration media

Filtered modified phosphate buffer saline (m-PBS, pH 7.1) with 3% (v/v) inactivated fetal calf serum (FCS); tablets soluble in distilled water (six times of distillation) with the addition of Na pyruvate (0.2 mM), glucose (5.56 mM), bovine serum albumin (BSA; 6 mg/mL), streptomycin sulfate (5 mg/100 mL) and penicillin (100 IU/mL) was used as aspiration medium.

2.2.2. Maturation media

Tissue culture media-199 (TCM-199) supplemented with gentamycin (1 μL/mL), Earl's salts, L-glutamine, and 25 mM HEPES with 10% heat-inactivated FCS (PAA, Austria, Linz) were used for oocytes maturation. The media was adjusted for pH 7.3–7.6, osmolarity 270–290 mOsm/kg, and then sterilized by filtration using a 0.22 μm Millipore membrane biological filter.

2.2.3. Sperm capacitation, fertilization, and embryo culture media

Sperm Tyroid's albumin lactate pyruvate medium (sp-TALP) was used for sperm capacitation (22), while IVF-TALP, which contains caffeine as a motility-enhancing substance, was used as fertilization media (23). Modified synthetic oviductal fluid (SOF) medium was prepared at the laboratory according to Tervit et al. (24).

2.3. Oocyte recovery

The aspirated oocytes with compact multi-layered cumulus cells and evenly granulated cytoplasm were selected for IVM. The recovered COCs were classified according to their quality into; good oocytes with homogenous granular ooplasm surrounded by more than three-compact layers of cumulus cells, fair oocytes with homogenous ooplasm surrounded by 1–3 layers of cumulus cells, and denuded oocytes with uneven ooplasm (14).

2.4. *In vitro* maturation of oocytes

Selected good COCs were washed four times in fresh warm aspiration medium and subjected to the final wash using an IVM medium. In 35-mm Petri dishes, groups of 5–15 COCs were cultured in 50–100 μL droplets of the filtrated IVM media (pH 7.4) covered with sterilized mineral oil for 24 h at 5% CO_2, 38.5°C, and 90–95% relative humidity (25).

2.5. Sperm preparation and capacitation

Post-thawing, semen aliquots were subjected to a swim-up procedure, as follows, in a 15-ml rounded-bottom centrifuge tube; (a) 1 mL of semen and (b) 1 mL of sp-TALP were added, (c) the tube was incubated at 37°C with 5% CO_2 for 60 min in a 45° angle, (d) the upper 0.85 mL of supernatant was collected and transferred in a conical tube and diluted with sp-TALP to reach a final volume of 5 mL, (e) the tube was centrifuged at 120 g for 10 min, (f) the supernatant was discarded, and the sperm pellet was diluted with 200 μl of sp-TALP, (g) the recovered spermatozoa were evaluated for progressive motility, and (h) the pellet containing motile population was resuspended in a sperm culture medium and used for culturing with the mature oocytes (26) (Figure 1).

2.6. *In vitro* fertilization

The matured oocytes were partially removed from the surrounding cumulus cells for IVF and then washed twice in a warm IVF-TALP medium. Microdroplet (100 μl) of the IVF-TALP containing caffeine (0.0194 g/mL) and BSA (6 mg/mL) with 10×10^6 sperm/mL was prepared as; 75 μL of IVF-TALP specified to 10 oocytes per 25 μL of sperm suspension. Co-culture of sperm-oocyte for fertilization was performed in a humidified atmosphere (22, 23, 27) (Figure 2).

2.7. *In vitro* embryo culture

The presumed zygotes were washed four times in the embryo culture medium, SOF, and cultured in a humidified incubator. Morula production rate was examined for the 8–16 cell stages from 94 to 96 h post-insemination. One day later, 120 h after fertilization, the blastocysts formed and began to appear. A part of SOF-medium was replaced with a fresh one 2–3 times (pre-incubated for at least 2 h) before the embryo transfer to minimize the harmful effect of the waste products elaborated by the embryos (28).

2.8. Effects of EGF on oocyte maturation, fertilization, and embryo development

One of four different concentrations of EGF; 0 (control), 10, 20, and 50 ng/mL was supplemented to TCM-199 media during the maturation stage only (first experiment), to IVF-TALP media during the fertilization stage only (second experiment), or to the SOF media during the embryo culturing and development stage only (third experiment).

2.9. Effects of βME on oocyte maturation, fertilization, and embryo development

One of the five concentrations of βME; 0 (control), 25, 50, 100, and 200 μM was supplemented to the IVM medium (TCM-199) during the maturation stage only for the first experiment, to the IVF medium (F-TALP) during fertilization only for the second experiment, or to the IVC medium (SOF) during the embryo development stage only for the third experiment.

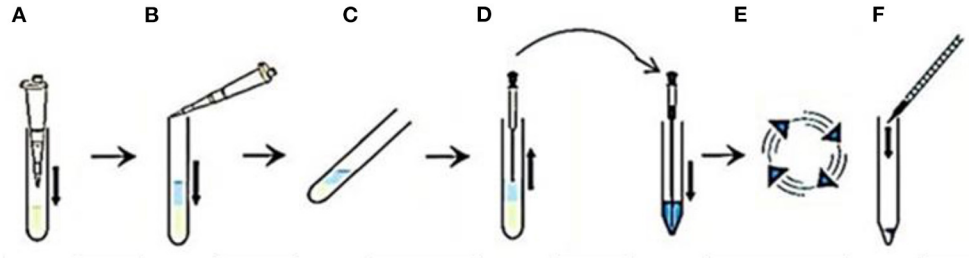

FIGURE 1
A flow-diagram describing sperm preparation by swim up method using sp-TALP in the following steps. **(A)** One milliliter of semen is added into a centrifuge tube. **(B)** One milliliter of the sp-TALP is added over semen. **(C)** The tube is incubated in an inclined position for 60 min. **(D)** The upper 0.85 mL layer of the motile sperm cells is removed to be placed in a conical tube with adding sp-TALP till reach a final volume of 5 mL. **(E)** Centrifugation for 120 g is performed for 10 min. **(F)** The sperm pellet is resuspended in the culture medium.

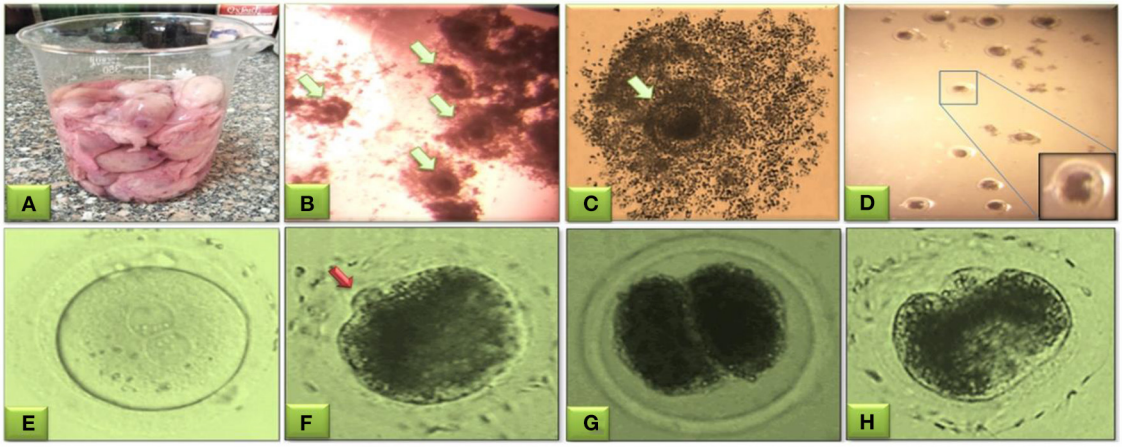

FIGURE 2
Buffalo oocytes obtained from slaughterhouse ovaries for aspiration of immature cumulus oocytes' complexes (COCs) from follicles of 3–8 mm size **(A)**. Microscopic selection of the good quality even-shaped nucleated oocytes surrounded by expanded cumulus cells (**B, C**, green arrow). Mature oocytes after washing three times in IVF-TALP medium **(D)**. Evaluation of fertilization and cleavage stages in buffalo embryos; fertilized oocyte presenting the male and female pronuclei **(E)**, fertilized oocytes with polar body (red arrow) **(F)**, start of cleavage showing the two-cell stage cleaved embryo **(G)**, and the four-cell stage cleaved embryo **(H)**. This figure is partially retrieved from another recent piolet study (27) based on previous studies (22, 23).

2.10. Effects of combined EGF and βME on the embryo development

After the data of the single use of different concentrations of either EGF or βME as a supplement to one of the three developmental stages, IVM, IVF, or IVC were statistically analyzed, a combined concentration of 20 ng/mL EGF + 50 μM βME was decided as the most effective concentration to be supplemented to the IVM medium (TCM-199) during the maturation stage.

2.11. Statistical analysis

Non-parametric data were expressed as percentages of progress, and rates of oocyte maturation and embryo production, morula, and blastocyst were calculated using the Chi-square for trend. Numerical data of the different groups were expressed as mean ± standard error of the mean (SEM) and compared among the three experiments using one-way ANOVA, and Tukey was used as a *post-hoc* test. Each experiment was repeated three times, and the differences were considered for the mean ± SEM for three replicates ($N = 3$). Differences were considered significant at $P < 0.05$. Graph-Pad Prism software was used for analysis (San Diego, USA, V.0.6).

3. Results

Effects of EGF; 10, 20, and 50 ng/mL, supplementation of TCM-199, IVF-TALP, and IVC/SOF media on the development of embryos were shown in Table 1 and Figure 3. The TCM-199, IVF-TALP, or SOF media supplemented with 10 ng/mL of EGF didn't significantly increase the rates of embryo development, morula, and blastocyst compared to each respective control (Table 1). However, supplementation of 20 ng/mL of EGF to the different media significantly increased the stages of embryonic development

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TABLE 1 Effects of epidermal growth factor (EGF) added to maturation (TCM-199), fertilization, (IVF-TALP), and culture (SOF) media on the embryo stages in buffaloes; oocyte maturation, fertilization, cleavage/morula and blastocyst development.

EGF concentrations	Media treatment with EGF											
10 ng/mL	TCM-199				IVF-TALP				SOF			
	CTL		10 ng/mL		CTL		10 ng/mL		CTL		10 ng/mL	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Total original oocytes	55		48		66		62		70		67	
Mature oocytes	39	70.9	37	77.1	46	69.7	48	77.4	55	78.6	52	77.6
Fertilized oocytes	30	76.9	29	78.4	36	78.3	38	79.2	38	69.1	39	75.0
Morula production	9	30.0	18	62.1	14	38.9	20	52.6	17	44.7	22	56.4
Blastocyst production	2	22.2	6	33.3	6	42.9	11	55.0	8	47.1	11	50.0
Total number (100%)	103				128				137			
Chi^2 (*P*-value)	3.8 (0.053)^{NS}				2.0 (0.157)^{NS}				0.95 (0.329)^{NS}			
20 ng/mL	CTL		20 ng/mL		CTL		20 ng/mL		CTL		20 ng/mL	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Total original oocytes	55		62		66		62		70		71	
Mature oocytes	39	70.9	53	85.5	46	69.7	47	75.8	55	78.6	57	80.3
Fertilized oocytes	30	76.9	48	90.6	36	78.3	39	83.1	38	69.1	45	78.9
Morula production	9	30.0	40	83.3	14	38.9	31	79.5	17	44.7	38	84.4
Blastocyst production	2	22.2	21	52.5	6	42.9	20	64.5	8	47.1	26	68.4
Total number (100%)	117				128				141			
Chi^2 (*P*-value)	19.0 (<0.0001)***				9.3 (0.002)**				10.0 (0.001)**			
50 ng/mL	CTL		50 ng/mL		CTL		50 ng/mL		CTL		50 ng/mL	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Total original oocytes	55		54		66		57		70		67	
Mature oocytes	39	70.9	38	70.4	46	69.7	46	80.7	55	78.6	54	80.6
Fertilized oocytes	30	76.9	28	73.7	36	78.3	35	76.1	38	69.1	41	75.9
Morula production	9	30.0	13	21.4	14	38.9	25	71.4	17	44.7	35	85.4
Blastocyst production	2	22.2	4	66.7	6	42.9	12	48.0	8	47.1	20	57.1
Total number (100%)	109				123				137			
Chi^2 (*P*-value)	0.54 (0.462)^{NS}				4.4 (0.037)*				7.5 (0.006)**			

The differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001; ^{NS} stands for non-significant difference. CTL, control. EGF was added to the three media in 10, 20, or 50 ng/mL. The values denotes sum of three experiments. Chi-square (Chi^2) for the trend of the morula and blastocyst production rates was calculated considering the sum of values scored for the three experiments.

up to the blastocyst stage compared to each respective control, as shown in the demographic trends of Chi^2 analyses (Table 1 and Figures 3A1, C1, E1, respectively; $P < 0.05$). Embryo production rates were also significantly increased in response to supplementing the IVF-TALP and SOF media compared to their controls' trend ($Chi^2 = 4.4$ and 7.5, respectively; $P < 0.05$), but not the TCM-199 media, with 50 ng/mL of EGF (Table 1).

Fertilized oocytes proportion with the extruded 2nd polar body was the highest in TCM-199 supplemented with 20 ng/mL of EGF (90.6%) compared to control (76.9%) (16.0 ± 6.0 vs. 10.0 ± 1.2; $P < 0.05$). Furthermore, morula production was significantly increased in response to the same concentration of EGF added to the TCM-199 media (83.3%) rather than the control (30.0%; 13.3 ± 0.7 vs. 3.0 ± 0.6; Table 1 and Figures 3A, A1; $P < 0.05$).

The effects of βME supplementation 25, 50, 100, and 200 μM to the TCM-199, F-TALP, or SOF media on the buffalo embryo development are shown in Table 2 and Figure 3. Interestingly, adding 50 μM βME to the maturation or fertilization media significantly increased the development trend of morula and blastocyst stages compared to their controls ($Chi^2 = 12.0$, $P = 0.0005$, and $Chi^2 = 9.5$, $P = 0.002$, respectively; Table 2 and Figures 3B1, D1, respectively). The other concentrations of βME, 25, 100, and 200 μM, didn't increase the rates of oocyte maturation, fertilization, or cleavages of embryos compared to those of the control (Table 2). According to Chi^2 analyses, 50 μM of βME significantly scored the highest trend for morula production after supplementation of the IVM (75.9 vs. 50.0%) or IVF medium (73.5 vs. 46.2%) compared to their

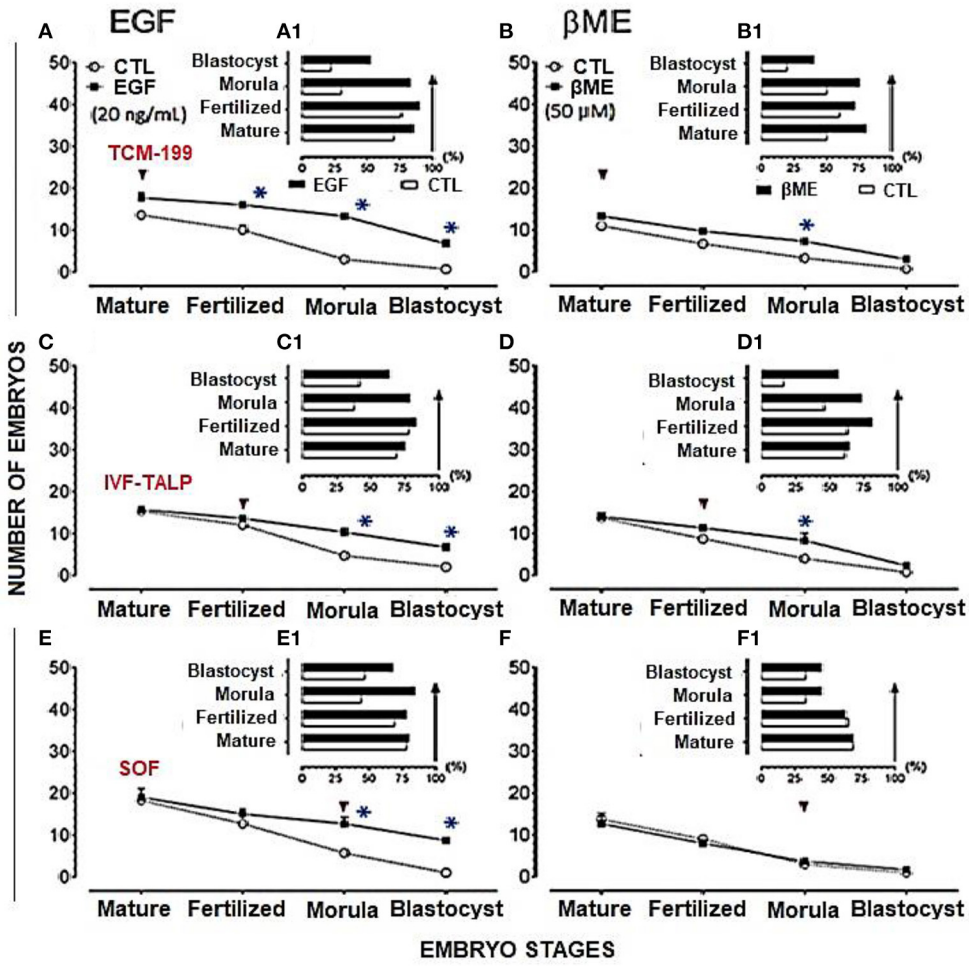

FIGURE 3
Effects of EGF (20 ng/mL) added to TCM-199, IVF-TALP, and SOF media, on buffalo embryo stages; oocyte maturation, fertilization, morula and blastocyst were shown in **(A, C, E)**, respectively. EGF (20 ng/mL) characteristics on the developmental stages (%) of those embryos *in vitro* were shown in **(A1, C1, E1)**, respectively. Effects of βME (50 μM) added to TCM-199, IVF-TALP, and SOF media, on buffalo embryo stages; oocyte maturation, fertilization, morula and blastocyst were shown in **(B, D, F)**, respectively. βME (50 μM) characteristics on the developmental stages of embryos *in vitro* were shown in **(B1, D1, F1)**, respectively. Values were presented as mean ± SEM for three experiments **(A–F)**. Arrows denotes the stage in treated medium. Asterisk (*) denotes significance difference between each respective values (treated vs. CTL) at $P < 0.05$.

controls (Table 2 and Figures 3B1, D1; $P < 0.001$). Morula production was significantly increased in response to the same concentration of βME added to the TCM-199 media (75.9%) rather the control (50.0%; 7.3 ± 0.3 vs. 3.3 ± 0.3; Table 2 and Figures 3B, D; $P < 0.05$).

Effective concentrations of either the EGF or βME showed higher potency in the TCM-199 media compared to the fertilization or culture media. Therefore, the effects of combined EGF (20 ng/mL) plus βME (50 μM) supplementation of IVM/TCM-199 media on embryo development were evaluated (Table 3 and Figure 4). Supplementation of the TCM-199 media with a combined concentration of EGF and βME significantly increased the fertilization and growth rates of morula and blastocyst compared to the control (Table 3 and Figures 4A, A1; $Chi^2 = 6.0$, $P = 0.015$).

The demographic characteristics of *in vitro* maturation, fertilization, and cleavage rates in addition to blastocyst production in response to EGF and/or βME added to the different media using Chi^2 analyses have shown that EGF in 20 ng/mL was the highly potent agent rather than βME 50 μM. Although the combined treatment of both EGF and βME significantly increased the growth rates of those embryos, it didn't score additional enhancement compared to those of EGF or βME, each alone ($Chi^2 = 19.0$ and 12.0, respectively; Tables 1, 2 and Figures 3, 4, respectively; $P < 0.05$). Accordingly, considering the blastocyst to morula ratio, the proportion of blastocyst production scored the highest trend in the TCM-199 medium supplemented with EGF (20 ng/mL) rather, in order, than those supplemented with βME (50 μM) and then the combined supplementation; EGF (20 ng/mL) plus βME (50 μM), compared to those of controls (52.5 vs. 22.2, 40.9 vs. 20.0, and 54.5 vs. 42.9%, respectively). However, considering the ratio of the blastocyst to the original oocytes, the proportion of blastocyst production also scored the highest trend after the treatment of TCM-199 medium with EGF rather, in order, than

TABLE 2 Effects of Beta-Mercaptoethanol (βME) added to TCM-199, IVF-TALP, and SOF media on the embryo stages in buffaloes; oocyte maturation, fertilization, cleavage/morula, and blastocyst development.

βME concentrations	Media treated with βME											
25 μM	TCM-199				IVF-TALP				SOF			
	CTL		25 μM		CTL		25 μM		CTL		25 μM	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Total original oocytes	65		58		67		63		59		60	
Mature oocytes	33	50.8	36	62.1	41	61.2	40	63.5	41	69.5	44	73.3
Fertilized oocytes	20	60.6	23	63.9	26	63.4	31	77.5	27	65.9	31	70.5
Morula production	10	50.0	15	65.2	12	46.2	20	64.5	9	33.3	12	38.7
Blastocyst production	2	20.0	5	33.3	2	16.7	6	30.0	3	33.3	3	25.0
Total number (100%)	123				130				119			
Chi^2 (*P*-value)	2.6 (0.107)^{NS}				3.4 (0.064)^{NS}				0.26 (0.61)^{NS}			
50 μM	CTL		50 μM		CTL		50 μM		CTL		50 μM	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Total original oocytes	65		49		67		65		59		55	
Mature oocytes	33	50.8	40	81.6	41	61.2	42	64.6	41	69.5	38	69.1
Fertilized oocytes	20	60.6	29	72.5	26	63.4	34	81.1	27	65.9	24	63.2
Morula production	10	50.0	22	75.9	12	46.2	25	73.5	9	33.3	11	45.8
Blastocyst production	2	20.0	9	40.9	2	16.7	14	56.0	3	33.3	5	45.6
Total number (100%)	114				132				114			
Chi^2 (*P*-value)	12.0 (0.0005)***				9.5 (0.002)**				0.38 (0.536)^{NS}			
100 μM	CTL		100 μM		CTL		100 μM		CTL		100 μM	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Total original oocytes	65		67		67		60		59		61	
Mature oocytes	33	50.8	42	62.7	41	61.2	42	70.0	41	69.5	42	68.1
Fertilized oocytes	20	60.6	25	59.5	26	63.4	33	78.6	27	65.9	27	64.3
Morula production	10	50.0	16	64.0	12	46.2	12	36.4	9	33.3	11	40.7
Blastocyst production	2	20.0	6	37.5	2	16.7	3	25.0	3	33.3	4	36.4
Total number (100%)	132				127				120			
Chi^2 (*P*-value)	2.2 (0.13)^{NS}				0.89 (0.35)^{NS}				0.08 (0.774)^{NS}			
200 μM	CTL		200 μM		CTL		200 μM		CTL		200 μM	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Total original oocytes	65		59		67		75		59		63	
Mature oocytes	33	50.8	33	55.9	41	61.2	42	56.0	41	69.5	45	71.4
Fertilized oocytes	20	60.6	15	45.5	26	63.4	28	66.7	27	65.9	29	64.4
Morula production	10	50.0	8	53.3	12	46.2	7	25.0	9	33.3	10	34.5
Blastocyst production	2	20.0	2	25.0	2	16.7	2	28.6	3	33.3	1	10.0
Total number (100 %)	124				142				122			
Chi^2 (*P*-value)	0.10 (0.75)^{NS}				0.77 (0.38)^{NS}				0.10 (0.746)^{NS}			

The differences were considered significant at $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. ^{NS}Stands for non-significant difference. βME was added to the three media in 25, 50, 100, or 200 μM. The values denotes sum of three experiments. Chi^2 for trend of embryo production was calculated considering the sum of values scored for the three experiments.

TABLE 3 Effects of combined EGF and βME supplementations to the TCM-199 medium on the embryo stages; cleavage and gastrulation.

Embryo stages	TCM-199 medium			
	CTL		EGF + βME	
	N	%	*N*	%
Total original oocytes	35		43	
Fertilized oocytes	14	40.0	26	60.5
Morula production	7	50.0	22	84.6
Blastocyst production	3	42.9	12	54.5
Total number (100%)	78			
Chi^2 (*P*-value)	6.0 (0.015)*			

EGF (20 ng/mL) and βME (50 μM) were added to the three media. Chi^2 for trend of embryo production was calculated. The differences were considered significant at *($P < 0.05$). Other explanations were given in Tables 1, 2. The values denotes sum of three experiments.

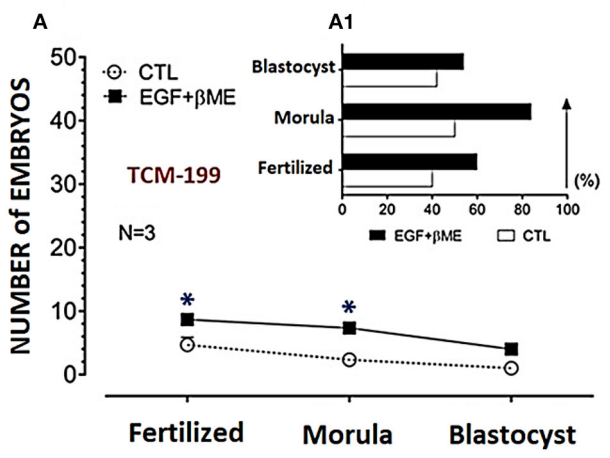

FIGURE 4
Effects of combined supplementation of EGF (20 ng/mL) and βME (50 μM) to the TCM-199 medium on the buffalo embryo stages; fertilized oocytes, morula, and blastocyst, were shown in **(A)**, and the trend demograph for the blastocyst production was shown in **(A1)**. Asterisk (*) denotes significance difference between each respective values (treated vs. CTL) at $P < 0.05$.

the combined supplementation of EGF plus βME, and then βME compared with each respective control (33.9 vs. 3.6, 27.9 vs. 8.6, and 18.4 vs. 3.1%, respectively; Tables 1–3 and Figures 3, 4, respectively; $P < 0.05$).

4. Discussion

The present study investigated the effects of supplementing EGF- and/or βME to the TCM-199, IVF-TALP, or SOF media on the *in vitro* growth rates of the buffalo embryos. The study demonstrated that the optimum concentration of EGF as 20 ng/mL supplemented to TCM-199, IVF-TALP, or SOF media, was potent for enhancing the expansion of COCs, inducing higher maturation, fertilization, cleavage, morula, and blastocyst production (19). However, Sadeesh et al. (20) reported that the optimum concentration of EGF was also 20 ng/mL added to the maturation/TCM-199 medium for buffalo's *in-vitro*-produced embryos. Earlier studies reported that oocyte competence and blastocyst development rate were improved at 5 ng/mL of EGF (29). Moreover, Other *in-vitro* embryo production studies in bovine found that the oocytes' maturation rates were increased at 10 ng/mL of EGF added to the maturation media (30). Previous studies were compatible with our findings that higher concentrations of EGF, 40 or 50 ng/mL, reduced the blastocyst development rather than the 20 ng/mL, which may be attributed to a suggested phenomenon of growth factor-induced receptor down-regulation. Generally, EGF was previously reported to enhance the cellular function causing early embryo development in mammals; more specifically, our study elucidated that the optimum way to add EGF only as a supplement to *in-vitro*-produced buffaloes' embryos was 20 ng/mL added to the TCM-199 media (14).

Our findings successfully determined that the optimum concentration of βME alone was 50 μM added to the TCM-199 media according to those reported by Songsasen and Apimeteetumrong (31), who showed that supplementation of TCM-199 medium with βME increased the proportion of buffaloes' oocytes exhibiting synchronous pronuclei formation and enhance the development of the produced blastocysts. Our results agreed with those of Furnus et al. (32), who provided evidence that the optimal development of cattle embryos *in vitro* partially depends on the presence of precursor amino acids for intracellular GSH production during IVM. Similarly, the present study provides the beneficial effect of βME as likely attributed to the function of βME in the production of GSH to improve the buffalo embryo maturation and fertilization by increasing the blastocyst production rates.

A previous study in porcine showed that supplementation of lower concentration of βME, 25 μM, in the IVM medium increased the oocyte maturation rate (33); however, 50 μM of cysteamine, or βME, could enhance not only the oocyte maturation but also the transformation of sperm pronuclei formation (34). Our findings revealed that supplementing 100 or 200 μM of βME to the maturation medium couldn't improve the growth of buffaloes' oocytes. Those findings agreed with Patel et al. (35), who reported similar findings *in-vitro*-produced buffaloes. Cleavage, morula, and blastocyst rates were increased in the media containing 50 μM of βME rather than those of control which agreed with Sadeesh et al. (20), who stated that supplementation of antioxidants such as βME to the culture medium enhanced the antioxidant systems within the embryo and stimulated the embryonic development. Thus, the oocyte developmental competence could be improved by increasing the antioxidant capacity of the oocytes during maturation *in vitro* (36, 37). The present results are supported by Sidi et al. (18) studies which reported that low molecular weight thiol-compounds like the cysteamine or βME could stimulate the synthesis of oocyte GSH. Both cysteamine and βME reduce cystine into cysteine and promote cysteine uptake to enhance the oocyte's GSH synthesis. This pathway explains the efficacy of βME in improving oocyte maturation (16).

Although βME added to SOF media showed no effect on the embryo's development, Sidi et al. (18) showed that

supplementation of βME into the culture media could protect the bovine embryos against oxidative stress and promote both amino acid transport, DNA synthesis, and development of embryos with increased the blastocysts viability. A previous report by Kobayashi et al. (38) found that adding βME in 100 μM to the TCM-199 improved the blastocyst formation and the cell numbers of porcine oocytes, which may likely be due to the species difference.

The present study reported that a combination of 20 ng/mL of EGF plus 50 μM of βME to the TCM-199 media significantly improved the maturation of buffalo oocytes *in vitro*. The combination of EGF and antioxidants could increase the maturation rate due to cumulus expansion, not nuclear maturation. A significant increase in cleavage and blastocyst rates in response to a combined treatment of EGF and cysteamine was reported (21). On the other hand, Singhal et al. (19) reported that the TCM-199 medium supplemented with EGF (20 ng/mL) plus cysteamine (50 μM) showed no effect on the buffalo oocyte maturation and embryo development. Still, in agreement with our study, the oocyte cleavage rate was improved (46.4%) after supplementation of the maturation medium with 20 ng/mL of EGF plus 50 μM of βME.

Sadeesh et al. (20) stated that combined supplementation of EGF; 20 ng/mL plus βME; 100 μM, in the maturation medium improved the maturation of buffalo oocytes with polar bodies and further embryo development *in vitro*. They also compared the TALP medium with the Bracket and Oliphant (BO) medium for culturing and blastocyst production. Their findings were consistent with our study, which used the combined treatments, but the βME was at 50 μM since all stages of the embryo development and cleavage rates were improved compared to their respective controls. Another study testing the use of BO medium instead of IVF-TALP should be carried out in the future. They also observed that increasing the dose caused a decrement in the cumulus expansion and the polar body formation rates.

Simultaneously, in our study, using EGF or βME each alone rather than combined use could be more effective for cleavage and blastocyst production. These results could be attributed to the embryos' receptor's down-regulation, supporting what Sadeesh et al. (20) reported, but the potency was different than ours.

Growth factors activate the receptor tyrosine kinases (RTKs) on the cell's surface, which triggers cellular proliferation by stimulating the tyrosine autophosphorylation of RTKs and recruiting the Src homology-2 (SH2) domains. Those activated RTKs are rapidly internalized into cells by endocytosis and eventually degraded by the lysosomes in a process termed receptor down-regulation, which plays a crucial role in terminating the cellular proliferation signals and preventing cellular overgrowth (39). The epidermal growth factor receptor (EGFR) is the RTK (40). Activation of the protein kinase C, which phosphorylates EGF-receptor threonine (41), or stimulation of the calmodulin-dependent protein kinase II, which phosphorylates serine in those receptors (42) leads to regulation of the EGFRs.

A recent study showed that EGF and EGFR are expressed in the bovine oocytes and their zona pellucida, in addition to the follicular granulosa, cumulus cells, follicular fluid, and theca folliculi (43). As far as we know, studies concerning the cellular effects and their RTKs in response to βME maybe not be available. However, our finding suggests a pivotal role of βME in the up/down-regulation of the EGFR, which explain the decreased growth rates of the buffalo embryos after supplementing the EGF with βME. Also, the regulatory mechanism of RTKs could be variable between the embryonic stages; oocyte, morula, and blastocyst, which explains the variant ratios detected between blastocysts vs. morulae or blastocyst vs. oocytes. The results of Sadeesh et al. (20) and our study are consistent but different in potency. We approve that the higher doses lead to adverse effects. They observed the adverse effect on the maturation, but in ours, it happened in the culturing and blastocyst production between the single and combined treatments.

5. Conclusions

In conclusion, EGF could efficiently improve the growth rates and development of the *in vitro* produced buffalo embryos after supplementation of the TCM-199, IVF-TALP, or SOF media with the potent concentration of 20 ng/mL, while the increased concentrations of EGF; up to 50 ng/mL, stimulate the embryo production only after treatment of the fertilization/ and/ or culture media, but not that the maturation media. However, βME was less efficient than EGF; it could only stimulate the embryos' growth rates after treatment of the TCM-199 and IVF-TALP media with a concentration of 50 μM. Furthermore, the combined treatment of both EGF and βME showed no enhancement effect on the buffalo embryo production compared to each alone. Our study strongly supports the phenomenon of growth factor-induced receptor downregulation.

Data availability statement

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

Ethics statement

This study was discussed and approved by the Ethical Research Committee members at the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt (No. 57/18.09.2022).

Author contributions

AA and NY: conceptualization, methodology, data curation, and writing original draft. RS, HH, MB, KS, FA-S, and AH: methodology, writing-review, and editing, and critical reading. MA and MEA: editing, critical reading, data curation, formal analysis. All authors listed in this paper have contributed to the preparation and execution of this research. All

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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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Molecular prevalence and associated risk factors of *Anaplasma ovis* in Pakistani sheep

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Background: Majority of Pakistani population lives in rural areas and raising animals, especially the small ruminants, is their primary source of income. *Anaplasma ovis* is known to infect small ruminants globally and causing significant economic losses to livestock owners, however prevalence of *Anaplasma ovis* has been least investigated from Pakistan despite having a huge sheep population.

Methods: The present study was conducted from June 2021 till December 2021 to report the PCR based prevalence of *Anaplasma ovis* in the blood samples of sheep ($n = 239$) that were collected from District Dera Ghazi Khan in Pakistan.

Results: Out of 239 samples, 30 (12.5%) amplified a 347 bp fragment specific for the *msp4* gene of *Anaplasma ovis*. Represented partial *msp4* gene sequences were confirmed by Sanger sequencing and deposited to GenBank (OP620757-59). None of the studied epidemiological factors (age, sex, breed, size of herd, dogs with herd, and composition of herd) showed an association ($P > 0.05$) with the *Anaplasma ovis* infection in enrolled sheep. Analysis of the amplified partial *mSP4* sequence of *Anaplasma ovis* revealed that this gene is highly conserved as all three sequences were identical and phylogenetically resembled with the *msp4* sequences amplified from small ruminants in China, Kenya, and Germany, Turkey, Portugal, Tunisia and India. In conclusion, for the first time, we are reporting a moderate prevalence of *Anaplasma ovis* prevalence in Pakistani sheep and this data will help in developing the integrated control policies against this newly reported tick-borne disease that is infecting our sheep breeds.

KEYWORDS

***Anaplasma ovis*, sheep, *msp4* gene, Pakistan, phylogeny**

Introduction

Diseases, including metabolic, infectious and vector bone, are a major constraint to small ruminants and livestock industry as diseases impose financial risks to the livestock owners due to morbidity and mortality of their animals (1, 2). Ecto parasites, especially ticks, are the major source of pathogen transmission to sheep (3, 4). Tick population as seen a substantial increase globally as well as in Pakistan in recent years due to global weather changes and due to introduction of the animal species in environments/countries where they did not exist

before (4). This rise in tick population has led to an increase in the incidence of tick borne diseases (TBDs) and hence the economic losses (5).

Anaplasma (*A.*) *ovis*, an intra erythrocytic gram negative rickettsial bacterium, that belongs to the genus *Anaplasma*, family Anaplasmataceae that infect sheep, goats and some wild ruminants causing anaplasmosis (6). *Anaplasma ovis* is frequently reported to be transmitted to sheep by ticks belonging to *Ixodes*, *Dermacentor*, *Rhipicephalus*, and *Amblyomma* genera (7). In sheep, infection due to *Anaplasma ovis* is very common and chances of infection increases when the weather is hot and dry or when the sheep are already co infected with some other parasite (8). In extreme cases, infection with *A. ovis* can lead to the death of infected animal (6). The most marked clinical signs of anaplasmosis are anemia and jaundice. Usually a persistent infection is developed in those animals that survive the acute phase of disease (9). Doxycycline is the treatment of choice for the treatment of anaplasmosis and all other tick borne rickettsial diseases (10).

Although Dera Ghazi Khan is known for its large sheep population but ovine anaplasmosis has never been reported from this District. Hence the present study was designed to report the molecular prevalence of *A. ovis* in enrolled sheep breeds and to report the association of this infection with the epidemiological risk factors, if any.

Materials and methods

Sample and data collection

Randomly selected herds in Dera Ghazi Khan District were targeted to collect 239 blood samples from apparently healthy sheep during June 2021 till December 2021. Enrolled sheep belonged to four breeds: Mundri ($N = 171$), Kajli ($N = 44$), Latti ($N = 14$), and Baluchi ($N = 10$). Following the informed consent from the owners, around 3–5 ml of blood sample was collected by pricking the Jugular vein of each sheep with a disposable syringe into a tube containing 0.5 M EDTA as an anticoagulant. This blood was later on used for the DNA extraction. In order to find out the epidemiological factors that are associated with *A. ovis* infect in enrolled sheep, a questionnaire was filled on the sampling site. Data regarding sex, age, herd size, composition of herd, and dogs present in herd was collected.

DNA extraction and PCR amplification

DNA was extracted from the collected blood by using a non-organic method as reported by Grimberg et al. (11). A pair of primers, AOF 5'-TGAAGGGAGCGGGGTCATGGG-3' and AOR 5'-GAGTAATTGCAGCCAGGCACTCT-3' was used to amplify a 347 bp fragment specific for *msp4* gene of *Anaplasma ovis* as previously reported by Torina et al. (12). A master mixture of 25 µl was prepared containing 3 mM $MgCl_2$, 10X PCR buffer, 5 µl of template DNA, 0.2 mM deoxy ribonucleotide triphosphates, 2 U of Taq DNA Polymerase (Parstous, Iran) and 0.5 mM of each primer. Reaction conditions comprised of an initial denaturation at 95°C for 5 min, 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 62°C and extension for 30 s at 72°C and a final elongation for 5 min at 72°C (12).

DNA sequencing and phylogenetic analysis

Randomly selected PCR products ($n = 3$) were sent to First Base Sequencing Service (Malaysia) for purification and DNA sequencing. The resultant partial *msp4* sequences from *A. ovis* isolates were deposited at GenBank under the accession numbers OP620757- OP620759. *Anaplasma ovis* sequences (having 96–100% similarity to the ones generated in present investigation) were download from the GenBank database (https://www.ncbi.nlm.nih.gov/) and all sequences were trimmed to 309 bp to be used in phylogeny. Maximum Likelihood method was applied in MEGA version 11 for evolutionary analysis (13). Kimura 2-parameter model with invariant sites was the top ranking substitution model according to lowest Bayesian Information Criterion score (14). Also, a bootstrap analysis with 1,000 replicates was used for the tree construction. *Anaplasma phagocytophilum*'s *msp4* partial gene sequence was used as an outgroup. Sequence alignment was performed by using ClustalW and visualizated with BioEdit (15).

Statistical analysis

Minitab (version 17, USA) was used for data analysis. $P \leq 0.05$ was selected as significant level. Comparison of *A. ovis* prevalence between various sheep breeds was made by applying one way analysis of variance (ANOVA). Association between *A. ovis* occurrence and various risk factors was assessed through the Fisher's exact test (for 2 × 2 tables). Tajima's D Fu and Li's D values were estimated with DnaSP v5 (16).

Results

Molecular investigation and risk factors' analysis

Analysis of the results revealed that PCR amplified a 347 base pair fragment specific for *msp4* gene of *A. ovis* in 30 out of 239 (12.5%) collected sheep blood samples during present molecular survey. When prevalence of *A. ovis* was compared between enrolled sheep breed, one way ANOVA results revealed that the bacterium prevalence was not restricted to a particular sheep breed ($P = 0.3$; Table 1). Fisher Exact test results revealed that all the epidemiological parameters investigated during this survey were not associated ($P > 0.05$) with the *A. ovis* infection in sheep (Table 2).

Phylogenetic study and genetic diversity

During phylogenetic analysis, we have compared the partial *msp4* (347 bp) gene sequences generated during this study (OP620757–OP620759) with those previously deposited GenBank from various parts of the World having sequence homology of 96%

TABLE 1 Comparison of *Anaplasma ovis* prevalence in blood samples of various sheep breeds enrolled from District Dera Ghazi Khan during present study.

Sheep Breeds	*N*	*Anaplasma ovis* +ve sheep	*Anaplasma ovis* −ve sheep	*P*-value
Mundri	171	18 (10.5%)	153 (89.5%)	0.3
Latti	14	2 (14.3%)	12 (85.7%)	
Kajli	44	8 (18.2%)	36 (81.1%)	
Baluchi	10	2 (20%)	8 (80%)	
Total	239	30 (12.6%)	209 (87.5%)	

N represents the total number of sheep samples collected from each breed. % prevalence of Anaplasma ovis is given in parenthesis. P-value represents the results of one way ANOVA test calculated for studied parameter.
$P < 0.05$ = Non-significant.

TABLE 2 Association of *Anaplasma ovis* prevalence with the studied epidemiological parameters describing sheep characters enrolled during the present study from District Dera Ghazi Khan.

Parameters		*Anaplasma ovis* +ve sheep	*Anaplasma ovis* −ve sheep	P-value
Sex	Male	1/9 (11.1%)	8/9 (88.9%)	1
	Female	29/230 (12.6%)	201/230 (87.4%)	
Age	<2 years	14/123 (11.4%)	109/123 (88.6%)	0.7
	>2 years	16/116 (13.8%)	100/116 (86.2%)	
Composition of herd	Sheep only	18/160 (11.3%)	142/160 (88.8%)	0.4
	Sheep and goat	12/79 (15.2%)	67/79 (84.8%)	
Dogs with herd	Present	7/55 (12.7%)	48/55 (87.3%)	1
	Absent	23/184 (12.5%)	161/184 (87.5%)	
Size of herd	<20	7/47 (14.9%)	40/47 (85.1%)	0.6
	>20	23/192 (12%)	169/192 (88%)	

N represents the total number of collected samples. % prevalence of Anaplasma ovis is given in parenthesis. P-value represents the results of Fischer Exact test calculated for each studied parameter.
$P > 0.05$ = Non-significant.

or more (Figure 1). Analysis revealed that all three *A. ovis* sequences generated during present study clustered in a single cluster as shown in Figure 1 along with those amplified from small ruminants in Kenya, China, Germany, Turkey, Portugal, Tunisia, and India (Figure 1).

Alignment of *msp4* partial sequences of *msp4* gene from Pakistani sheep revealed a single genotype (Figure 2). All the three sequences generated during present study showed 100% genetic similarity with one another indicating that this *msp4* sequence is highly conserved (Figure 2). While these sequences had 96–100% identity with the *msp4* sequences submitted in GenBank from various countries (Figure 2). The phylogeny did not indicate a population structure that was based on geography. Thereof, the set of sequences generated in this study was taken as a single population for neutrality test. Calculated values for Fu and Li's *D*-test was −3.49615 with a statistical significance of $P < 0.02$ and the value for Tajima's *D* was −2.16609 with a statistical significance of $P < 0.01$.

Discussion

Ovine anaplasmosis is among the most common TBDs reported in sheep from worldwide especially in tropical and sub-tropical regions (17). Since its identification in 1912, *Anaplasma ovis* has been reported from Asia, Europe, Africa, and the United States (8). Usually infection caused by *Anaplasma ovis* is not severe but cases with severe pathology in small ruminants have been documented from Northern United States and from Africa (18). In Haibei County of western China, during 2008, infection due to *A. ovis* resulted in 17% mortality and 40–50% sheep morbidity (19). Due to increased tick infestation, the incidence of ovine and caprine anaplasmosis is rising worldwide (20). Data regarding the prevalence of *A. ovis* in sheep and goats of Pakistan is very limited and only one previous report is available in literature to date.

In present study, we have reported that 12.5% of collected blood samples from sheep were infected with *Anaplasma ovis*. The only study from Pakistan is reported by Niaz et al. (21) in which sheep were enrolled from northern areas of Pakistan and they have used molecular tool (PCR) for the detection of *Anaplasma spp*. and they had found that 21.7% of enrolled sheep were infected with *A. spp*. and DNA sequencing of the amplified PCR products confirmed the presence of *A. ovis* in enrolled sheep. The prevalence of *A. ovis* has been reported from various parts of the world. The prevalence of *A. ovis* was reported to be 70.1% in sheep of Tunisa (22), 69% in sheep of Mogolia (23), 54.5% in sheep of Qinghai, China (24), 34.2% in sheep of central and Western Kenya (25), 29.7% in sheep of Turkey (26), 20.8% in sheep of Iran (27), 10% in sheep of West

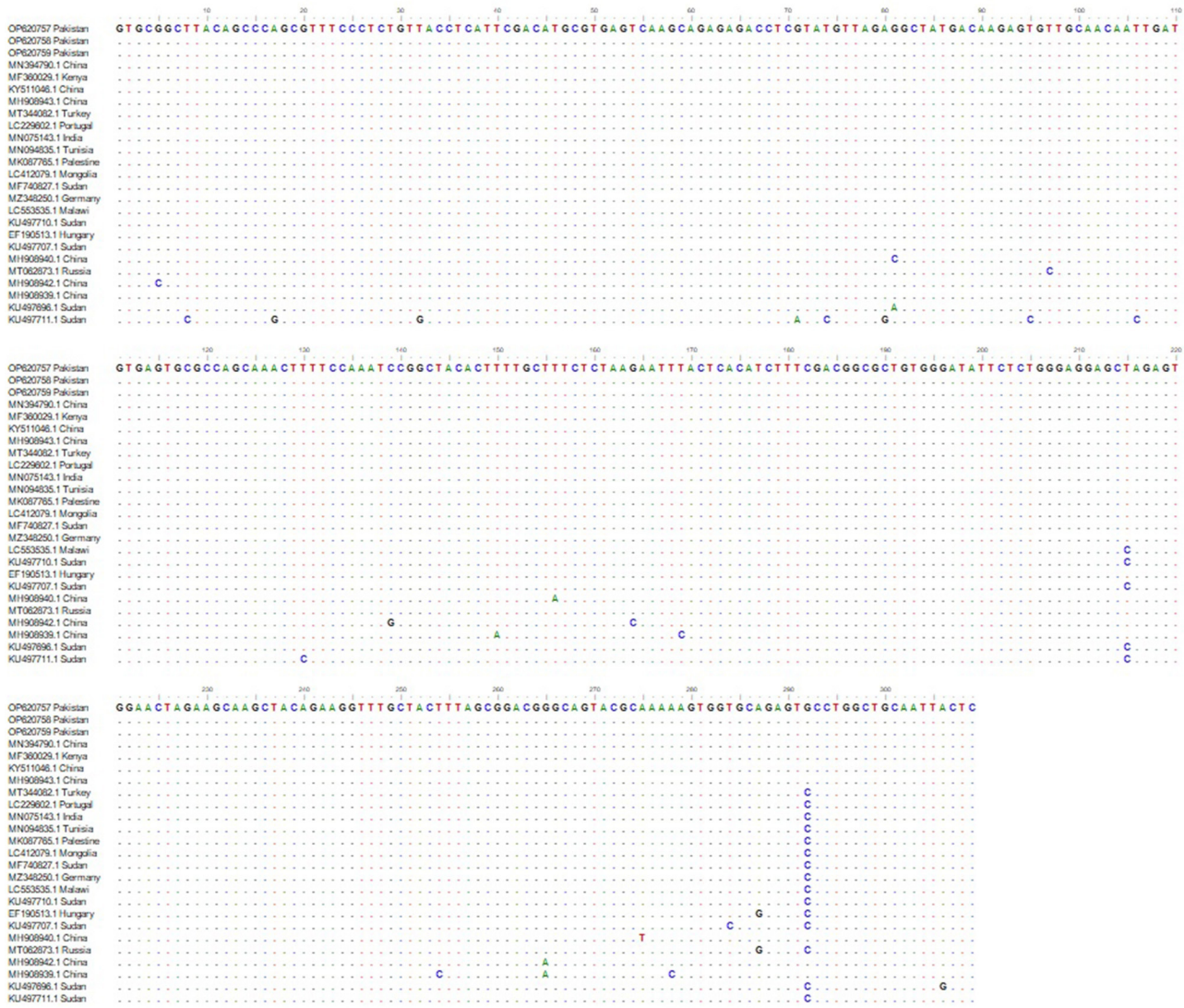

FIGURE 1
Phylogenetic analysis of *Anaplasma ovis* based on *msp4* partial gene sequence. Sequences in the box were determined in this study. GeneBank accession numbers and country are shown for all sequences. Values at the nodes represent the number of occurrences of clades in 1,000 bootstrap replications of the taxa. The *msp4 partial gene* sequence of *Anaplasma marginale* from Mexico, *Anaplasma centrale* from Israel were and *Anaplasma phagocytophilum* from Italy were used as the outgroup. Maximum Likelihood method and Kimura 2-parameter model were used to infer the evolutionary history. The tree with the highest log likelihood (−1,168.91) is shown. Evolutionary analyses were conducted in MEGA11.

Iran (28), 5.7% in sheep of South Western China (29), and 2.6% in sheep of North East China (30). The variation in the prevalence of *Anaplasma ovis* between different studies is due to difference in the geographical and climatic conditions of sampling sites, age, gender, immunity of the host animal, tick density in a specific area, and also depends upon type of farm management technique that was followed during a specific study (31).

Phylogenetic and sequence analysis of *msp4* partial sequence amplified during this study revealed that this gene sequence highly conserved (Figures 1, 2). Phylogenetic analysis revealed that our amplified DNA sequences are placed in stable monophyletic with 100% homology with *msp4* partial sequences from China, Kenya, Germany, Turkey, Portugal, Tunisia, and India (Figure 1). However, our isolates are relatively distant from the strains isolated from small ruminants in Sudan, Hungry and Russia. The genetic variations between *msp4* sequences of *A. ovis* that we have generated and those deposited in GenBank are probably due to the difference of geographic conditions of the areas from the bacterium samples were detected as not only the tick diversity and density varies with the geographic and climatic conditions but also the pathogenicity of *A. ovis* strains is also affected (32). Negative values of Tajima's *D* and Fu's *F* were obtained during present study that indicates an excess of rare variation, deviations from neutrality and a recent expansion of the population. This is consistent due to the origin of the sequences used. However, there number of DNA sequences that were used in the analysis was limited and use of large number of DNA sequences are recommended in future studies for a thorough evolutionary analysis. Further, we are not excluding that other *msp4* variants may be circulating in the small ruminants of Pakistan.

FIGURE 2
msp4 sequence alignment from *Anaplasma ovis* isolates amplified from Pakistani sheep and the sequences deposited in GenBank from various parts of world. Dashes indicate the conserved nucleotide positions. The positions with substitutions in DNA sequence of various *Anaplasma* spp. are represented by different colored nucleotides.

In present study, all the epidemiological factors (age, sex, breed, size of herd, dogs with herd, and composition of herd studied) were not found associated with the prevalence of *A. ovis*. Contrary to our result, it is reported that eve are more susceptible to *A. ovis* infection as compared to ram. This is because of the fact that eve faces more hormonal fluctuations due to their reproductive cycles that makes them more susceptible to infections (22, 28). It is also reported that adult sheep were more affected than lambs. This is probably due to the fact that adults are more exposed to the environment (for grazing and for marketing) and hence they have higher chances to encounter vectors rather than lambs that are mostly kept at farms (22, 27).

Conclusions

In conclusion, we are reporting a moderate prevalence of *A. ovis* in sheep blood samples that were collected from Dera

Ghazi Khan in Punjab (Pakistan). None of the enrolled sheep breed was specifically susceptible to *A. ovis*. Data generated in this study will pave the way for the prophylactic detection and control of ovine anaplasmosis in Pakistan. We recommend that similar and large scale studies must be conducted in all those areas of Pakistan that are unexplored for the incidence and prevalence of *A. ovis*. This will significantly help in control of tis bacterium and will improve the output of livestock sector in Pakistan.

Data availability statement

The data presented in the study are deposited in the GenBank, accession numbers OP620757-59.

Ethics statement

The animal study was reviewed and approved by Ethical Committee of Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan (Pakistan). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

FI and AdK designed and supervised the study. MN, MA, and ATar collected blood samples from sheep and noted epidemiological data. MN, MS, AsK, ATaq, and SA extracted DNA from blood samples and carried out PCR assays. IA-E, AS, and AA performed or assisted with the statistical analysis, sequence alignment, and phylogenetic study. MN, AS, AA, and FI wrote the text and edited and finalized the article. All authors approved the final version of the article.

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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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Situational analysis on fluoroquinolones use and characterization of high-level ciprofloxacin-resistant *Enterococcus faecalis* by integrated broiler operations in South Korea

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Fluoroquinolones are classified as "critically important antimicrobials for human medicine"; however, their extensive use in livestock poses a significant health risk to humans as it leads to the rapid spread of antimicrobial resistance. This study confirmed that 40.0%–71.4% of the farms in three of the five integrated broiler operations were administered ciprofloxacin (CIP). Moreover, preventive purposes (60.9%), veterinarian prescriptions (82.6%), drinking water route (100%), and 1 to 3 days (82.6%) of age were significantly highest ($P < 0.05$). 194 high-level ciprofloxacin-resistant (HLCR) *Enterococcus faecalis* (*E. faecalis*) were found in 65 of 74 farms, and of which, the prevalence of *qnrA* (63.9%), *tetM* (60.3%), *ermB* (64.9%), *blaz* (38.7%), and *catA* (34.0%) was significantly highest ($P < 0.05$). 154 (79.4%) isolates showed MDR, and the distribution of MDR was significantly differences among the operations ($P < 0.05$). All HLCR *E. faecalis* possessed double mutations in *gyrA* and *parC*, and S83I/S80I (90.7%) mutations were most commonly identified. Interestingly, the distribution of isolates with MICs $\geq$ 512 for both CIP and moxifloxacin was significantly higher in CIP–administered farms (56.5%) than in non-CIP–administered farms (41.4%) ($P < 0.05$). Also, the prevalence of strong or moderate biofilm formers in HLCR *E. faecalis* was significantly higher than that of weak and no biofilm formers ($P < 0.05$). HLCR *E. faecalis* were heavily distributed in the broiler farms in Korea; therefore, it is necessary to minimize the prevalence of resistant bacteria *via* structural management regulations such as cleaning and disinfection of farm environments.

KEYWORDS

high level ciprofloxacin resistance, *Enterococcus faecalis*, broiler, Korea, biofilm, antimicrobial resistance genes, multidrug resistance, mutation

1. Introduction

Antimicrobials play a critical role in the prevention and treatment of disease in humans and animals. However, the extensive use of antimicrobials poses a significant health risk to humans, leading to the rapid spread of antimicrobial resistance (1). Fluoroquinolones, including ciprofloxacin (CIP), are classified as "critically important antimicrobials for human medicine" by the World Health Organization (2), but they have been widely used for treatment and prevention of livestock because of their strong antibacterial activity against gram-negative bacteria, gram-positive bacteria, and mycoplasma (3). Fluoroquinolones inhibit DNA replication by inhibiting bacterial DNA topoisomerase (*parC* and *parE* subunits) and DNA gyrase (*gyrA* and *gyrB* subunits), which act as essential enzymes in bacteria (4). Therefore, single and double point mutations in the quinolone resistance determining region (QRDR) of these subunits reduce susceptibility and lead to high resistance to fluoroquinolones in the future (5, 6).

Because the risk promoting fluoroquinolones-resistant bacteria could be harmful to humans, US FDA has finally decided to withdraw approval of enrofloxacin for use in poultry in 2005 (7, 8), and in Ireland, fluoroquinolones have been prescribed in <1% of antimicrobials for livestock since 2009 (9). Moreover, in Australia, fluoroquinolones have never been approved for food production animals due to strict regulations on antimicrobial use. Although the manufacture and import of enrofloxacin in the poultry industry in Korea have been only banned since October 2021 (10), the rate of resistance to CIP, a major metabolic product of enrofloxacin *in vivo*, increased by ∼4.5% in 2021 compared with that in 2020 (10).

In Korea, 96.4% of the broilers are produced in integrated operations (11). However, antimicrobial resistance patterns may differ by operation depending on biosecurity and hygiene practices, vaccine programs, and antimicrobial applications (12). Therefore, this study was conducted to analyze the current status of fluoroquinolones-based prescriptions and characterize high-level CIP-resistant (HLCR) *E. faecalis* from five major integrated broiler operations which produce 50.2% of chicken meat in Korea (11).

2. Materials and methods

2.1. Data source

A situational analysis of CIP use was performed using a questionnaire on CIP administration patterns during the broiler grow-out period. The questionnaire areas included purpose, proponent, route, age, and duration of CIP administration.

2.2. Sample collection

Fecal and dust samples from 74 farms of five integrated broiler operations were collected in 2021 according to the standard set of Processing and Ingredients Specification of Livestock Products by the Ministry of Food and Drug Safety (2018) (13). Briefly, approximately 10 g of the dust was swabbed using a sterile gauze moistened with sterile double-strength skim milk, and feces were sampled to disposable boots in five different sites from each flock. All samples were placed in whirl pak bags (Whirl-Pak, Nasco, Madison, WI) and transported to the laboratory at 4°C.

2.3. Bacterial identification

The isolation and identification of *E. faecalis* were performed following the standard microbiological protocols published by the Ministry of Food and Drug Safety (2018). In brief, each dust and fecal samples were cultured in 90 mL of buffered peptone water (BPW; BD Biosciences, San Jose, CA, USA) for 18–24 h at 37°C. The pre-enriched BPW was transferred to Enterococcosel broth (BD Biosciences) at a ratio of 1:10 and streaked onto Enterococcosel agar (BD Biosciences) after incubation at 37 °C for 18–24 h. At least three presumptive *Enterococcus* spp. colonies were selected from each sample, and *E. faecalis* was confirmed using polymerase chain reaction (PCR) with a specific primer for *ddl*, as described previously (14). If two or more isolates from the same farm showed the same antimicrobial susceptibility pattern, only one isolate was selected. A total of 351 *E. faecalis* isolates were analyzed in this study.

2.4. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of CIP and moxifloxacin (MOX) were determined by standard agar dilution methods with Mueller–Hinton agar (BD Biosciences) according to the guidelines of the Clinical and Laboratory Standards Institute (15). The breakpoint for HLCR was set at ≥64 μg/mL against CIP (16). Moreover, based on the guidelines of the Clinical and Laboratory Standards Institute (15), antimicrobial resistance of all HLCR *E. faecalis* isolates was determined using the disc diffusion method with the following discs (BD Biosciences): ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (CIP, 5 μg), doxycycline (30 μg), erythromycin (15 μg), penicillin (10 units), rifampin (5 μg), tetracycline (30 μg), and vancomycin (30 μg). Multidrug resistance (MDR) was defined as the acquired non-susceptibility to at least one agent in three or more antimicrobial classes (17).

2.5. Identification of mutations in QRDRs

PCR was performed to amplify *gyrA* and *parC* in HLCR *E. faecalis* isolates using primers and conditions described previously (18). The amplified DNA was purified by a PCR purification kit (Bioneer Inc., Seoul, Republic of Korea) and sequenced using an automatic sequencer (Cosmogenetech, Seoul, Republic of Korea). The sequences were confirmed with those in the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) program available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

2.6. Detection of antimicrobial resistance genes

DNA extraction was performed using the boiling method as described previously (19). The presence of resistance genes associated with fluoroquinolones (*qnrA*, *qnrB*, *qnrD*, and *qnrS*), β-lactams (*blaZ*, and *mecA*), phenicols (*cfr*, *catA*, and *catB*), glycopeptides (*vanA*), macrolides (*ermA*, *ermB*, and *ermC*), tetracyclines (*tetL*, *tetM*, and *tetO*), and rifamycin (*rpoB*) was detected using PCR as described previously (20–29).

2.7. Biofilm formation assay

All HLCR *E. faecalis* were determined for biofilm formation by using the standard microtiter plate test, as described previously (30). All HLCR isolates were cultured overnight on Brain–Heart Infusion agar (BD Biosciences, Sparks, MD, USA) at 37°C. Then, 500 μL of bacterial suspension adjusted for turbidity with 0.5 McFarland standard was inoculated into 5 mL of tryptic soy broth (BD Biosciences) with 0.25% (wt/vol) glucose, and 200 μL of the mixture was transferred into three wells of a 96-well microtiter plate. After overnight incubation at 37°C, the 96-well microtiter plates were washed with phosphate–buffered saline (BD Biosciences) to remove the planktonic bacteria. After air-drying, the attached bacteria were fixed with 200 μL of methanol for 15 min, and the bacterial biomass was quantified by measuring the absorbance at 595 nm (OD_{595}) after staining with crystal violet (1% wt/vol) for 10 min and destaining with 50% ethanol-50% glacial acetic acid solution. The ability to form biofilms was classified as negative ($OD_{595} < 0.12$), weak ($0.12 \leq OD_{595} < 0.24$), moderate ($0.24 \leq OD_{595} \leq 0.48$), and strong ($OD_{595} > 0.48$). To verify the analysis, *E. faecalis* OG1RF and *E. faecalis* ATCC 29212 were used as reference strains for weak and no biofilm formers, respectively. A sterile medium was used as the contamination control as described previously (31, 32).

2.8. Statistical analysis

Statistical analysis was performed using Pearson's chi-square tests and Fisher's exact tests with Bonferroni correction in Statistical Package for the Social Science version 25 (IBM, Korea). Significant differences were considered at $P < 0.05$.

3. Results

3.1. Analysis of the prescription status of CIP

The situational analysis of the prescription status of CIP in 74 farms of five integrated broiler operations is shown in Table 1. Among the five integrated broiler operations, the prevalence of CIP–administered farms was significantly highest in operation D (71.4%), followed by operations E (50.0%) and B (40.0%) ($P < 0.05$). No farms administered CIP in operations A and C. In the analysis for the purpose of CIP administration, operation D was significantly higher for the prevention (80.0%) than treatment (20.0%), whereas operation E were significantly higher for the treatment (60.0 %) than prevention (40.0 %) ($P < 0.05$). There were no significant differences between the rates of prevention and treatment in operation B. CIP administration *via* veterinarians (82.6%) and drinking water route (100%) was significantly highest ($P < 0.05$). Moreover, CIP was administered significantly highest at 1 to 3 days of age (82.6%), and significantly higher on duration of CIP administration in 2 days (43.5%) to 3 days (39.1%) ($P < 0.05$).

3.2. Distribution of HLCR *E. faecalis* and antimicrobial resistance genes

The distribution of HLCR *E. faecalis* and antimicrobial resistance genes in 74 farms of five integrated broiler operations are shown in Table 2. HLCR *E. faecalis* were found in 65 (87.8%) of 74 farms. In particular, HLCR *E. faecalis* were significantly higher at 95.0% and 100% in farms of operations B and E, respectively ($P < 0.05$). In addition, HLCR *E. faecalis* was found in a total of 194 (55.3%) of 351 *E. faecalis* isolates, which was significantly highest in farms of operation B ($P < 0.05$). In the distribution of fluoroquinolones resistance genes of HLCR *E. faecalis*, the prevalence of *qnrA* (63.9%) and *qnrB* (57.7%) genes were significantly higher than that of *qnrD* (20.1%) and *qnrS* (18.0%) ($P < 0.05$). In particular, *qnrA* showed a significantly high prevalence in the farms of four operations A (66.7%), B (60.7%), D (78.9%), and E (73.3%) except for operation C (45.0%) ($P < 0.05$), and *qnrB* was found in 50.0%−66.7% of farms in all five operations without significant differences.

All HLCR *E. faecalis* also showed the highest resistance to tetracyclines (82.0%), followed by macrolides (79.4%), β-lactams (52.1%), phenicols (43.3%), rifamycin (32.5%), and glycopeptides (1.0%) without no significant differences among the five integrated broiler operations. Moreover, the prevalence of *tetM* (60.3%), *ermB* (64.9%), *blaz* (38.7%), and *catA* (34.0%) was significantly highest among tetracyclines, marcrolides, β-lactams and phenicols resistance-related genes, respectively ($P < 0.05$). MDR was found in 154 (79.4%) of 194 HLCR *E. faecalis* isolates; however, the distribution of MDR was significantly highest in operation B (87.5%) and lowest in operations A (73.3%) and C (70.0%) ($P < 0.05$).

3.3. Detection of mutations in the QRDRs of HLCR *E. faecalis*

The presence of mutations in *gyrA* and *parC* and distribution of MICs in 194 HLCR *E. faecalis* isolates are shown in Table 3. All HLCR *E. faecalis* isolates possessed double mutations in *gyrA* and *parC*. Moreover, S83I/S80I (90.7%) mutations were most commonly identified. The distribution of isolates with MICs ≥ 512 for both CIP and MOX was significantly higher in CIP–administered farms (56.5%) than in non-CIP–administered farms (41.4%) ($P < 0.05$).

TABLE 1 Analysis of the prescription status of ciprofloxacin (CIP) by five integrated broiler operations.

Variables	No. of farms included / No. of CIP–administered farms by five integrated broiler operations (%)					
	A	B	C	D	E	Total
No. of CIP–administered farms/No. of farms tested (%)	$0/15\ (0.0)_{c}$	$8/20\ (40.0)_{b}$	$0/15\ (0.0)_{c}$	$10/14\ (71.4)_{a}$	$5/10\ (50.0)_{b}$	23/74 (31.1)
Purpose of CIP administration						
Prevention		$4/8\ (50.0)_{b}$		$8/10\ (80.0)_{a}^{A}$	$2/5\ (40.0)_{b}^{B}$	$14/23\ (60.9)^{A}$
Treatment		$4/8\ (50.0)_{a}$		$2/10\ (20.0)_{b}^{B}$	$3/5\ (60.0)_{a}^{A}$	$9/23\ (39.1)^{B}$
Who proposed CIP						
Veterinarian		$7/8\ (87.5)_{a}^{A}$		$9/10\ (90.0)_{a}^{A}$	$3/5\ (60.0)_{b}^{A}$	$19/23\ (82.6)^{A}$
Veterinary drug dealer		$1/8\ (12.5)^{B}$		$2/10\ (20.0)^{B}$	$2/5\ (40.0)^{B}$	$5/23\ (21.7)^{B}$
Others*		$0/8\ (0.0)^{C}$		$0/10\ (0.0)^{C}$	$0/5\ (0.0)^{C}$	$0\ (0.0)^{C}$
Route of CIP administration						
Drinking water		$8/8\ (100.0)^{A}$		$10/10\ (100.0)^{A}$	$5/5\ (100.0)^{A}$	$23/23\ (100.0)^{A}$
Feed		$1/8\ (12.5)_{a}^{B}$		$0/10\ (0.0)_{b}^{B}$	$0/5\ (0.0)_{b}^{B}$	$1/23\ (4.3)^{B}$
Age of CIP administration (days)						
$1\leq\ \leq 3$		$6/8\ (75.0)^{A}$		$9/10\ (90.0)^{A}$	$4/5\ (80.0)^{A}$	$19/23\ (82.6)^{A}$
$4\leq\ \leq 6$		$2/8\ (25.0)^{B}$		$1/10\ (10.0)^{B}$	$1/5\ (20.0)^{B}$	$4/23\ (17.4)^{B}$
$15\leq\ \leq 18$		$0/8\ (0.0)_{b}^{C}$		$0/10\ (0.0)_{b}^{C}$	$1/5\ (20.0)_{a}^{B}$	$1/23\ (4.3)^{C}$
Duration of CIP administration (days)						
1		$1/8\ (12.5)_{a}^{B}$		$2/10\ (20.0)_{a}^{B}$	$0/5\ (0.0)_{b}^{B}$	$3/23\ (13.0)^{B}$
2		$5/8\ (62.5)_{a}^{A}$		$3/10\ (30.0)_{b}^{B}$	$2/5\ (40.0)_{b}^{A}$	$10/23\ (43.5)^{A}$
3		$2/8\ (25.0)_{b}^{B}$		$5/10\ (50.0)_{a}^{A}$	$2/5\ (40.0)_{a,b}^{A}$	$9/23\ (39.1)^{A}$
4		$0/8\ (0.0)_{b}^{C}$		$0/10\ (0.0)_{b}^{C}$	$2/5\ (40.0)_{a}^{A}$	$2/23\ (8.7)^{B}$

The superscript letter represents significant differences of the column, while the subscript letter represents significant differences of the row ($P < 0.05$).
*Others included farm owners and pharmaceutical company employees.

3.4. Distribution of biofilm-forming ability of HLCR *E. faecalis*

The distribution of biofilm-forming ability of 194 HLCR *E. faecalis* isolates is shown in Table 4. Although there were no significant differences between CIP–administered farms and non-CIP–administered farms, the prevalence of strong or moderate biofilm formers (60.5% and 60.0%, respectively) in all HLCR *E. faecalis* showed significantly higher than that of weak and no biofilm formers ($P < 0.05$).

4. Discussion

Enterococci are harmless to healthy individuals, but are considered opportunistic pathogens associated with hospital-acquired infections in humans and animals (33). In particular, the ability to cause infection is critical to avoid the action of most commonly used antimicrobials. Recently, antimicrobial-resistant enterococci isolated from poultry farms have become a problem in many countries, including Korea (34–37), because antimicrobial-resistant enterococci can easily spread within pathogenic strains or between pathogenic and non-pathogenic strains (38). Moreover, resistance to fluoroquinolones, which are critically important in human medicine, has been continuously reported worldwide (39).

Antimicrobial resistance in enterococci is described as intrinsic resistance found within a species genome as well as acquired resistance by obtaining new genetic material or acquiring sporadic mutations in intrinsic genes (40, 41). In particular, the prevalence of acquired fluoroquinolones-resistant enterococci has increased with the increased use of fluoroquinolones (36, 42). In this study, CIP was administered in 23 (31.1%) of 74 broiler farms of five integrated broiler operations. Two integrated broiler operations did not use CIP, but the other three used CIP for prevention and treatment, mostly under the proposal of veterinarian. Moreover, the prevalence of 1 to 3 days of age (82.6%) was the highest among the age of CIP administration in the three integrated broiler operations. It is related to the high incidence of colibacillosis in young chicks causing mortality in Korea (43). CIP was also mainly administered *via* drinking water for 2 or 3 days because it is easier than injection administration, but it has a disadvantage that the time when the drug appears varies depending on individual even within the same flock (44).

In this study, HLCR *E. faecalis* were isolated in 65 (87.8%) of 74 farms. Farms of operations A and C were not administered CIP at all, but the distribution of HLCR *E. faecalis* isolates in these two

TABLE 2 Distribution of high-level ciprofloxacin-resistant (HLCR) *E. faecalis* and antimicrobial resistance genes by five integrated broiler operations.

Resistance gene	No. of isolates carried gene / No. of HLCR *E. faecalis* isolates by five integrated broiler operations (%)					
	A	B	C	D	E	Total
No. (%) of farms with HLCR *E. faecalis* isolates / No. of farms tested (%)	11/15 (73.3)$_{b}$	19/20 (95.0)$_{a}$	12/15 (80.0)$_{b}$	13/14 (92.9)$_{a,b}$	10/10 (100.0)$_{a}$	65/74 (87.8)
No. of HLCR *E. faecalis* isolates / No. of *E. faecalis* isolates (%)	30/68 (44.1)$_{b}$	56/81 (69.1)$_{a}$	40/79 (50.6)$_{b}$	38/65 (58.5)$_{a,b}$	30/58 (51.7)$_{a,b}$	194/351 (55.3)
qnrA	20/30 (66.7)$_{a}$	34/56 (60.7)$_{a,b}$	18/40 (45.0)$_{b}$	30/38 (78.9)$_{a}$	22/30 (73.3)$_{a}$	124/194 (63.9)^{A}
qnrB	17/30 (56.7)	30/56 (53.6)	20/40 (50.0)	25/38 (65.8)	20/30 (66.7)	112/194 (57.7)^{A}
qnrD	3/30 (10.0)$_{b}$	11/56 (19.6)$_{a,b}$	8/40 (20.0)$_{a,b}$	9/38 (23.7)$_{a,b}$	8/30 (26.7)$_{a}$	39/194 (20.1)^{B}
qnrS	5/30 (16.7)	8/56 (14.3)	8/40 (20.0)	6/38 (15.8)	8/30 (26.7)	35/194 (18.0)^{B}
No. (%) of tetracyclines resistant HLCR *E. faecalis* isolates	25/30 (83.3)	48/56 (85.7)	34/40 (85.0)	29/38 (76.3)	23/30 (76.7)	159/194 (82.0)
tetL	12/30 (40.0)$_{a,b}$	25/56 (44.6)$_{a}$	10/40 (25.0)$_{b}$	12/38 (31.6)$_{a,b}$	10/30 (33.3)$_{a,b}$	69/194 (35.6)^{B}
tetM	20/30 (66.7)	32/56 (57.1)	22/40 (55.0)	25/38 (65.8)	18/30 (60.0)	117/194 (60.3)^{A}
tetO	1/30 (3.3)	0/56 (0.0)	0/40 (0.0)	0/38 (0.0)	0/30 (0.0)	1/194 (0.5)^{C}
No. (%) of macrolides resistant HLCR *E. faecalis* isolates	22/30 (73.3)	48/56 (85.7)	30/40 (75.0)	30/38 (78.9)	24/30 (80.0)	154/194 (79.4)
ermA	1/30 (3.3)	1/56 (1.8)	2/40 (5.0)	3/38 (7.9)	3/30 (10.0)	10/194 (5.2)^{B}
ermB	19/30 (63.3)	40/56 (71.4)	24/40 (60.0)	25/38 (65.8)	18/30 (60.0)	126/194 (64.9)^{A}
ermC	0/30 (0.0)	0/56 (0.0)	0/40 (0.0)	0/38 (0.0)	0/30 (0.0)	0/194 (0.0)^{B}
No. (%) of β-lactams resistant HLCR *E. faecalis* isolates	13/30 (43.3)	22/56 (55.4)	16/40 (50.0)	15/38 (50.0)	14/30 (60.0)	101/194 (52.1)
blaZ	10/30 (33.3)	23/56 (41.1)	15/40 (37.5)	14/38 (36.8)	13/30 (43.3)	75/194 (38.7)^{A}
mecA	2/30 (6.7)	1/56 (1.8)	2/40 (5.0)	0/38 (0.0)	1/30 (3.3)	6/194 (3.1)^{B}
No. (%) of phenicols resistant HLCR *E. faecalis* isolates	10/30 (33.3)	24/56 (42.9)	18/40 (45.0)	16/38 (42.1)	16/30 (53.3)	84/194 (43.3)
cfr	2/30 (6.7)	2/56 (3.6)	0/40 (0.0)	2/38 (5.3)	0/30 (0.0)	6/194 (3.1)^{B}
catA	12/30 (40.0)	18/56 (35.7)	13/40 (45.0)	12/38 (47.4)	11/30 (36.7)	66/194 (34.0)^{A}
catB	2/30 (6.7)	1/56 (1.8)	0/40 (0.0)	2/38 (5.3)	1/30 (3.3)	6/194 (3.1)^{B}
No. (%) of rifamycin resistant HLCR *E. faecalis* isolates	10/30 (33.3)	16/56 (28.6)	15/40 (37.5)	12/38 (31.6)	10/30 (33.3)	63/194 (32.5)
rpoB	10/30 (33.3)	13/56 (23.2)	13/40 (32.5)	9/38 (23.7)	8/30 (26.7)	53/194 (27.3)
No. (%) of glycopeptides resistant HLCR *E. faecalis* isolates	1/30 (3.3)	0/56 (0.0)	0/40 (0.0)	0/38 (0.0)	1/30 (3.3)	2/194 (1.0)
vanA	0/30 (0.0)	0/56 (0.0)	0/40 (0.0)	0/38 (0.0)	0/30 (0.0)	0/194 (0.0)
No. (%) of multidrug resistant HLCR *E. faecalis* isolates	22/30 (73.3)$_{b}$	49/56 (87.5)$_{a}$	28/40 (70.0)$_{b}$	30/38 (78.9)$_{a,b}$	25/30 (83.3)$_{a,b}$	154/194 (79.4)

The superscript letter represents significant differences of the column, while the subscript letter represents significant differences of the row ($P < 0.05$).

farms showed a high prevalence of 73.3% and 80.0%, respectively. Burbarelli et al. (45) reported that antimicrobial–resistant bacteria could survive and spread throughout the breeding period regardless of CIP administration unless cleaning and disinfection of the broiler environment is followed for farm hygiene. In particular, antimicrobial–resistant bacteria in feces, which are generally considered a natural reservoir, have the potential to remain in the environment or bind to dust and spread throughout the farm (46–48).

Moreover, in this study, the antimicrobial resistance genes *qnrA* and *qnrB*, which are related to fluoroquinolones resistance, were present in 63.9% and 57.7%, respectively, of the distribution of HLCR *E. faecalis* in the five integrated broiler operations. In particular, the prevalence of *qnrA* in HLCR *E. faecalis* isolates in operation A was 66.7%, but the prevalence of *qnrA* in operation C was 45.0%, which was significantly lowest in these operations, even though CIP was not administered in operation A and C. It is thought that environmental cleaning and the use of CIP, and it is

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TABLE 3 Characteristics of amino acid changes in *gyrA* and *parC* and distribution of MICs in 194 high-level ciprofloxacin-resistant (HLCR) *E. faecalis* from 74 broiler farms of five integrated broiler operations.

Amino acid change		MIC (μg/mL)[a]		No. (%) of HLCR *E. faecalis* isolates		
gyrA	*parC*	CIP	MOX	CIP–administered farms ($n = 124$)	Non–CIP–administered farms ($n = 70$)	Total ($n = 194$)[b]
S83I	S80I	≥512	≥512	70 (56.5)$_a$	29 (41.4)$_b$	99 (51.0)
		256	≥512	17 (13.7)	14 (20.0)	31 (16.0)
		≥512	256	11 (8.9)	11 (15.7)	22 (11.3)
		256	256	6 (4.8)	6 (8.6)	12 (6.2)
		256	128	2 (1.6)	3 (4.3)	5 (2.6)
		128	256	2 (1.6)	0 (0.0)	2 (1.0)
		128	128	2 (1.6)	0 (0.0)	2 (1.0)
		128	64	1 (0.8)	0 (0.0)	1 (0.5)
		64	64	1 (0.8)	1 (1.4)	2 (1.0)
S83F	S80I	≥512	256	5 (4.0)	2 (2.9)	7 (3.6)
		256	256	2 (1.6)	1 (1.4)	3 (1.5)
		256	128	2 (1.6)	0 (0.0)	2 (1.0)
		128	64	2 (1.6)	1 (1.4)	3 (1.5)
S83V	S80I	≥512	256	1 (0.8)	0 (0.0)	1 (0.5)
S83Y	S80I	≥512	256	0 (0.0)	1 (1.4)	1 (0.5)
S83I	S84K	256	256	0 (0.0)	1 (1.4)	1 (0.5)

[a] MIC, Minimum Inhibitory concentrations; CIP, ciprofloxacin; MOX, moxifloxacin.
[b] All isolates possessed double mutations in *gyrA* and *parC*.
The subscript letter represents significant differences of the row ($p < 0.05$).

necessary to continuously investigate the distribution of these genes in farms that do not use CIP.

The plasmids carrying mobile resistance genes are easily transferable from one bacteria to another by conjugation, sharing genetic information (49, 50). Moreover, the externalization of antimicrobials *via* efflux pumps responsible for the outflow of the antimicrobials can simultaneously confer resistance to several antimicrobials, and their overexpression can lead to high levels of resistance and MDR (51–53). In this study, HLCR *E. faecalis* isolated from five broiler integrated operations were mostly resistant to tetracyclines (82.0%), macrolides (79.4%), β-lactams (52.1%), phenicols (43.3%), and rifamycin (32.5%), without significant differences among the operations. In addition, the prevalence of *tetM*, *ermB*, *blaZ*, *catA*, and *rpoB*, which are related to resistance to tetracyclines, macrolides, β-lactams, and phenicols, was 60.3%, 64.9%, 38.7%, and 34.0%, respectively, without significant differences among the operations. However, the distribution of MDR was significantly highest in operation B (87.5%) and lowest in operations A (73.3%) and C (70.0%). These results are consistent with the fact that the distribution of HLCR *E. faecalis* isolates was significantly highest in farms of operation B and lowest in farms of operations A and C. Moreover, this is consistent with the reports that the efflux pump is overexpressed as the number of HLCR isolates increases (54, 55), which is thought to be related to the increased distribution of MDR.

The mechanism of resistance to fluoroquinolones includes the formation of mutations in the two bacterial essential enzymes, DNA gyrase and DNA topoisomerase (56). DNA gyrase comprises *gyrA* and *gyrB* subunits, and DNA topoisomerase comprises *parC* and *parE* subunits. In particular, HLCR is associated with double mutations of *gyrA* and *parC* (57). Kim et al. (36) reported that all tested HLCR enterococci isolates showed double mutations in *gyrA* and *parC*, and the double mutations were the most frequently identified in S83I/S80I at 94.1%. Similarly, all HLCR *E. faecalis* isolates in this study

TABLE 4 Distribution of biofilm-forming ability in 194 high-level ciprofloxacin-resistant (HLCR) *E. faecalis* from 74 broiler farms of five integrated broiler operations.

Biofilm former	No. (%) of HLCR *E. faecalis* isolates		
	CIP–administered farms ($n = 124$)	Non-CIP–administered farms ($n = 70$)	Total ($n = 194$)
No biofilm former	19 (15.3)^B	10 (14.3)^B	29 (14.9)^B
Weak biofilm former	30 (24.2)^B	18 (25.7)^B	48 (24.7)^B
Strong or moderate biofilm former	75 (60.5)^A	42 (60.0)^A	117 (60.3)^A

The superscript letter represents significant difference of the column ($p < 0.05$).

possessed double mutations in *gyrA* and *parC*. In particular, 90.7% of HLCR isolates showed S83I/S80I mutations. Moreover, 99 (56.3%) of 176 HLCR *E. faecalis* with S83I/S80I mutations showed MICs of ≥512 for CIP and MOX. However, the distribution of these HLCR *E. faecalis* with MIC of ≥512 for both CIP and MOX in S83I/S80I mutations was found to be significantly higher in CIP–administered farms (56.5%) than in non–CIP–administered farms (41.4%). These results indicate that higher CIP administration leads to higher MIC values, just as fluoroquinolones-resistant enterococci increase with increasing fluoroquinolones use.

Biofilms of bacteria protect them from host immunological responses, phagocytosis, and antimicrobials (58). The ability to form biofilms is related to the ability of bacteria to adhere to a surface and form a layer, and the density of the layer indicates the strength of the biofilms (59). In addition, the thicker the layer, the less effective the antimicrobials against bacteria, suggesting a correlation between antimicrobial resistance and biofilm-forming ability (60). Al-Shammary (61) reported that 93.8% of MDR *E. faecalis* isolates from raw milk samples were biofilm formers. Sadek and Koriem (62) also reported that all MDR *E. faecalis* isolates from dairy products were biofilm formers. In this study, 165 (85.0%) of 194 HLCR *E. faecalis* isolates showed strong or moderate biofilm-forming ability. However, there were no significant differences between the isolates from CIP–administered farms and non–CIP–administered farms. Therefore, these results suggest that if already classified as HLCR, isolates can have strong and moderate biofilm-forming ability regardless of CIP administration, and the increased biofilm density also allows infections to persist chronically despite antimicrobial treatment strategies for broiler diseases. In this study, it was confirmed that HLCR *E. faecalis* were heavily distributed in the broiler operations in Korea, therefore, it is necessary to minimize the prevalence of resistant bacteria *via* structural management regulation, such as cleaning and disinfection of farm environments, and reduce the use of antimicrobials *via* prescriptions based on scientific disease evidence rather than disease prevention.

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

YuL, SK-L, and YoL conceived of the presented idea. YuL, H-RJ, and SY developed the theory and performed the experiments. YuL and H-RJ verified the analysis. YuL wrote the manuscript with supervision from S-KL and YoL. 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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Modification of the drug resistance of emerging milk-borne pathogens through sodium alginate-based antibiotics and nanoparticles

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Streptococcus agalactiae and *Klebsiella pneumoniae* are emerging as major milk-borne pathogens. Additionally, resistance to antibiotics of pathogens is of concern. Therefore, this study investigated the prevalence and drug resistance of *S. agalactiae* and *K. pneumoniae* in mastitis milk samples and assessed the antimicrobial potential of sodium alginate (G)-stabilized MgO nanoparticles (M) and antibiotics (tylosin [T] and ampicillin [A]) against both of these pathogens. A total of $n = 200$ milk samples from cattle were collected using purposive sampling, and standard microbiological approaches were adopted to isolate target bacteria. Parametric and non-parametric statistical tests were used to analyze the obtained data. Four preparations, GT (gel-stabilized tylosin), GA (gel-stabilized ampicillin), GTM (tylosin and MgO nanoparticles stabilized in gel), and GAM (ampicillin and MgO nanoparticles stabilized in gel), were evaluated against both bacteria through well diffusion and broth microdilution method. The analysis revealed that 45.24% (95/210) of the milk samples were positive for mastitis, of which 11.58% (11/95) were positive for *S. agalactiae* and 9.47% (9/95) were positive for *K. pneumoniae*. *S. agalactiae* had a significantly higher zone of inhibition (ZOI) than *K. pneumoniae* against penicillin, tetracycline, and amoxicillin, whereas the opposite was observed against imipenem and erythromycin. All gel (G)-based preparations showed an increase in the percentage of ZOI compared with antibiotics alone, with GTM presenting the highest of all, i.e., 59.09 and 56.25% ZOI compared with tylosin alone against *S. agalactiae* and *K. pneumoniae*, respectively. Similarly, in a broth microdilution assay, the lowest MIC was found for *K. pneumoniae* ($9.766 \pm 0.0\,\mu g/mL$) against GTM, followed by GT, GAM, and GA after incubation for 24 h. A similar response was noted for preparations against *S. agalactiae* but with a comparatively higher MIC. A significant reduction in MIC with respect to incubation time was found at 8 h and remained until at 20 h against both pathogens. The cytotoxicity of the MgO nanoparticles used in this study was significantly lower than that of the positive control. Overall, this study found that *K. pneumoniae*

and *S. agalactiae* appeared higher in prevalence and antimicrobial resistance, and sodium alginate-based antibiotics and MgO nanoparticles were effective alternative approaches for tackling antimicrobial resistance.

KEYWORDS

***Klebsiella pneumoniae*, *Streptococcus agalactiae*, dairy milk, antibiotic resistance, MgO nanoparticles, sodium alginate, tylosin, ampicillin**

1. Introduction

The gap between milk production and supply is increasing despite the fact that Pakistan is one of the top milk-producing countries. The average shortfall in Karachi, a major cosmopolitan city, has already been estimated to reach 4 million liters per day. Milk consumption is expected to increase by a minimum of 5% annually in the future (1). Additionally, it is notable that an increase in milk yield is hampered by the bacterial attack on the udder of animals, which in turn, compromises animal and public health. There are several bacteria involved, salient of which are *Staphylococcus aureus, Escherichia coli, Corynebacterium* spp., *Streptococcus* spp., and *Klebsiella* spp. In recent years, minor species of bacteria have increased because of drug resistance. *Klebsiella pneumoniae* has been designated as a "critical" bacterium for the research and development of novel antibiotics due to its weak antibacterial susceptibility (2). *Streptococcus agalactiae*, now known as group B *Streptococcus* (GBS), was differentiated from other *Streptococci* in the 1930s after it was identified in milk and cows suffering from bovine mastitis. Additionally, this pathogen is an intracellular parasite of the cow mammary gland and is highly infectious. Both of these pathogens have recently been reported to be increasing in dairy cattle in China and Egypt (3, 4). Furthermore, the development of antiobiotic resistance is an inevitable phenomenon. Recently, studies have reported considerable variations in the efficacy of antibiotics against both pathogens, clearly indicating their increasing resistance (5).

There is a dire need to counter antimicrobial resistance, for which reduction, refinement, replacement, review, and responsible use of antibiotics has been the recent approach. Nanoparticles are emerging as an effective replacement for antimicrobials against a wide range of pathogens. The use of nanoparticles in biomedicine has not only been applied for antimicrobial replacement (6, 7) but also for the improvement of meat quality (8) and has a significant role in antioxidant enzymes (9). A wide range of metallic and non-metallic nanoparticles are being applied in various products and other applications have also being evaluated in recent studies. Metallic MgO nanoparticles play a significant role in biological and applied sciences. Additionally, it is important to use antibiotics by coupling with a non-antibiotic source, such as chitosan or sodium alginate, to enhance efficacy at reduced quantity. Sodium alginate is a natural and hydrophilic anionic polysaccharide extracted from brown marine algae (*Phaeophyceae*), and it has been extensively applied in the food, bioengineering, and pharmaceutical fields. The inclusion of alginate in pectin-based formulations improves the strength of the zinc ions crosslinking network, which means it increases the capacity of the nanoparticle (10). The use of alginates has been proven to be safe, has excellent biocompatibility and biodegradability, and is highly effective at thickening and gelling products. They are hypothesized to enhance the antimicrobial activity of antimicrobial candidates. Recently, sodium alginate has been successfully used as a reducer and stabilizer of metal nanoparticles at different reaction times and temperatures in studies to examine other potential applications. Sodium alginate-stabilized silver nanoparticles have served as an effective antibacterial composite against gram-positive and gram-negative pathogens and have thus increased the shelf life of food (11, 12).

The bacteria selected in this study have commonly been overlooked for their prevalence and emerging resistance to drugs which is now being otherwise. On the other hand, the application of composite therapy consisting of conventional and non-conventional potential antimicrobial candidates is highly desirable. With this scenario in mind, the current study planned to estimate the prevalence of *K. pneumoniae* and *S. agalactiae* in mastitis milk samples, assessing their response to antibiotics, and examining their resistance modulation potential through sodium alginate-stabilized antibiotics and nanoparticles.

2. Results

2.1. Prevalence and antimicrobial susceptibility profile

This study revealed that 45.24% (95/210) of the dairy cattle milk samples were positive for mastitis. Of these mastitis samples, 11.58% (11/95) were positive for *S. agalactiae* and 9.47% (9/95) were positive for *K. pneumoniae*. Regarding the resistance of these bacteria, there was variation in the responses of *K. pneumoniae* and *S. agalactiae* against antibiotics in general (Table 1). For penicillin, tetracycline, and amoxicillin, *S. agalactiae* had considerably higher zones than *K. pneumoniae*. On the other hand, *K. pneumoniae* showed significant differences in zones of inhibition to those of *S. agalactiae* against imipenem and erythromycin (Figure 1). There were minor variations in antibacterial responses between both bacteria against ceftriaxone. Standard deviation from the mean value of the ZOI was higher for *K. pneumoniae* against cetirizine (6.14 mm) and imipenem (5.12 mm) than *S. agalactiae* against ceftriaxone (4.97 mm), penicillin (3.56 mm), and erythromycin (3.24 mm). This reflects the fact that the latter pathogen is much more inclined toward a single susceptibility category, whereas the former exhibits variable responses against antibiotics. The response of *S. agalactiae* (2.75 ± 0.83 mm) was nearly 10 times lower than that of *K. pneumoniae* (21.5 ± 2.96 mm) against aztronam.

TABLE 1 Zone of inhibitions (mm) shown by *K. pneumoniae* and *S. agalactiae* against antibiotics.

Name of antibiotic	Abbreviation	*K. pneumoniae*	*S. agalactiae*
		Mean ± SD	Mean ± SD
Imipenem	IMI	29.75 ± 5.12	19 ± 1.73
Penicillin	P10	19 ± 3.00	27.75 ± 3.56
Tetracycline	TE	20.75 ± 2.38	26.25 ± 1.09
Ampicillin	AMP	21 ± 2.12	28.25 ± 1.48
Ceftriaxone	CRO	22 ± 3.54	22.25 ± 4.97
Amoxicillin	AUG	20.5 ± 0.87	27 ± 2.12
Cefepime	FEP	20.25 ± 1.64	26 ± 2.92
Aztronam	ATM	21.5 ± 2.96	2.75 ± 0.83
Erythromycin	ERY	26.25 ± 2.86	23 ± 3.24

Zones of inhibitions are measured in millimeters (mm). SD, standard deviation.

2.2. Characterization of nanoparticles

The FTIR pattern of nanoparticles is shown in Figure 2A. The characteristic peak of the Mg-O bond was observed at a wavelength of 680 nm. This confirms that the metal-oxygen bond was present in the nanoparticles. Surfactant sodium dodecyl sulfate is an organic molecule and contains a C-H bond. Its band was observed at ∼1,400–1,500 cm^{-1} due to the adsorption of surfactant on the surface of the nanoparticles. Water molecules present in the atmosphere are quickly adsorbed on the surface of the nanoparticles as soon as they are exposed to the atmosphere. The band at ∼3,700 cm^{-1} shows the presence of an O-H bond due to water molecules.

EDX analysis of nanoparticles is shown in Figure 2B. Characteristic peaks of magnesium and oxygen are present in the plot. This confirms that magnesium and oxygen are present in the nanoparticles, which are MgO. Carbon tape was used for sample handling; hence, a peak of carbon can be observed.

STEM analysis of the nanoparticles is shown in Figure 2C. Spherical or oval-shaped nanoparticles were formed, ranging from 50 to 300 nm in size. The surface of the nanoparticles appeared to be smooth. The size distribution plot was drawn using data from STEM and shows that most of the nanoparticles ranged from 50 to 150 nm in size (Figure 2D), although a few nanoparticles were larger. Very small nanoparticles may have been attached to the surface of the other nanoparticles, thus making them larger than the others. XRD produced diffracted peaks for the nanoparticles that were narrow at higher temperatures. The Miller index at 2 theta angles 43.0°, 46.0°, 63.0°, 75.0°, and 78.0° were noted at 111, 200, 220, 311, and 222, respectively. These nanoparticles belonged to the face-centered cubic structure and Fm-3m space group, thus confirming the synthesis of MgO nanoparticles. SEM analysis showed a rounded cubical-shaped structure with corners that were not pointed but rather rounded or curved. The boundaries of these nanoparticles were smooth and clear. The size of the prepared nanoparticles ranged between 80 and 200 nm (approximately), and they had a well-dispersed shape rather than an aggregate form (Figure 2).

FIGURE 1
Zone of inhibitions (black arrows) for different preparations against *K. pneumoniae* **(left)** and *S. agalactiae* **(right)**.

2.3. Toxicity analysis

The cytotoxicity study revealed a significant difference between MgO-treated groups and the controls (Table 2). DNA damage was significantly ($p < 0.05$) lower in the MgO-treated groups than in the positive control after 24 and 48 h of contact time. However, DNA damage was directly proportional to the concentration of MgO nanoparticles, with a significant difference between each other, except between concentrations of 2.5 mg/mL and 5 mg/mL after 48 h of contact time. On the other hand, the phase index increased with an increase in the concentration of MgO up to 2.5 mg/mL; the phase index decreased beyond this concentration. This finding thus suggests that MgO nanoparticles are safe as their toxicity is significantly lower than the positive control.

2.4. Resistance modulation through gel-stabilized preparations

2.4.1. Comparison of composites with single antibiotics (well diffusion assay)

This study showed a significant difference in the ZOI (mm) between the sodium alginate-stabilized antibiotics and/or MgO

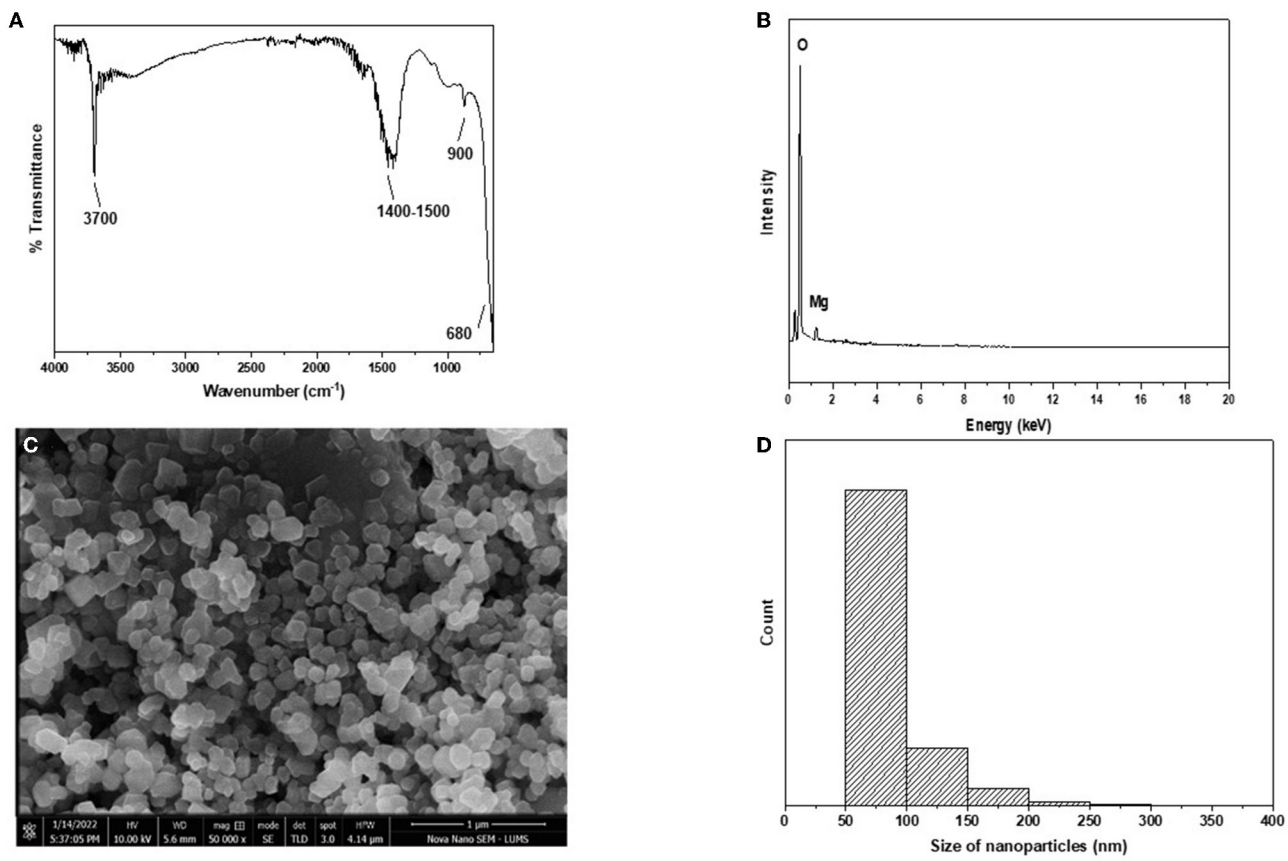

FIGURE 2
(A–D) Characterization of MgO nanoparticles. **(A)** FTIR. **(B)** EDX. **(C)** STEM. **(D)** Size distribution histogram.

nanoparticles compared with the antibiotics alone. GT and GTM were non-significantly different with each other in terms of antibacterial activity but were significantly different ($p < 0.05$) from tylosin alone (Table 3, Figure 1). A similar response was noted when gel-stabilized antibiotic and antibiotic plus MgO nanoparticles were compared with ampicillin (A) alone. There was a more than 50% increase in antibacterial activity in both gel-impregnated tylosin and tylosin plus MgO nanoparticles compared with T alone. On the other hand, GA and GAM showed a 47.28 and 53.112% increase in antibacterial activity compared with that of A alone. GT and GTM showed significantly higher responses compared with tylosin alone and an increase in the ZOI of 54.93 and 56.25%, respectively, against *S. agalactiae*. A comparison between ampicillin and GA and GAM revealed a significant difference ($p < 0.05$) among all in that the latter showed a significantly higher ZOI than A and GA. GA and GTM increased the ZOI by 43.91 and 53.80%, respectively.

2.4.2. Comparison of composites with single antibiotics (broth microdilution assay)

This study indicated the efficacy of antibiotics at lower concentrations when stabilized in sodium alginate in combination with nanoparticles. The lowest MIC against *K. pneumoniae* was observed with GTM (9.766 ± 0.000 μg/mL), followed by GT, GAM, and GA after 24 h of incubation (Table 4). There were significant differences ($p < 0.05$) in MICs between GT and GAM or GTM, whereas GTM and GA were not significantly different ($p > 0.05$) from each other, as were GA and GT ($p > 0.05$). A similar response of alginate-stabilized products was observed against *S. agalactiae* (Table 5). However, the magnitude of MICs was relatively higher than those noted in the case of *K. pneumoniae*. The lowest MIC was observed with GTM (26.04 ± 11.28 μg/mL) followed by GT (39.06 ± 0.00 μg/mL), whereas GA and GAM had the same MIC (78.13 ± 0.00 μg/mL). The MIC of GT and GTM was significantly different to that of GAM and GA. However, GA and GAM were not significantly different ($p > 0.05$) from each other, as were GT and GTM ($p > 0.05$).

2.4.3. Effect of incubation interval on the activity of composite

The efficacy of composite to respond at various time intervals of incubation differed significantly thus reflecting the availability of several effective dose to response time options. Each preparation at different time intervals showed a variable response (Figure 3). GT showed a significant reduction in MIC after 8 h of incubation that continued to differ until 12 h of

TABLE 2 Effect of magnesium oxide nanoparticles on the phase index and DNA damage in *A. cepa*.

Concentration (mg/mL)	CCN	Phase index (%) ± SD				DNA damage
		Prophase	Metaphase	Anaphase	Telophase	
24 h of contact time						
Control (negative control)	506	90.09 ± 0.420[a]	3.02 ± 0.11[a]	3.62 ± 0.28[a]	6.98 ± 0.19[a]	18.11 ± 0.12[e]
MMS (positive control)	507	84.130 ± 0.02[b]	1.01 ± 0.16[d]	1.99 ± 0.01[b]	3.88 ± 0.87[b]	135.0 ± 0.22[a]
1.25 mg/mL M	540	81.120 ± 0.240[c]	2.150 ± 0.12[b]	1.810 ± 1.260[b]	2.320 ± 0.22[c]	110. ± 0.10[d]
2.5 mg/mL M	550	80.22 ± 0.12[d]	2.09 ± 0.01[b]	1.800 ± 0.220[b]	2.13 ± 0.41[c]	112. ± 0.45[c]
5 mg/mL M	565	78.23 ± 0.110[e]	1.76 ± 0.11[c]	1.22 ± 0.19[b]	1.99 ± 0.31[c]	115.0 ± 0.35[b]
48 h of contact time						
Control	506	90.090 ± 0.420[a]	3.02 ± 0.11[a]	3.620 ± 0.280[a]	6.98 ± 0.190[a]	18.11 ± 0.12[d]
MMS	507	84.130 ± 0.020[b]	1.01 ± 0.16[c]	1.99 ± 0.01[ab]	3.88 ± 0.87[b]	135.0 ± 0.22[a]
1.25 mg/mL M	555	78.99 ± 0.55[c]	1.89 ± 0.03[b]	1.99 ± 1.46[ab]	2.65 ± 0.45[bc]	112.0 ± 0.11[c]
2.5 mg/mL M	507	83.12 ± 0.33[b]	1.69 ± 0.04[b]	1.86 ± 0.31[ab]	2.450 ± 0.50[c]	120.0 ± 0.550[b]
5 mg/mL M	540	80.03 ± 0.56[c]	1.66 ± 0.11[b]	1.77 ± 0.17[b]	1.53 ± 0.28[c]	120.0 ± 0.99[b]

Different superscript letters in the same column show a statistically significant difference ($p < 0.05$) at 24 and 48 h. MMS, methyl methane sulphonate; CCN, counting cell numbers; M, MgO nanoparticles. NB: The averages of the positive and negative controls were first assessed for the whole duration and set as the standard for the comparison of MgO concentrations after 24 and 48 h of contact time. SD, Standard deviation.

TABLE 3 Comparison of the antibacterial activity (zone of inhibition) of sodium alginate-stabilized antibiotic and nanoparticles against bacteria.

Antibiotic	Combinations	*S. agalactiae*		*K. pneumoniae*	
		Mean ± SD	% change	Mean ± SD	% Change
T	Alone	12.47 ± 0.950[b]	–	13.80 ± 0.608[b]	–
	GT	27.67 ± 1.528[a]	54.93%	31.17 ± 2.75[a]	54.74%
	GTM	28.500 ± 1.000[a]	56.25%	33.73 ± 0.681[a]	59.09%
A	Alone	9.47 ± 1.002[c]	–	10.37 ± 0.850[b]	–
	GA	16.83 ± 0.764[a]	43.91%	19.67 ± 2.08[a]	47.28%
	GAM	20.50 ± 1.000[b]	53.80%	22.33 ± 1.258[a]	53.112%

The percentage change in the ZOI of gel-stabilized preparations was compared with the antibiotic alone. It was measured as (e.g., GT compared with T alone) = (GT-T/GT)* 100. SD, Standard deviation.
Different superscript letters within column for T (tylosin) indicate significant difference ($p < 0.05$) for bacteria. Same is in the case of A (ampicillin).

TABLE 4 Comparison of the minimum inhibitory concentrations (μg/mL) of different gel-based preparations against *K. pneumoniae* at each time interval.

Preparations	4 h	8 h	12 h	16 h	20 h	24 h
GT	625.0 ± 0[a]	260.4 ± 90.2[a]	130.2 ± 45.1[a]	78.13 ± 0.00[a]	52.1 ± 226[a]	19.53 ± 0.00[a]
GA	625.0 ± 0[a]	260.4 ± 90.2[a]	156.25 ± 0.0[a]	130.2 ± 45.1[a]	52.1 ± 22.6[a]	39.06 ± 0.00[ab]
GTM	417 ± 180[a]	156.25 ± 0.0[a]	78.13 ± 0.00[a]	65.1 ± 22.6[a]	19.53 ± 0.00[b]	9.766 ± 0.000[bc]
GAM	521 ± 10[a]	260.4 ± 90.2[a]	130.2 ± 45.1[a]	78.13 ± 0.00[a]	39.06 ± 0.00[c]	26.04 ± 11.28[c]

GT, gel-stabilized tylosin; GA, gel-stabilized ampicillin; GTM, tylosin and MgO nanoparticles stabilized in gel; GAM, ampicillin and MgO stabilized in gel.
Different superscript letters within a column indicate a significant difference ($p < 0.05$).

incubation, indicating that GT can achieve the highest response after 12 h of incubation. GA showed variable means of MICs at different time intervals showing significant differences ($p < 0.05$) at initial hours and last hours while the middle hours of incubation remained non-significant ($p > 0.05$) different to each other. The same kind of response was observed for GAM, while GTM exhibited a comparatively smoother decline in MICs, showing a significant time-dependent response of MICs against *K. pneumoniae* (Figure 3). The antibacterial activity of GTM showed the lowest MIC (26.04 ± 11.28 μg/mL) followed by GT (39.06 ± 0.00 μg/mL), while GA and GAM showed the same MIC (78.13 ± 0.00 μg/mL) against *S. agalactiae* (Figure 4). The incubation period showed a significant reduction in MIC after 8 h of incubation for each preparation, which was sustained until 16 h

TABLE 5 Comparison of the minimum inhibitory concentrations (μg/mL) of different gel-based preparations against *S. agalactiae* at each time interval.

Preparations	4 h	8 h	12 h	16 h	20 h	24 h
GT	833 ± 361[a]	313 ± 271[a]	156. 25 ± 0.0[a]	156.25 ± 0.0[a]	65.1 ± 22.6[ab]	39.06 ± 0.00[b]
GA	1042 ± 361[a]	625.0 ± 0.0[a]	469 ± 271[a]	130.2 ± 45.1[a]	130.2 ± 45.1[a]	78.13 ± 0.00[b]
GTM	521 ± 180[a]	260.4 ± 90.2[a]	208.3 ± 90.2[a]	130.2 ± 45.1[a]	39.06 ± 0.00[b]	26.04 ± 11.28[b]
GAM	625.0 ± 0.0[a]	312.5 ± 0.0[a]	208.3 ± 90.2[a]	130.2 ± 45.1[a]	104.2 ± 45.1[ab]	78.13 ± 0.00[a]

GT, gel-stabilized tylosin; GA, gel-stabilized ampicillin; GTM, tylosin and MgO nanoparticles stabilized in gel; GAM, ampicillin and MgO stabilized in gel.
Different superscript letters within a column indicate significant differences ($p < 0.05$).

of incubation. GTM and GT showed a uniform response of non-significant reduction in MIC after 8 h of incubation, indicating that effective antibacterial activity was obtained in a short incubation period.

2.5. Comparision of minimum inhibitory concentration (μg/mL) of *K. pneumoniae* and *S. agalactiae* against different gel-based preparations

The mean and standard deviation of the comparison of the MIC between *K. pneumoniae* and *S. agalactiae* showed a non-significant difference. GT, GA, GTM, and GAM had means and standard deviations (μg/mL) of 39.06 ± 0.00 and 19.53 ± 0.00; 78.13 ± 0.00 and 39.06 ± 0.00; 26.04 ± 11.28 and 9.766 ± 0.000; and 78.13 ± 0.00 and 26.04 ± 11.28, respectively, at 24 h against *K. pneumoniae* and *S. agalactiae*, respectively (Table 6).

3. Discussion

3.1. Prevalence and resistance

China in a study published in year 2022 has reported 105 strains of *S. agalactiae* out of 313 clinical mastitis samples thus accounting for 33.55% prevalence on phenotypic basis of identification. The prevalence in the current study was lower than Kurjogi and Kaliwal (13), i.e., 28.10%. The pathogen is said to cause infection not only in bovines but also in humans and aquatic animals (3), so the rise in prevalence is justifiable as the pathogen spreads at one health interface. The current study showed a high prevalence of *S. agalactiae* compared with the study by Permatasari et al., (14). A study from Bangladesh reported 62.5% *Klebsiella* sp. in mastitic milk (15), which is much higher than that observed in the current study.

Previous studies conducted in the USA on *Klebsiella* spp. showed 5–19.5% resistance against the tetracycline group and up to 6.9% resistance against β-lactam. Similarly, up to 32% resistance against cefquinome, kanamycin, ceftiofur, polymyxin B, and tetracycline was found against *Klebsiella* spp (14). However, our findings reported otherwise by demonstrating the effective response of *K. pneumoniae* against penicillin, tetracycline, and amoxicillin. Contrary to the findings of our study, *S. agalactiae* showed 90% resistance against chloramphenicol, 78% against clindamycin, and 72% against enrofloxacin in previous studies, while the most sensitive response was shown against gentamicin (15).

3.2. Characterization of nanoparticles and their efficacy

The characterization of nanoparticles in the current study were in line with previously reported by Zaheer et al. (16). Similar findings were made in recent studies. Some studies reported smooth surfaces with a well-dispersed form, whereas others showed some nanoparticles clumped together. The average size of spherical nanoparticles ranged between 7 and 38 nm. Azam et al., (17) reported nanoparticles that ranged between 30 and 80 mm and were compactly aggregated. MgO nanoparticles have shown significant antibacterial activity, reducing growth by more than 90% at doses >5 mg/mL (18). In our study, there was a significant reduction in MIC with GAM after 8 h of incubation for each preparation, which was sustained until 16 h of incubation; this is in line with the findings from a previous study in which MgO was tested against *S. aureus* (19). Similar to our study, Aymen et al., (5) reported that MgO showed significant differences in MIC ($P < 0.05$) at the 20th hour of incubation compared with the 4th hour. According to Dehkordi et al. (20), CuO and MgO nanoparticles inhibit a wide variety of bacterial species. Siddiqi and Husen (19) reported that the antibacterial activity of ZnO nanoparticles was 1.8 ± 0.1 mg/mL. Additionally, the antibacterial activity of Fe_2O_3 has been shown to be 0.3125 ± 0.00 mg/mL (21, 22).

3.3. Efficacy of gel-based composites of antibiotics and nanoparticles

The antibacterial activity of ampicillin and florfenicol has been reported to increase when combined with silver nanoparticles against gram-positive and gram-negative bacteria. The enhancement in antimicrobial potential of antibiotics and nanoparticles stabilized in sodium alginate was also noted against bacterial pathogens (23, 24). They reported a significant reduction in the growth of bacteria with sodium alginate gel in combination with propolis. The current study was in line with the findings of (25), who reported a time-dependent increase in the antibacterial activity of sodium alginate-stabilized composites of antibiotics and MgO nanoparticles. The composites significantly reduced the MIC

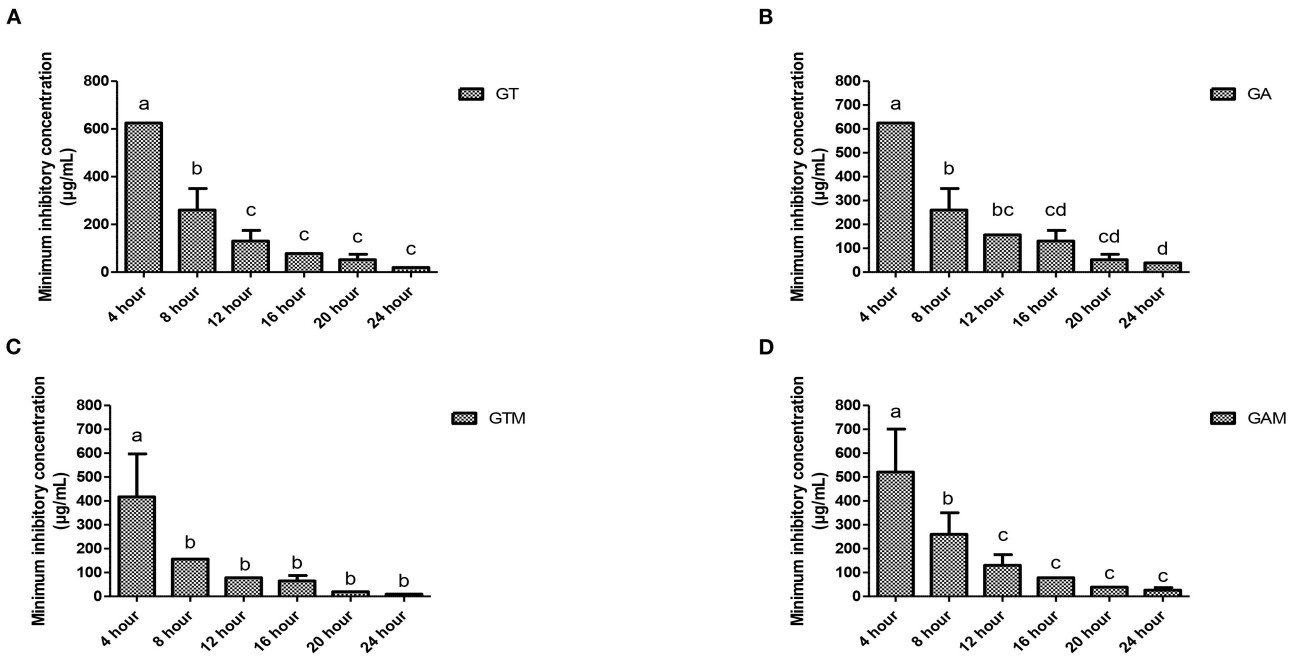

FIGURE 3
(A–D) Comparison of minimum inhibitory concentrations (µg/mL) among different incubation times for each preparation against *K. pneumoniae*. **(A)** GT, sodium alginate-stabilized tylosin. **(B)** GA, sodium alginate-stabilized ampicillin. **(C)** GTM, sodium alginate-stabilized tylosin and MgO nanoparticles. **(D)** GAM, sodium alginate-stabilized ampicillin and MgO nanoparticles.

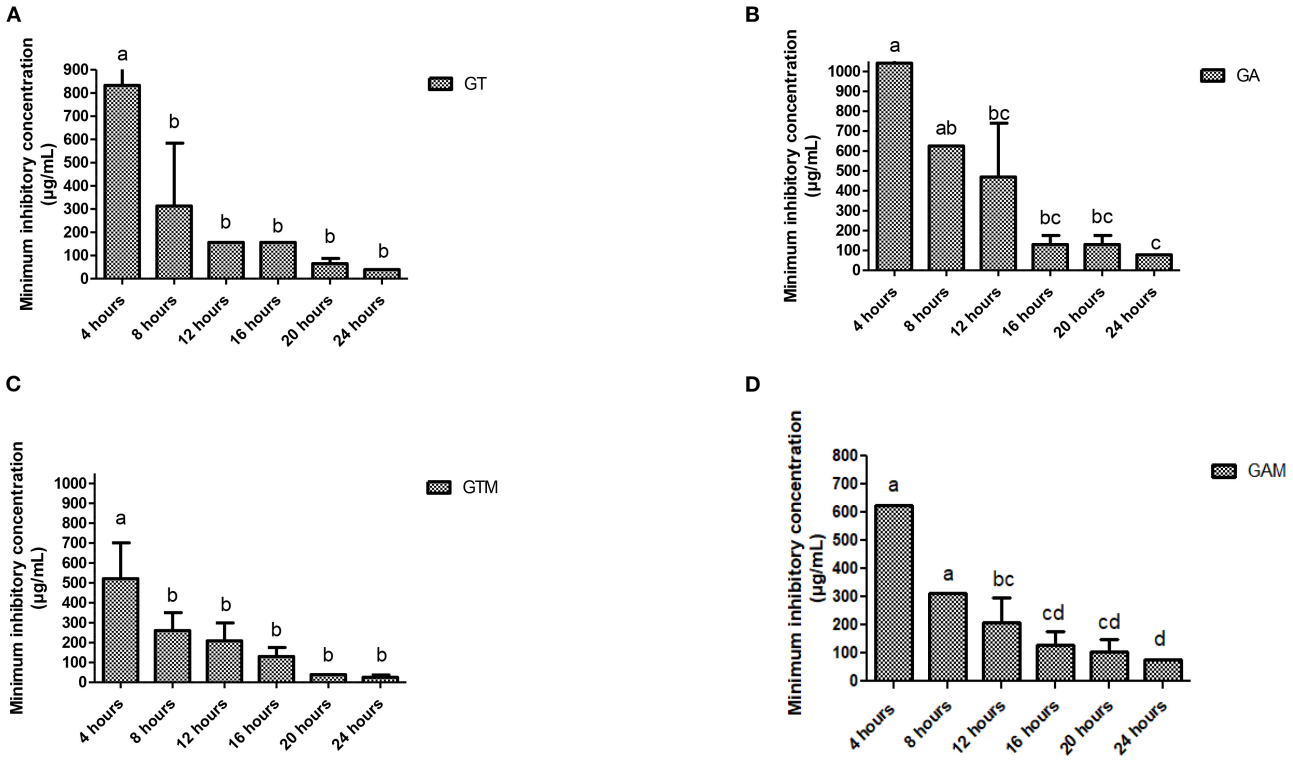

FIGURE 4
(A–D) Comparison of minimum inhibitory concentrations (µg/mL) among different incubation times for each preparation against *S. agalactiae*. **(A)** GT, sodium alginate-stabilized tylosin. **(B)** GA, sodium alginate-stabilized ampicillin. **(C)** GTM, sodium alginate-stabilized tylosin and MgO nanoparticles. **(D)** GAM, sodium alginate-stabilized ampicillin and MgO nanoparticles.

TABLE 6 Minimum inhibitory concentration (μg/mL) of *K. pneumoniae* and *S. agalactiae* against different gel-based preparations.

Nanoparticle and antibiotic	Bacteria	4 h	8 h	12 h	16 h	20 h	24 h
GT	*Streptococcus*	625.0 ± 0.0^a	260.4 ± 90.2^a	130.2 ± 45.1^a	78.13 ± 0.00^a	52.1 ± 22.6^a	19.53 ± 0.00^a
	Klebsiella	833 ± 361^a	313 ± 271^a	156.25 ± 0.0^a	156.25 ± 0.0^a	65.1 ± 22.6^a	39.06 ± 0.00^a
GA	*Streptococcus*	625.0 ± 0.0^a	260.4 ± 90.2^a	156.3 ± 0.0^a	130.2 ± 45.1^a	52.1 ± 22.6^a	39.06 ± 0.00^a
	Klebsiellae	1042 ± 361^a	625.0 ± 0.0^b	469 ± 271^a	130.2 ± 45.1^a	130.2 ± 45.1^b	78.13 ± 0.00^a
GTM	*Streptococcus*	417 ± 180^a	156.25 ± 0.0^a	78.13 ± 0.00^a	65.1 ± 22.6^a	19.53 ± 0.00^a	9.766 ± 0.000^a
	Klebsiella	521 ± 180^a	260.4 ± 90.2^a	208.3 ± 90.2^a	130.2 ± 45.1^a	39.06 ± 0.00^a	26.04 ± 11.28^a
GAM	*Streptococcus*	521 ± 180^a	260.4 ± 90.2^a	130.2 ± 45.1^a	78.13 ± 0.00^a	39.06 ± 0.00^a	26.04 ± 11.28^a
	Klebsiella	625.0 ± 0.0^a	312.5 ± 0.0^a	208.3 ± 90.2^a	130.2 ± 45.1^a	104.2 ± 45.1^a	78.13 ± 0.00^b

Different superscript letter between *S. agalactiae* and *K. pneumoniae* against each preparation at each incubation hour indicate a significant difference ($p < 0.05$). GT, tylosin stabilized in sodium alginate gel; GTM, tylosin and MgO nanoparticles stabilized in sodium alginate gel; GAM, ampicillin and MgO nanoparticles stabilized in gel.

after incubation for 8–12 h. By forming reactive oxygen species (ROS), magnesium oxide inactivates pathogens. However, the adsorption process and direct cell membrane penetration may also be effective means of inactivating bacteria (26).

One theory for the primary mechanism of bacterial lysis is the action of ROS produced by MgO nanoparticles. This idea is reinforced by the inclusion of ascorbic acid, a ROS scavenger, in the formula (27). Therefore, it can be inferred that these ROS play a significant role in the breakdown of microorganisms and that the bacterium continues to grow and reproduce normally in their absence. According to the literature, MgO nanoparticles can control ROS formation by adjusting their size, shape, surface charge, surface area, ion release, and alkalinity (28, 29). A review on MgO nanoparticles as an antibacterial (29) reads alkaline earth metallic oxides being covered with OH^- layers. As MgO solution is naturally alkaline and superoxide ions are chemically stable in alkalis. The concentrated layers of superoxides may be present on the surface of the MgO powder while equilibrium of superoxide with hydroperoxyl radical ($HO_2^{\cdot}$) is maintained as $O_2^- + H^+ \leftarrow HO_2^{\cdot}$. The $HO_2^{\cdot}$ is created when the MgO powder interacts with substances such as a bacterial cell would improve antibacterial activity. Additionally, the Mg^{2+} ions produced from the mitochondria might deactivate cellular enzymes or inhibit mitochondrial respiration, which raises the levels of ROS in the mitochondria (30). The increased ROS generation puts the cells under oxidative stress, oxidizing the components of their membranes and ultimately jeopardizing their integrity, resulting in necrosis. The mechanism underlying the ability of these nanoparticles to kill cells is not yet fully understood. To increase the antibacterial activity of nano-MgO, other polymeric additions have been used, such as chitosan (31), polylactic acid (32), or starch (33). However, the mechanism behind their synergistic effects is also not yet known. It is evident that the innovation of this work rests in its original synthesis method, its in-depth understanding of the quantity of ROS created, and the reduction of activity in the presence of a radical scavenger.

3.4. Toxicity analysis

The findings of the current study were in line with those of Ge et al. (26), who found that MgO nanoparticles were only slightly toxic to human cells at low concentrations (0.3 mg/mL). As a result of advanced strategies, antimicrobial activities have been improved and undesirable nanoparticle side effects have been reduced. The toxcicity of nanoparticles not only depend upon physical and chemical characteristics of nanoparticles but also on cell types being tested (34). This outcome is a result of the packaging of multiple antimicrobial agents into one nanoparticle, the coating of nanoparticles with biodegradable materials, and the engineering of target-specific nanoparticles for delivery to infection sites (35). However, further studies are required on the development of effective regimens against pathogens.

4. Materials and methods

4.1. Toxicity analysis using *Allium cepa*

For the telophase test, small onions were merged in solutions with different concentrations for 48 h. The test involved the following three groups: (1) a negative control group, in which only distilled water was used; (2) a positive control group, in which methyl methanesulfonate (MMS) (10 μg/mL) was used; and (3) a treatment group, in which different concentrations of MgO nanoparticles (M) were used. The onions were incubated in a dark room at room temperature for two different periods, 24 h and 48 h, and the concentrations used in the treatment groups were 1.25, 2.5, and 5 mg/mL. The root tips, after incubation, were fixed in 3:1 (v/v) ethanol:acetic acid, following which root tips were washed with distilled water and finally fixed in 70% ethanol. A total of eight root tips from every treatment were hydrolyzed with 1N HCl at 60°C for 10 min and then rinsed with water. Root tips were stained with Schiff's reagent at room temperature for 30 min. Apical tips were crushed on slides with acetic acid (45%) and examined

under a microscope using cover slips placed on the tips. To check for mitotic activity, more than 500 cells were counted for each treatment and the mitotic index and phase index were calculated as follows:

$$MI = \frac{Number\ of\ cells\ in\ division}{Number\ of\ total\ cells} x100$$

$$Phase\ index = \frac{Particular\ phase}{Number\ of\ cells\ in\ division} x100$$

4.1.1. Comet assay on *A. cepa* root tips

The treated and control groups were compared using the root tips of onion bulbs. Root tips were crushed with nuclear isolation buffer (600 μL, pH 7.5) to isolate nuclei. Following centrifugation (1,200 rpm, 4°C for 7 min), the nuclear suspension was placed on slides coated with 1% normal melting point agarose at 37°C. Slides were kept on ice for 5 min, following which coverslips were removed and slides were immersed in fresh buffer in an electrophoresis tank for 20 min at 300 mA. Staining of slides was carried out for 5 min with ethidium bromide. A fluorescence microscope was used to analyze the slides (three from each sample) for DNA damage, which was classified using a 0–4 qualitative scale, with a particular focus on head integrity and tail length (36). The following formula was used to calculate DNA damage in arbitrary units:

$$Arbitrary\ Unit = \sum_{i=0}^{4} Ni \times i \quad (1)$$

Ni = Number of cells
i = degree of damage (0–4).

4.2. The isolation and characterization of bacteria

The dairy farms located at the border of Lodhran District and Bahawalpur District consented to sample collection. A dairy farm greater than $n = 20$ animals in production was selected for sampling. Convenient sampling techniques were used to collect milk samples from 210 cattle. Milk samples presenting clinical or subclinical mastitis were collected using standard protocols. Screening for subclinical mastitis was carried out following the protocol described by Muhammad et al. (37). *S. agalactiae* and *K. pneumoniae* were identified using pooled information obtained through a series of biochemical tests described in Bergey's Manual of Determinative Bacteriology (38).

4.3. Antibiogram of bacteria

Biochemically characterized *S. agalactiae* and *K. pneumoniae* were tested for antibiotic susceptibility using the disc diffusion method described by Bauer et al. (39). Briefly, freshly grown bacteria (24 h) were spread over Mueller Hinton agar (10^8 CFU/mL), upon which 10 antibiotics were placed following the guidelines of the Clinical and Laboratory Standard Institute (40). ZOI were recorded following incubation at 37°C for 24 h. Means and standard deviations were measured to assess the antibacterial potential of *S. agalactiae* and *K. pneumoniae*.

4.4. Preparation and characterization of magnesium oxide nanoparticles

MgO nanoparticles were prepared using the method described by Parashar et al. (41) using $MgCl_2$ $0.6H_2O$, sodium dodecyl sulfate, and 2.5 M NaOH solution. The preparation protocol has been described in our previous study. XRD and SEM analysis was performed to determine the shape and dimensions of MgO nanoparticles. XRD analysis of nanoparticles was carried out using a powder diffractometer Rigaku D/max Ultima III operated at 40 kV and 0.130 A. Cu-Kα radiation was applied as a source emitting at a wavelength of 0.15406 nm, and a Quanta 250 SEM operating at 30 kV was used to obtain images of the nanoparticles (42).

4.5. Stabilization of MgO nanoparticles and antibiotics in sodium alginate

Sodium alginate solution (2% m/v) and gelatin solution (2% m/v) were made in water. These solutions were mixed at an 80:20 ratio (sodium alginate:gelatin) and homogenized for 2 h at 500 rpm with a mechanical stirrer to obtain sodium alginate gel (G). MgO nanoparticles (1.5 g) were added to 20 mL of sodium alginate gel and stirred for 4 h at 500 rpm to stabilize nanoparticles in the gel. Antibiotic (0.035 g) was dissolved in 20 mL of distilled water mixed with 20 mL of gel, and stirred for 4 h at 500 rpm. Then, the product was dried and ground to a fine powder. Nanoparticles and antibiotics were mixed with 20 mL of gel and stirred for 4 h at 500 rpm (23).

The following products/composites were prepared for further study: tylosin stabilized in sodium alginate gel (GT); ampicillin stabilized in sodium alginate gel (GA); MgO nanoparticles and tylosin simultaneously stabilized in sodium alginate gel (GTM); and MgO nanoparticles and ampicillin simultaneously stabilized in sodium alginate gel (GTA).

4.6. Resistance modulation assays

For this trial, *S. agalactiae* and *K. pneumoniae* were selected based on their minimum ZOI against more than three different classes of antibiotics. The selected isolates were used in well diffusion and broth microdilution assays to estimate resistance modulation and determine the least effective dose of gel-based preparations, respectively.

4.6.1. Agar-based assay

Fresh growth of *S. agalactiae* and *K. pneumoniae* was adjusted to 10^8 CFU/mL. Preparation of assembly for agar based was the same as described in the previous section "4.3. Antibiogram of bacteria". The modification done included making 6–8 mm wells

by well borer and followed by swabbing of bacteria. Lastly, the preparations were poured in the wells aseptically and the put to incubation for 24 hours at 37°C. Zone of inhibitions were measured following the incubation to find comparative antibacterial potential of the preparations.

Preparation of assembly for agar based was the same as described in the previous section of "antibiogram of bacteria," except wells of 6–8 mm were made by well borer for the pouring of preparations (39).

4.6.2. Broth microdilution method

The broth microdilution method was carried out to determine the MIC of gel-based preparations every 4 h during incubation. For this purpose, sterile Mueller Hinton broth (50 μL) was added to every well of a 96-well microtitration plate, following which a twofold dilution (starting from 10,000 μg/mL) of composites (sodium alginate-stabilized nanoparticles and sodium alginate-stabilized antibiotics) were added to all wells except for the positive control. Bacteria adjusted to 10^5 CFU/mL were aseptically poured into all 96-well titration plate except the negative control well. The positive control well consisted of broth and bacteria and the negative control only consisted of broth. The optical density (OD) value was obtained at a wavelength of 695 nm using an ELISA reader at 0, 4, 8, 12, 16, 20, and 24 h of incubation. The net optical density value was calculated by subtracting the OD value at 0 h of incubation from the OD value at the different time intervals. MICs of different preparations were compared among each other, and within each preparation at different time intervals were calculated and compared similar as described in previous studies (43, 44).

4.7. Statistical analysis

Univariate data were analyzed through descriptive statistics and parametric tests were applied for quantitative data from two or more than two groups using a t-test and ANOVA, respectively. For comparison of means, Tukey's test was applied as a *post hoc* test. All the data were analyzed with a 5% probability using SPSS version 22.

5. Conclusion

The current study found an increase in the prevalence and drug resistance of *K. pneumoniae* and *S. agalactiae*. Sodium alginate-stabilized nanoparticles and antibiotics showed significant antibacterial activity against both bacteria. The preparation with antibiotics and MgO nanoparticles stabilized in sodium alginate gel exhibited a higher antibacterial response. In addition, the study concluded that stabilized preparations have the highest activity at the earliest stages of incubation, suggesting their effectiveness in outbreaks. The nanoparticles used in this study were found to be safe based on their cytotoxicity trials compared with the positive control. To overcome the difficulties associated with treating milk-borne pathogens, we should shift from the conventional worldview to the nanoworld view. A combination of non-antibiotic and antibiotic sources is a desirable candidate to replace, refine, and reduce the use of antibiotics to counter the spread of resistance. Further studies on various molecular aspects of responses of pathogens and the formulation of effective therapeutic regimens are required.

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 Cholistan University of Veterinary and Animal Sciences Bahawalpur Pakistan Ethical review committee, No. ORIC No. 211. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

Conceptualization and project administration: AIA, AMn, HM, and KA. Methodology: AMn, AIA, AS, MA, and SRK. Software: KA, CM, KN, MM, AM, and AIA. Formal analysis and supervision: AIA, MS, IA, and HM. Investigation and data curation: AIA and AMn. Resources: MS, IA, and AIA. Writing—original draft preparation: AMn, MM, and AIA. Writing—review and editing: AIA, AS, IA, MS, HM, SRK, AMn, CM, and KN. All authors have read and agreed the published 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.

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Botanicals: A promising approach for controlling cecal coccidiosis in poultry

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Avian species have long struggled with the problem of coccidiosis, a disease that affects various parts of the intestine, including the anterior gut, midgut, and hindgut. Among different types of coccidiosis, cecal coccidiosis is particularly dangerous to avian species. Chickens and turkeys are commercial flocks; thus, their parasites have remained critical due to their economic importance. High rates of mortality and morbidity are observed in both chickens and turkeys due to cecal coccidiosis. Coccidiostats and coccidiocidal chemicals have traditionally been added to feed and water to control coccidiosis. However, after the EU banned their use because of issues of resistance and public health, alternative methods are being explored. Vaccines are also being used, but their efficacy and cost-effectiveness remain as challenges. Researchers are attempting to find alternatives, and among the alternatives, botanicals are a promising choice. Botanicals contain multiple active compounds such as phenolics, saponins, terpenes, sulfur compounds, etc., which can kill sporozoites and oocysts and stop the replication of *Eimeria.* These botanicals are primarily used as anticoccidials due to their antioxidant and immunomodulatory activities. Because of the medicinal properties of botanicals, some commercial products have also been developed. However, further research is needed to confirm their pharmacological effects, mechanisms of action, and methods of concentrated preparation. In this review, an attempt has been made to summarize the plants that have the potential to act as anticoccidials and to explain the mode of action of different compounds found within them.

KEYWORDS

cecal coccidiosis, botanicals, herbal extracts, *Eimeria*, *E. tenella*, poultry, immune response, oxidative stress

Introduction

Coccidiosis is a widespread parasitic disease caused by multiple species of protozoan parasites (1, 2). *Eimeria* spp. are among the most important coccidian parasites, with hundreds of species infecting vertebrates (3, 4). Avian coccidiosis is an absolute intestinal disease characterized by bloody or mucoid diarrhea (5–8). *Eimeria* species are host- and site-specific, and this specificity is below the organ level, i.e., they infect only the intestine, and within the intestine, they have their reserved regions (9, 10). Various species of *Eimeria* show symptoms of the disease from the anterior portion of the intestine to the hindgut, depending on their specified predilection sites (11, 12). Cecal coccidiosis is the most dangerous disease among all types of coccidiosis in avian species (13–15). A high and rapid onset of mortality was only observed in cecal coccidiosis (16, 17). The main causative agent for cecal coccidiosis in broiler chickens is *Eimeria tenella*, which resides in the cecum and causes hemorrhages in it, leading to bloody diarrhea (18–20). Other agents that cause cecal coccidiosis are

mentioned in Table 1. This pressing problem highlights the need for scientists to create measures to control coccidiosis, particularly cecal coccidiosis (25, 26).

Currently, multiple drugs are being used in poultry on a daily basis to control coccidiosis (27–29). Due to the acute nature of cecal coccidiosis, preventive measures for controlling this disease are the focus of attention (28–30). Multiple coccidiocidal and coccidiostat drugs are being given in the feed to prevent coccidiosis (31, 32). Anticoccidial drugs target different stages of the Eimeria life cycle, aiming to arrest the parasite at that stage and ultimately control coccidiosis (33).

While chemical anticoccidial drugs have been effective in fighting against coccidiosis, the multiple problems related to this disease have raised doubts about their continued use in the future (34–36).

The primary issue that is being faced with the use of chemical anticoccidials is drug resistance (37–39). Resistance is the ability of the pathogen to escape from the medicine (40, 41). Anticoccidial resistance is a major problem for commercial farmers, as it can result in the wastage of resources and capital on disease control (42, 43). *Eimeria* are resistant to multiple drugs because of multiple mechanisms (21, 27, 44–47). They have developed genetic modifications (48, 49), altered their metabolism, cell membrane permeability, transport channels, and many other ways to escape the drug interaction, leading to the development of resistance (45, 50, 51). Anticoccidial drugs are becoming less effective in treating coccidiosis due to the emergence of increasingly resistant strains of *Eimeria* (52–55).

Resistance is not the only issue with anticoccidial drugs. Multiple scientists also have reported public health issues related to anticoccidial drugs (38, 56, 57). The anticoccidial drug metabolites escape the circulatory system and accumulate in various body parts (58–62). These secondary metabolites are transferred to consumers when they consume meat from animals that have been treated with anticoccidial drugs (58, 63). These drug residues cause several problems and may even be carcinogenic or teratogenic (64–66). Anticoccidial drug residues may cause heart, liver, and kidney failure, leading to death and chronic problems in consumers (67–69). Due to these problems, the European Union has banned the routine use of chemical coccidiostats in feed and allowed a limited amount of use only with the veterinarian's prescription (70–72). These issues and the economics of chemical anticoccidial drugs are forcing researchers to investigate alternatives (36, 73).

Commercially, vaccines are one of the most commonly used alternatives for treating cecal coccidiosis (74–77). Anticoccidial vaccines have been developed to prevent various types of coccidiosis (77). Vaccines are being developed to target various stages of the *Eimeria* and are effective against multiple species of the parasite (56, 78, 79). Anticoccidial vaccines usually use killed parasites or particles of pathogens (80–82). Multiple vaccines, such as Immucox®, Livacox,® Coccivac®, Hatchpack Cocci-III®, Paracox®, etc., are being used in routine farming (17, 56, 83). Anticoccidial vaccines can provide immunity, but some issues limit their use (84–87). Vaccine failure is the primary issue observed with anticoccidial vaccines (30, 85, 88). Vaccine failure results in vaccine-induced coccidiosis (89). Moreover, anticoccidial vaccines provide only temporary protection and require frequent repetition, especially in breeders and layer birds (90, 91). The high cost of anticoccidial vaccines limits their widespread use, making them accessible to only breeding and grandparent flocks of chickens (78). These issues necessitate finding a proper alternative to combat cecal coccidiosis.

Several strategies are being explored for the prevention and control of cecal coccidiosis, including the use of organic acids, amino acids, their derivatives, and so on. Botanical substances are also among these proposed alternatives for controlling coccidiosis, as they are gaining attention from scientists for their antioxidant, anti-inflammatory, immunomodulatory, and anti-infectious properties (92–94). Recently, multiple reviews that summarize botanical products that have been proven to have anticoccidial effects have been published (95–101). These reviews have provided valuable insights into how plants can effectively control various forms of coccidiosis. However, there is a need to further investigate the mechanism of action and pharmacological properties of these botanicals. In this review, we have summarized the effective agents of botanicals and their modes of action, as well as the properties that make them beneficial for use against cecal coccidiosis.

Methodology

This review used Google Scholar (www.scholar.google.com) as the primary search engine. More websites, i.e., ResearchGate (www.researchgate.com) and ScienceDirect (https://www.sciencedirect.com/), and keywords "Cecal coccidiosis;" "*Eimeria*

TABLE 1 The species responsible for cecal coccidiosis in avian species.

Parasite	Host	Predilection sites	Pathogenicity	References
E. tenella	Chicken	Absolute cecal	+++	(21, 22)
E. brunetti	Chicken	Ceca, Rectum	++	(22)
E. necatrix	Chicken	Ceca, Jejunum, and ileum	+++	(22)
E. gallopavonis	Turkey	Ceca, Posterior illum and rectum	+	(23)
E. adenoeides	Turkey	Absolute cecal	+++	(24)
E. meleagridis	Turkey	Ceca, Small intestine	+	(24)
E. meleagrimitis	Turkey	Ceca, Small intestine, rectum	+	(24)

+: Less pathogenic; ++: moderate pathogenic; +++: highly pathogenic.

"*tenella*" "*E. tenella;*" "Herbal control of *E. tenella;*" "Use of essential oils for cecal coccidiosis;" "Use of plant extracts for cecal coccidiosis;" "Botanicals for the control of cecal coccidiosis," "Plants for the control of cecal coccidiosis" were used. The review articles/secondary data were used as a source for the original articles. The data were not quantified, and the statistical comparison was also not performed (102). Table 2 presents the qualitative effects.

TABLE 2 Botanical compounds, their mechanisms of action, and their use for cecal coccidiosis.

Sr. No	Botanical compound	Mechanism of action	Representative plants/ Products	Anticoccidial activities	References
1.	Saponins	They attach to the sterol molecules present in the cell membrane. Stop the metabolism of *Eimeria*. Immunomodulatory and antioxidant activities	Peptasan® (*Acacia concinna*)	Improvement of weight gain and feed intake. Reduction in fecal oocyst count and lesion score.	(101)
			Y. schidigera	Improvement in immunomodulatory response and changes in the cecal structure	(103)
			Quillaja saponaria, Y. schidigera	Lesion scores reduction and improvement in carcass parameters	(104)
			NorponinXO2 ® (*Y. schidigera* and *Trigonella foenum-graecum*)	The improvement in weight gain and lesion scores	(105)
2.	Tannins	They can penetrate the oocyst wall of *Eimeria*. Deteriorate the cytoplasm and lead to the inactivation of sporulation-inducing enzymes. Potent antioxidants	*Emblica officinalis*	Lesion scores were reduced, and improved weight gain of carcass weight and immune-boosting effects were observed	(106)
			Tannic acid extract product (*Rhus chinensis* gallnuts)	Improved feed conversion ratio, weight gain, and reduced coccidiosis parameters	(107)
			Pinus radiata	Showed *in vitro* and *in vivo* anticoccidial activities	(108, 109)
3.	Flavonoids	Prevention of development of *Eimeria* and reduction in the multiplication of merozoites. Strong antioxidants	*Musa paradisiaca*	Reduce the severity of the infection and coccidial parameters	(110)
			Moringa oleifera	Lesion scores were reduced, and improved body parameters and immune-boosting effects were observed	(111)
			Olea europeaea	The sporulation was infected, and the anticoccidial parameters were reduced in broiler chicks	(111, 112)
			Ficus racemosa, Syzygium cumini, Cassia fistula	Antioxidant activities killed the oocyst of *E. tenella*	
4.	Essential oils	Interact membrane sterols. Sporicidal activities by penetration into cell wall Antioxidant and immunomodulatory activities	*M. fragrans*	Stopped sporulation of *E. tenella,* improved body weight gain of organ ratios, and had anticoccidial effects in *in vivo* experiments	(113, 114)
			P. lentiscus	Reduction in lesion scores and destruction of oocysts	(115)
			Combination of *O. vulgare* and *Citrus* sp.	Decreased lesion of coccidiosis and improved the body characteristics, improved antibody response.	(116)
			OregoStim® (Based on 5% *Origanum vulgare* essential oil)	Signs of coccidiosis are reduced	(117)
5.	Sulfur compounds	Antioxidants, anti-inflammatory, interact with the cell membrane to destroy cell structure and immunostimulatory agents	Garlicon 40 ® (*Allium* spp.)	*E. acervulina* sporozoites were killed. Antibody response was improved	(118)
			Commercial Herbal Formula (*A. sativum, Satureja hortensis, Chelidonium majus*)	Improved carcass characteristics and decreased oocyst output	(119)
			A. sativum and *Z. officinale*	Improved body weight and feed intake reduced the coccidial signs and symptoms	(120)

Pathology and methods for controlling cecal coccidiosis

It is necessary to identify the points at which coccidiosis can be effectively controlled before reviewing the plants and plant products used to control cecal coccidiosis. Cecal coccidiosis starts with the ingestion of sporulated oocysts of *Eimeria,* which then release sporozoites in the stomach (21, 121, 122). Eimeria releases sporozoites. These sporozoites invade the bird's cecum and penetrate the cecal epithelium (17, 123–126). These sporozoites enter epithelial cells and undergo asexual division named merogony/schizogony, resulting in the formation of schizonts inside the cell (127). Cells containing mature schizonts of *Eimeria* then rupture and release the merozoites (128, 129). These merozoites of *Eimeria* undergo multiple cycles of schizogony, and then, due to unknown reasons, they mature into male and female gametes (micro- and macrogametes) (130, 131). These gametes fuse to form zygotes that develop into unsporulated oocysts, which are then shed in the bird's feces (132, 133).

The pathogenesis of coccidiosis symptoms arises when the merozoites start infecting the cell and causing the destruction of the epithelium of the cecum (134–136). The epithelium destruction due to schizogony and merozoite invasion induces an immune response (21, 137, 138). This immune response and epithelial destruction result in oxidative stress (139, 140). The reactive oxygen species are produced, and they interact with nearby cells (141–143). These reactive oxygen species extend the damage of coccidiosis to the blood supply to the epithelium, resulting in a blood eruption (144). The repeated cycles of merogony cause extensive damage, leading to bloody diarrhea and death (145). Identifying the different stages of this cycle and their corresponding pathologies provide some points where coccidiosis can be controlled (Figure 1).

First, cecal coccidiosis can be controlled by reducing the intake of sporulated oocysts (21, 146). These sporocysts can be controlled by *in vitro* sporicidal substances, which may kill the sporocysts or stop the sporulation of oocysts (147). The second method of controlling cecal coccidiosis is by reducing the ingestion of sporozoite/merozoite inside the cell (4, 148). Another important method of controlling cecal coccidiosis is reducing the damage caused by *Eimeria.* This can be achieved by stopping oxidative stress and inflammation in the cells and supporting the immune system in its efforts to eliminate *Eimeria* (149, 150). In addition, the number of merogony cycles was reduced and the parasite was forced to enter the sexual phase of the cycle (151, 152).

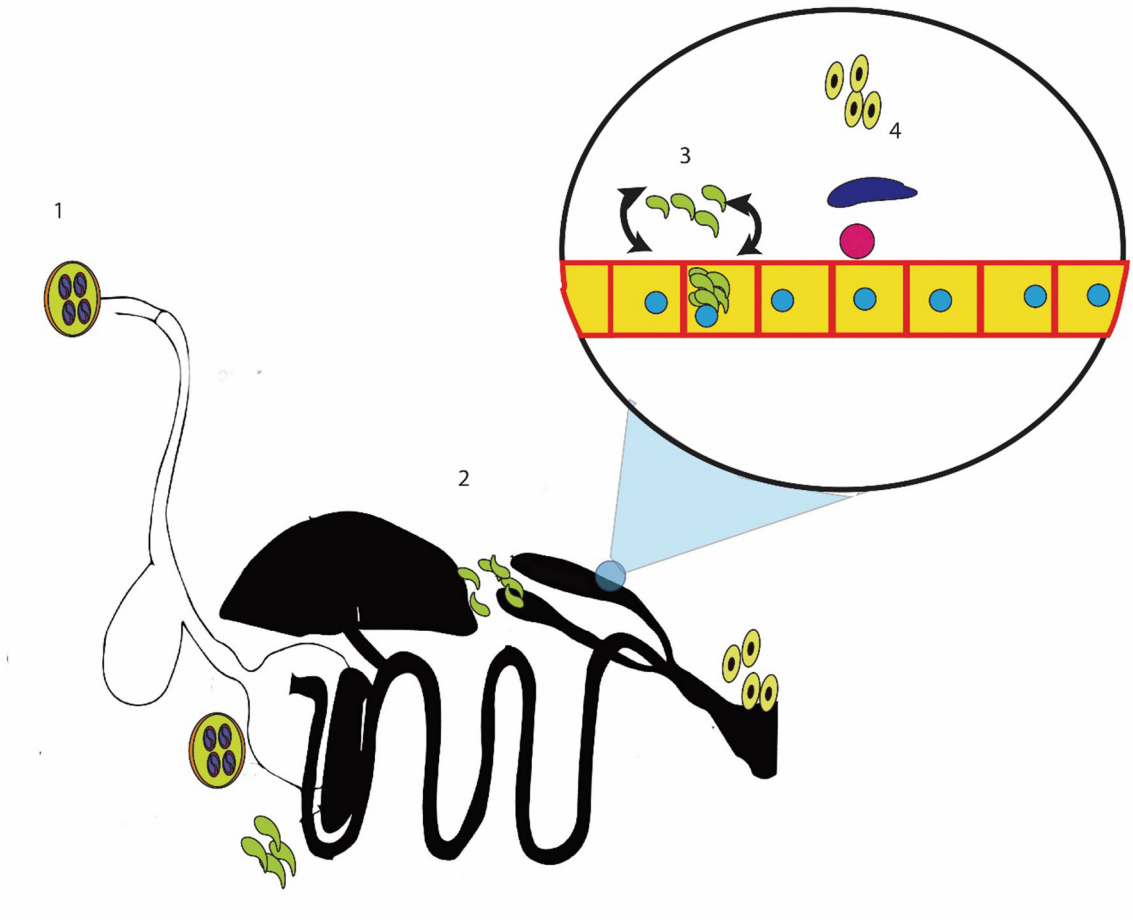

FIGURE 1
Methods for controlling cecal coccidiosis; 1: killing the sporocyst or reducing the intake of sporocyst; 2: stopping the entry of sporozoites into the cecal epithelium; 3: killing the sporozoites, merozoites, or schizont-containing cells; and 4: controlling the reproductive stage.

Multiple researchers have explored the use of herbal remedies for controlling cecal coccidiosis, and a few of these remedies have been commercially marketed (119, 153). The active compounds of the plant determine their efficacy in controlling the disease. The active compounds, modes of action, and plants that contain these active ingredients are given in the following sections.

Botanical compounds for controlling cecal coccidiosis

Saponins

Saponins are a group of phytochemicals that are commonly found in many plants (154, 155). They derive their name from their soapy nature, as they can create a foamy texture in aqueous solutions (156–158). Saponins are well known for their antimicrobial, antioxidant, and anticoccidial properties (159–164).

Saponins in botanicals perforate the cell membrane because they interact with cholesterol (158, 165). Cholesterol is the main functional and structural unit of the cell membrane of *Eimeria* (166, 167). In this way, saponins can kill the sporozoites or merozoites of *Eimeria* (55, 168–170). Saponins can directly interact with the sporozoite and stop reproduction (171). Saponins have a high antioxidant capacity, i.e., they can control reactive oxygen species and reduce the pathologies associated with oxidative stress, which is highly present in cecal coccidiosis (172–175).

Botanical saponins have an interesting ability to act as an immune booster, thereby making them suitable candidates for use as vaccine adjuvants (176–178). Multiple researchers have reported that plants containing high amounts of saponins can boost the immune system by affecting the immune organ maturation and increasing the antibody levels in the body, providing better defense against cecal coccidiosis (164, 179–182). Plants containing saponins also function as astringents, i.e., they reduce surface tension inside the body and help nutrients enter the cells (183, 184). Due to these activities, multiple saponin-containing plants have been used to control coccidiosis (101, 105, 119, 185–190).

Tannins

Tannins, or tannic acids, are a ubiquitous class of molecules that occur naturally in plants as defense compounds (191–193). Tannins are classified as phenolic compounds (Figure 2), and they contain various functional groups (194–196). They act as pesticides in plants, protecting them from invading pests and microbes (197, 198). They help regulate plant growth by protecting them from infectious agents (199–203). Tannins can coagulate bacterial cell walls by affecting their peptides (196, 204, 205). Peptide units also make the oocyst wall of *Eimeria* (206, 207); thus, they perforate the oocyst wall of *Eimeria* oocysts and denature them (188). Tannins can also protect the epithelium cells from damage and maintain the integrity of nucleic acid during microbial infections (208–210). Tannins also show immunomodulatory activities and potentiate the immune response, helping control infections (211–214). Tannins are also antioxidants and reduce oxidative stress, a condition commonly associated with numerous diseases, especially cecal coccidiosis (21, 215). Numerous tanniferous plants have been used to control coccidiosis (30, 216–222). The efficacy of these plants in controlling cecal coccidiosis can be attributed to their immunomodulatory, antioxidant, and direct anticoccidial activities (187, 222–225).

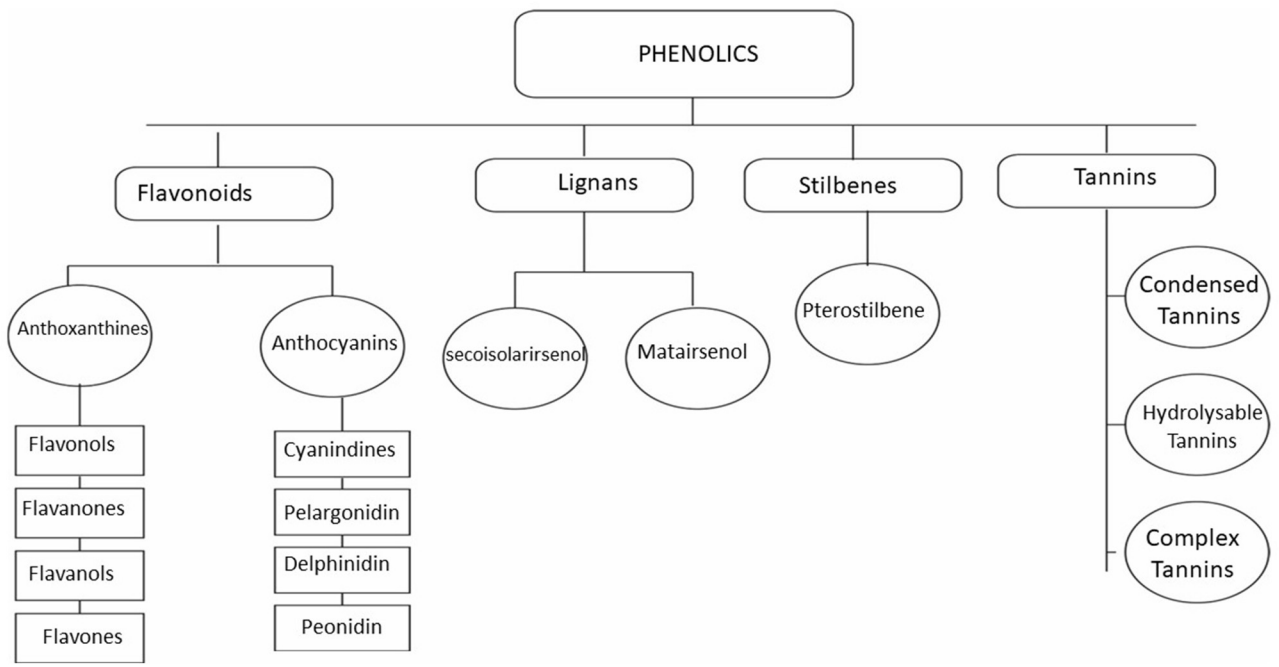

FIGURE 2
Classification of phenolics.

Flavonoids

Flavonoids are a diverse and broad class of plant phenolic compounds (Figure 2) (226–228) and constitute one of the most prevalent phenolic compounds (229–232). They are further divided into flavonols, flavanols, flavanones, flavones, etc., as described in Figure 2 (233–235). They have multiple modes of action depending on the type and class of flavonoids (236–238). Flavonoids have the potential to control coccidiosis due to their well-known oxidative stress reduction activities (239–241). Flavonoids can also penetrate the cell membranes and cause sporozoite and oocyst death (171, 242). Multiple plants, such as, *Moringa oliefera* and *Syzygium aromaticum*, with active flavonoids have anticoccidial activity (170, 188, 240, 243–245).

Essential oils

Essential oils are volatile, short-chain, lipophilic portions of plants that can be extracted using hydro or steam distillation techniques (246–249). They usually contain terpenes, terpene derivatives, aldehydes, and other compounds (250–252). Essential oils are highly active antioxidants and immunomodulators (253–257). These oils can kill the oocyst of *Eimeria* (15, 258–260) and stop sporulation by penetrating the walls of the *Eimeria* oocyst (147, 261–263). Essential oils can also help reduce coccidial signs and symptoms because of their direct and indirect anticoccidial activities (264). Essential oils have been widely used to control coccidiosis, especially the cecal kind (15, 116, 117, 259, 262, 265–272).

Vitamins and minerals

Vitamins and minerals are the crucial micronutrients needed by the body to regulate multiple functions and the metabolism of the body (273, 274). Vitamins and minerals are naturally present in plants. They play an important role in many metabolic reactions and help the body maintain growth. Similarly, multiple minerals act as co-factors of the enzymes and have been used to control coccidiosis (259). Vitamins and minerals act as antioxidants and immune stimulators, helping control cecal coccidiosis (275–277). Vitamins and minerals, i.e., vitamin E, selenium (278, 279), vitamin K (280), vitamin A, vitamin D (281, 282), etc., have been shown to be effective in reducing the signs and symptoms of coccidiosis in various multiple experiments.

Sulfur compounds

Sulfur compounds are usually present in the garlic family (*Allium spp.*) and cannabis (283, 284). Allicin, diallyl disulfide, propyl thiosulfinate oxide, allyl methyl thiosulfate, etc., are commonly found sulfur compounds in plants (285, 286). They have been shown to have high antimicrobial, antioxidant, and anti-aging properties in multiple experiments (287, 288). They have shown multiple bioactivities in many experiments. Sulfur compounds can potentially kill *Eimeria* by destroying the sporozoites (21). Garlic in multiple forms, i.e., essential oils, extracts, and powders, has been used to treat coccidiosis, and it has shown anticoccidial activity due to these compounds (89, 120, 124, 217, 289, 290).

Pharmacological interactions of botanicals

In a single plant, hundreds of compounds may show multiple activities (291, 292). In multiple experiments, more than one plant was used to control cecal coccidiosis (119, 120, 293). When we added multiple bioactive compounds, there was a possibility of multiple pharmacological interactions occurring among them (294). These interactions may be synergistic, additive, antagonistic, etc. Thus, it is necessary to determine whether these plant products exhibit synergistic or additive effects or if they demonstrate antagonistic properties (295–297). Several experiments have demonstrated that botanical compounds can exhibit additive and synergistic effects against caecal coccidiosis (56, 244, 298). Due to the presence of multiple compounds, plants can employ multiple mechanisms to control *Eimeria*, thereby reducing the likelihood of drug resistance (27, 299). Although reports suggest that interactions exhibit synergistic or additive effects, further research is needed to explore their potential antagonistic effects.

Conclusion

Studies have shown that plants contain multiple types of botanical compounds that differ in their quantities and ratios. These compounds have demonstrated antioxidant and anticoccidial properties against avian caecal coccidiosis. Phenolics, saponins, essential oils (including terpenes and derivatives), sulfur compounds, etc., have been proven to exhibit anticoccidial effects through diverse mechanisms of action. Moreover, it should be noted that these plant compounds may possess antinutritional properties, which can lead to reduced feed intake, growth inhibition, and adverse effects on body growth. However, studies on their potential toxicity are lacking with respect to coccidiosis. Before considering their therapeutic use, it is imperative to investigate their toxicological profiles. Furthermore, further research is needed to evaluate their pharmacological interactions within the body.

Author contributions

ZS wrote the article and created the illustrations. KA managed the references and edited the manuscript. 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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Recent trends in the use of bacteriophages as replacement of antimicrobials against food-animal pathogens

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A major public health impact is associated with foodborne illnesses around the globe. Additionally, bacteria are becoming more resistant to antibiotics, which pose a global threat. Currently, many scientific efforts have been made to develop and implement new technologies to combat bacteria considering the increasing emergence of multidrug-resistant bacteria. In recent years, there has been considerable interest in using phages as biocontrol agents for foodborne pathogens in animals used for food production and in food products themselves. Foodborne outbreaks persist, globally, in many foods, some of which lack adequate methods to control any pathogenic contamination (like fresh produce). This interest may be attributed both to consumers' desire for more natural food and to the fact that foodborne outbreaks continue to occur in many foods. Poultry is the most common animal to be treated with phage therapy to control foodborne pathogens. A large number of foodborne illnesses worldwide are caused by *Salmonella* spp. and *Campylobacter*, which are found in poultry and egg products. Conventional bacteriophage-based therapy can prevent and control humans and animals from various infectious diseases. In this context, describing bacteriophage therapy based on bacterial cells may offer a breakthrough for treating bacterial infections. Large-scale production of pheasants may be economically challenging to meet the needs of the poultry market. It is also possible to produce bacteriophage therapy on a large scale at a reduced cost. Recently, they have provided an ideal platform for designing and producing immune-inducing phages. Emerging foodborne pathogens will likely be targeted by new phage products in the future. In this review article, we will mainly focus on the Bacteriophages (phages) that have been proposed as an alternative strategy to antibiotics for food animal pathogens and their use for public health and food safety.

KEYWORDS

antimicrobials resistance, food security, bacteriophages, public health, food-animal pathogens, quality of life, pathogenesis

1. Introduction

Food safety is always a topmost priority in terms of public health and becomes more significant while considering animal-origin protein including eggs and meat. Many food-borne pathogens can cause illness in poultry birds starting from the initial hatch to the meal preparations (1). Antibiotics are used in the livestock and poultry industry for a century to treat

diseases, parasites, and animals, promote growth at subtherapeutic doses in animal feed, and improve animal products (2). Antibiotics are highly efficient against bacterial infections, saving millions of lives and drastically reducing mortality rates. However, multidrug-resistant bacteria (MDR), extensively drug-resistant bacteria (XDR), and even pan-resistant bacteria (PDR) have evolved because of antibiotic overuse, abuse, and misuse. Antibiotics are currently ineffective against infections caused by drug-resistant bacteria (3). As the antibiotic drug discovery pipeline is failing, finding new drugs is becoming increasingly critical. Consumer awareness about preservation in a chemical way in the food and on processing surfaces has also led to an increase in interest in natural antimicrobial agents (4). They can boom in a wide variety as pathogens emerge all through treatment, have the simplest minor results on regular flora, are similarly powerful against antibiotic-resistant bacteria, are without problems detected, break bacterial biofilms, and are often non-toxic (5).

A variety of pathogens have been found in soil, ground and surface water, food (e.g., sauerkraut and wine), sewage, and sludge (6). In addition to humans and animals, they have also been isolated from feces, urine, saliva, spit, rumens, and serum (7). With their bacterial hosts, phages are part of the intestinal flora because of their ability to penetrate different organs and tissues (8). The trophic effect of these bacteria limits bacterial populations in aquatic ecosystems by 10 to 80% (9). In terms of their morphology and size, bacteriophages are classified into different families. The majority of them have tails, but they also have filaments and pleomorphic (10). There are two basic components of a phage virion: nucleic acid (dual- or single-stranded RNA or DNA) and a protein envelope. Lipids are sometimes part of the envelope or of the lipid wall (11). In the course of bacteriophages' occurrence, viability, and storage, various external physical and chemical factors, including temperature, acidity, salinity, and ions, play a significant role. Damage to a phage's structural elements, loss of lipids, and/or changes in DNA structure can inactivate a phage (12).

In recent years, there has been a lot of interest in using phages to control pathogens (13). Studies have examined the use of phages as indicators of fecal contamination and as antimicrobials (14). They are currently one of the promising antibiotic alternatives with the most potential because of their ability to effectively combat bacterial infections. Phages are a new alternative therapy under the "one health" approach that can be used to control bacteria in plants, animals, food, and humans and they are very common in all natural environments and play an important role in bacterial evolution (15). Phages can be categorized into two types, namely virulent (exclusively undergo the lytic cycle) and temperate (are able to endure the lysogenic cycle). Phages are very specific as each type generally attacks different bacterial species. Virulent phages enter the bacterial cell, replicate using the host machinery and finally lyse the host cell, leading to the disintegration of the bacteria (16). Viral phages replicate much faster than host bacteria when they infect a bacterium. Within 30–40 min, the entire cycle can be completed. Phages are parasites that propagate by taking advantage of their hosts, which can be affected by temperature, nutrient levels, and various environmental factors (17). The purpose of this review is to discuss various issues that are related to antibiotic resistance as well as the use of bacteriophages as a cure against antimicrobial resistance against food-animal pathogens.

2. Recent antimicrobial resistance status in food animal pathogens

Antimicrobial resistance is a serious public health issue that poses a grave threat to the spread of incurable infections. Many emerging infectious diseases around the world are now caused by antibiotic-resistant bacteria (18). Although antimicrobial resistance is a natural result of how microbes evolve, human production abuse of antimicrobial drugs has accelerated resistance to transfer in a variety of pathogenic and microbiome pathogens (19). Even medical and public health professionals understand the significance of antimicrobial use in agriculture as a component of antimicrobial resistance (20). It is well known that antibiotic resistance is one of the most significant issues affecting bacterial pathogens around the world (21). Infections caused by Gram-positive and Gram-negative bacteria have developed multidrug resistance patterns that make conventional antimicrobials ineffective or even untreatable. There is a lack of early identification of causative microorganisms and antimicrobial susceptibility patterns in many healthcare settings, so wide-spectrum antibiotics are routinely and largely unnecessarily administered to patients with bacteremia and other serious infections (21). As a result of poor infection control practices and escalating resistance rates, bacteria can spread rapidly from patient to patient and into the environment. A practical antimicrobial stewardship program in hospitals can be developed when updated epidemiological data are available on antimicrobial resistance in frequently encountered bacterial pathogens (21). There is an alarming rise in multidrug-resistant bacteria and resistance to common antimicrobial therapies. Bacterial infections and their associated diseases pose a challenge, and since there are currently fewer effective drugs, fewer effective prevention measures, and only a few new antibiotics in clinical trials, novel treatment options, and alternative antimicrobial therapies are required (22).

The intensity of antimicrobial use in agriculture is insufficient and dwarfs the scale of clinical use and misuse, even though clinical concerns are not trivial. Additionally, the growing issue of drug-resistant food-borne infections is rarely linked to the production of food animals, which reduces the efficacy of public health initiatives to prevent food-borne illness (23). Most signification, rather than conceptualizing issues ecologically in terms of resistance gene reservoirs that may spread throughout the microbial ecosystem, the problem is frequently conceptualized in terms of resistance to specific antimicrobials in clinically important pathogens (24). As agricultural antibiotic use changed, researchers tracked the prevalence of antimicrobial resistance in ecological studies. Research on cross-sectional groups is considered to be cross-sectional if it focuses on specific groups that are in close contact with antimicrobial-treated food animals or farming families (e.g., farmers) (25). Bacteria that are resistant to antibiotics can be found in the home, animal waste, and the environment. Thirdly, researchers examined antibiotic resistance in bacteria isolated from conventional producers and those who did not use antibiotics (26).

3. Mechanism of bacteriophages

There are billions of viruses on the planet. The number of viruses in the universe is estimated to be 1,031, which is far greater than the

number of stars (27). As part of the viral population, bacteria are infected and devoured by bacteriophages, or phages. Phyto phages are unique in their potential antibacterial properties (28). There are many phages in every ecosystem, and they are considered the most abundant and diverse organisms on the planet (29). In addition to hunting for a specific bacterium species, phage particles can hunt for a subset of the same species. Phages replicate inside bacteria after they infect them. Phages multiply exponentially after infecting a bacterial cell, taking advantage of the bacteria's protein synthesis and energy-producing machinery. The protein synthesis and energy-producing machinery of bacteria are used by phages to multiply exponentially after infecting a bacterial cell. Nevertheless, lysis and lysogenesis are two ways of propagating bacteria. In the case of lytic phages, the bacterial cell is lysed before the virus is released (30). A lysogenic phage integrates its genome into the bacterium that replicates it and acquires new characteristics for the bacteria. It shows that phage is important for treating both local and systemic infections in humans (31). It is worth noting that the development of antibiotic resistance in bacteria has not kept the development of different stages of resistance in bacteria, instead, the fact that phage resistance may be a significant issue in bacteria. More therapy of phage has been shown to be safe and relatively free of side effects (32). In antimicrobial phage therapy, bacteriophages are used because they can recognize, adsorb, multiply, and cause lysis of bacteria only inside their cells (33). In its most basic form, phage therapy refers to the use of infectious bacteria-specific viruses to inhibit the growth of pathogenic bacteria (34). In most cases, bacteriophage may be effective against Gram-positive and negative bacteria that are resistant to antibiotics (35) (Figure 1).

3.1. Lytic phage

For the lytic cycle to begin, the phage must attach to the bacterium using a protein (13). The genetic material of the bacteriophage is incorporated into the bacterial host cell after the attachment of the viral particle. Phages use bacterial metabolic machinery to make copies of DNA or RNA from their genetic material upon entry (36). DNA viruses copy themselves directly into messenger RNA (mRNA) molecules that are used to control the host's ribosomes. Retroviruses (RNA viruses) are transcribed into DNA by reverse transcriptase, which then follows the pathway followed by the DNA virus to transcribe viral RNA. In the later stages of the translation, the newly translated proteins are assembled to form the capsid and tail of the phages, which are released from the host cell. Infecting and multiplying new host cells are the next steps for the newly formed particles. During phage replication, the host chromosome is sometimes packed into the capsid instead of the phage genome, representing an example of horizontal gene transfer (37). Direct applications of lytic-cycle-only phages include addressing antibiotic-resistant pathogenic bacteria using their appropriate lytic cycles (Figure 2).

3.2. Lysogenic phage

A lysogenic cycle characterized by the integration of viral genetic fabric into the bacterial genome (referred to as a prophage) and persevered replication of viral genetic cloth without deadly outcomes for the inflamed host (38), is particularly prevalent in temperate phages. Infection with viral genetic material can change the bacteria's phenotype, however, because the virus incorporates genetic material into the host. It is evident that many common bacterial strains are pathogenic as a result of this conversion. Hydrogen peroxide can also be used to prevent such lysogenic conversion by producing reactive oxygen species, glutathione, and overexpressing transcriptional repressors (39) (Figure 3).

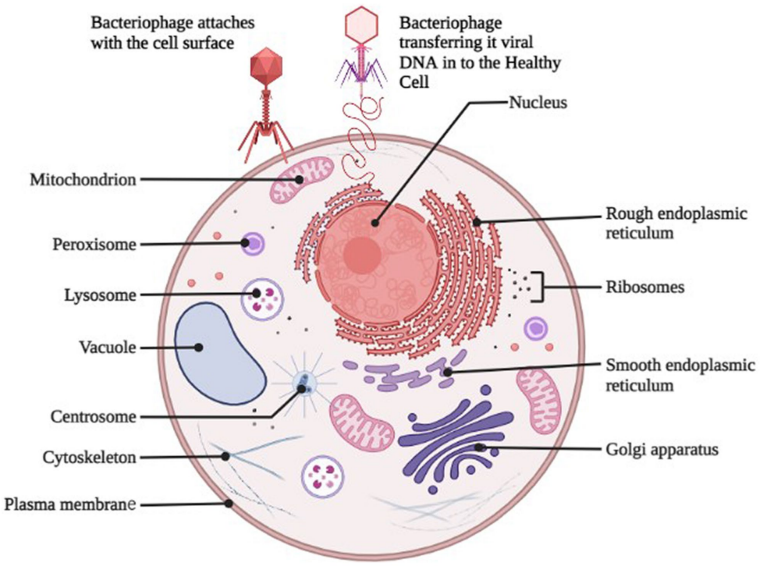

FIGURE 1
Mechanism of bacteriophage against bacterial cell. Phage attaches to the surface of the cell, breakdowns the cell wall of the bacteria, and transfers its viral DNA into the bacterial cell using the protein machinery of the bacteria and multiplies exponentially and infects the bacterial cell.

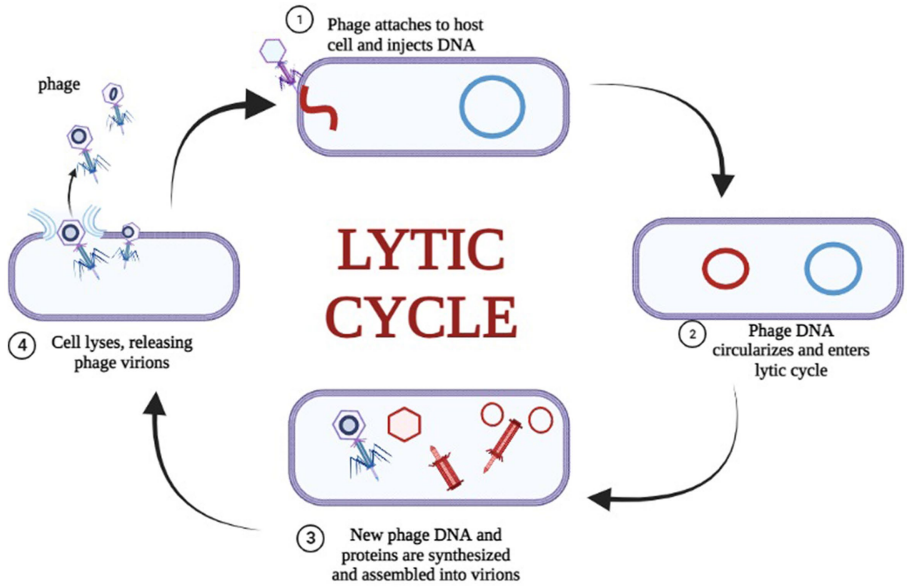

FIGURE 2
Lytic phages of phage. The phage attaches to the bacterial host cell and injects the viral DNA into the host, the phage DNA circulates in the host cell to form virions after the virions are assembled, cell lysis occurs and the phage virions are released.

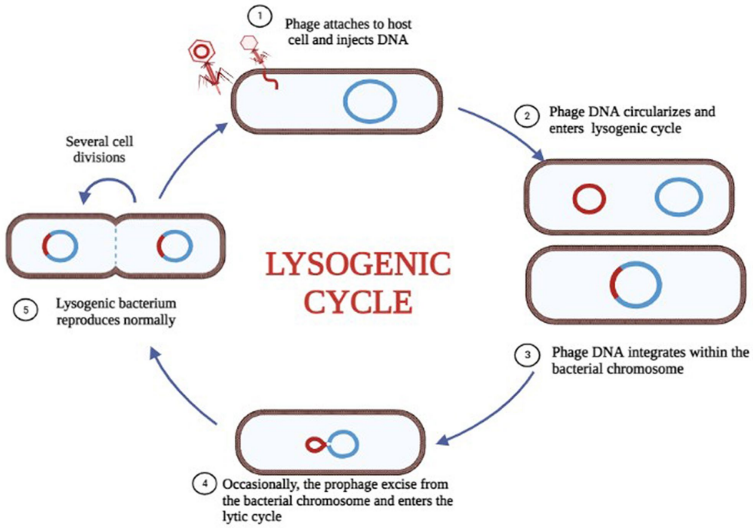

FIGURE 3
The lysogenic cycle of phages.

4. Bacteriophages against food animal pathogens-mechanisms and *in-vitro* studies

Escherichia coli, Campylobacter, Salmonella, and *Listeria* are the four most common foodborne pathogens of animal origin (40). Most of the time, these bacteria are carried asymptomatically in the gastrointestinal tracts of ruminants, poultry, and swine. To prevent animal disease and/or reduce gastrointestinal pathogen transport, which prevents pathogen entry into the food supply, phage administration to cattle is used as a direct pre-harvest strategy. The application of phages directly to animal carcasses is the basis of post-harvest strategies, aimed at cleaning the product (41).

4.1. *Escherichia coli*

The bacterium *E. coli* is Gram-negative. Food poisoning is frequently caused by *E. coli*, particularly the serotype O157:H7 strain, which produces the Shiga toxin (42). Its primary reservoir is ruminants, and because it thrives in intestinal environments, improper handling during slaughter may result in the contamination of meat

TABLE 1 Pre-and post-harvest use of *E. coli, Campylobacter, Salmonella*, and *Staphylococcus aureus* phage.

Animal	Plan	Phage	References
Postharvest application			
Meat	Applied on top	e11/2, e4/1c, pp01	(48)
Current produce (lettuce, cantaloupe)	Sprayed	Bacteria cocktail (ECP-100)	(49)
Food is exposed (spinach blades)	Sprayed	Bacteria cocktail	(50)
Food is exposed (steel, ceramic chips)	Applied on top	BEC8	(51)
Chicken	Put on top	Φ2	(52)
Chicken skin	Put on top	Φ29C	(53)
Meat (Beef)	Cj6	Cj6	(54)
Foods that are ready to eat and chocolate milk	Milk is mixed in and added to foods.	FO1-E2	(55)
Meat (pig)	Bacteria cocktail (PC1)	Phage cocktail (PC1)	(55)
Meat (raw/cooked beef)	Applied on top	P7	(56)
Meat (Skin chicken)	Applied on top	P22, 29C	(57)
Prepared meals (cheese)	Included in the pasteurized milk vat	vB_SauS-phi-IPLA35, vB_SauS-phi-SauS-IPLA88	(58)
Prepared food (milk curd)	To whole milk that has been pasteurized	Cocktail (Φ88 and Φ35)	(58)
Raw dairy products like whey	Added to raw milk whey	K	(59)
ready-to-eat foods that are raw (milk products and derivatives)	Add to milk	K	(59)
Preharvest application			
Ruminant (sheep)	Oral delivery	CEV1, CEV2	(60)
Ruminant (cattle)	Oral delivery	e11/2, e4/1c	(61)
Ruminant (cattle)	Oral/rectal delivery (via drinking water)	KH1, SH1	(62)
Poultry (broiler chicken)	Injection into the left thigh	SPR02 and DAF6	(52)
Poultry (Chickens broiler)	oral transmission (oral gavage and in feed)	Phage cocktail	(52)
Poultry (Broiler chickens)	Poultry (Broiler chickens)	69, 71	(63)
Swine (Weaned pigs)	Oral delivery	Phage cocktail	(64)
Chickens	Oral delivery	Phage cocktail (CB4ϕ, WT45ϕ)	(65)
Poultry	Antacid suspension	Φ151, Φ25, Φ10	(56)
Pig	Oral delivery	Felix01	(66)

with feces, dust from the hide, or intestinal contents. *E. coli* is most transmitted to humans through raw contaminated food, and raw milk and water are thought to be associated with cases of cross-contamination involving direct or indirect contact with feces. These microbes are highly toxic and a public health hazard because they can spread infections when ingested in concentrations as low as 10 cells (43). The application of phages to poultry has been successful to prevent fatal respiratory infections in broiler chickens (44). Several different approaches have been used; however, aerosol spraying and intramuscular (a.m.) injection have given the best results and reduced significantly the mortality of broiler chicken. Despite these results, phage administration in addition to bird drinking water proved to be inefficient in protecting the birds from fatal *E. coli* respiratory infections (45). The main speculated causes for the failure of oral treatments have been reported to be (i) nonspecific binding of phages to food particles and other debris in the rumen and gastrointestinal tract (46); (ii) phage inactivation upon contact with the acidic conditions of the abomasum (iii) causing an insufficient number of oral phages reaching the gastrointestinal tract. An interesting approach to reducing coliphage inactivation has been described by Stanford and colleagues in 2010. These authors successfully encapsulated phages in polymeric matrices which resisted *in vitro* acidic conditions and furthermore, once delivered orally to steers caused the reduction of *E. coli* levels (47) (Table 1).

4.2. *Campylobacter*

The ideal growth temperature for Gram-negative, spiral, motile, and microaerophilic bacteria belonging to the genus *Campylobacter* is 41°C (61). Members of *C. jejuni* and *E. coli* are considered the main etiological agents of enteric diseases worldwide. Due to the possibility of campylobacteriosis, which is usually characterized by fever, bloody diarrhea, and excruciating abdominal pain, when low doses (400–500

cells) are administered, this widespread infection has been described (52). Because Campylobacter can colonize the intestines of chickens and cattle, infection usually occurs through mouth-to-mouth contact, consumption of contaminated food (such as raw meat and milk that has been contaminated with feces), and contaminated drinking water. Spread by water. Several strategies to control this infection using bacteriophages have been developed because of the disease's widespread occurrence and financial impact on the agriculture and food industries (67). It is known that *Campylobacter* attaches and forms biofilms on surfaces as a measure to overcome environmental stresses, such as aerobic conditions, desiccation, heating, disinfectants, and acidic conditions frequently encountered in food environments (36); however, data, we were only able to find one report evaluating the efficacy of Campylobacter phages of disrupting biofilm formed on the glass. In this study, phages were able to reduce by 1 to 3 log the viable cell counts under microaerobic conditions; however, after treatment above 84% of the surviving bacteria were resistant to the two phages applied (68).

4.3. *Salmonella*

A genus of facultative intracellular species that are Gram-negative known as *Salmonella* is believed to account for a significant proportion of zoonotic diseases reported worldwide. It is frequently linked to consuming tainted food of animal origin because *Salmonella* serovars have the ability to colonize and survive in the human gastrointestinal tract (55). Each year, *Salmonella* infections cost the Europe Union (EU) healthcare system around three million euros. Most *Salmonella* outbreaks are attributed to the consumption of tainted meat and eggs from poultry, pork, and cattle, respectively. *Salmonella* is another bacterium that can spoil processed foods. This microorganism, when ingested, can cause diseases, fever, diarrhea, and pain (40). Unlike phage preharvest strategies on animals, several postharvest strategies have adopted the use of only one phage and not a cocktail. All *Salmonella* phages reported have been able to decrease the number of viable cells present in raw meats, D processed and ready-to-eat foods, and fresh produce (69). Furthermore, the combined treatment of phage and *Enterobacter asburiae*, a strain exhibiting antagonistic activity against *Salmonella*, to control this pathogen on tomatoes, mung bean sprouts, and alfalfa seeds, represents a highly promising, chemical-free approach. However, in some settings phages were found to be readily immobilized by the food matrix and, although retaining infectivity, they lost the ability to diffuse and infect target cells (55).

4.4. *Staphylococcus aureus*

Gram-positive *S. aureus* is a typical way for mastitis in dairy cows and is considered a serious threat to food safety. According to the Central Depository Company (CDC), there are 242,148 cases of staphylococcal food poisoning in the United States annually. Eating foods that have an adequate amount of one or more enterotoxins in them causes this condition (59). *S. aureus* contamination of food has been linked to a variety of mechanisms, including human handling of food products, livestock infection and colonization, and human infection and handling of farm animals. Although most people recover in 1–3 days after experiencing mild symptoms, *S. aureus* can cause poisoning within 1 to 6 h after consuming contaminated food. It is estimated that mastitis in adult dairy cows causes losses of US$ 35 billion per year. Dairy food products and the majority of lactating cows have been the focus of phage research (70).

4.5. *Clostridium*

Clostridium perfringens is spore-forming rods, non-motile and Gram-positive which are normally found in poultry's intestinal microbiota. As low as 10^4 CFU are present, it is not pathogenic, but its pathogenicity is primarily due to its toxins. Necrotic enteritis (NE) occurs both in acute clinical and subclinical forms in chickens and is one of the most economically important diseases of poultry caused by *Clostridium perfringens* type A and type C-producing toxins. Additionally, infected poultry meat may cause human illness when it is infected with *C. perfringens* produces enterotoxins (71). Bacteriophages derived from *C. perfringens* strains isolated from poultry intestines and soils, as well as those isolated from soils and sewage, were identified as *Siphoviridae* and *Podoviridae* bacteria. Despite the phages, many strains of *C. perfringens* remained resistant to them. Specific isolation of this bacterium was also shown to be resistant to the activity of phages. *C. perfringens* phages encode endolysin, which may be particularly useful for controlling this bacterium, according to some authors. Despite differences in sensitivities between strains of *C. perfringens*, the results indicate that endolysin is active against all strains tested (62, 72). reported a decreased incidence of necrotic enteritis (NE) in broiler chickens after bacteriophage (INT-401) treatment. Experimentally infected broiler chickens also gained more weight and had a better feed conversion ratio with phage treatment *via* drinking water or feed.

5. Field and lab animal trials of bacteriophages in infectious diseases of food animals

The most popular model in studies on phage therapy is poultry. It was found to be very successful to combine two bacteriophages that are lytic for *Campylobacter jejuni*. The experiment was conducted on first-caught, 25-day-old broiler chickens. Broilers were kept separate from Jejuni. It was found that the type and dosage of bacteriophages used had an impact on phage therapy's efficacy (34). According to Wagenaar et al. (73), prophylactic oral administration of a mixture of phages (phage strains 69 and 71) to chickens delayed colonization of the gastrointestinal tract by *C. jejuni* and resulted in this level of colonization within 1 week. As viewed, stabilized. 2.0 in the treatment group following a first log reduction. Contrarily, after several days of phage therapy, *C. jejuni* counts had initially decreased by 3.0 logs, but they had only increased by 1 log compared to the untreated control group of chickens was lower (74). *E. coli* infection is a common problem for sheep farmers. Bacteriophages and their potential applications in the defense of sheep against *E. coli* infection were described by Bach et al. (75) Experimental sheep were exposed to four different strains of *E. coli* on day 0. On days 2, 1, 0, 6, and 7 of the trial, three bacteriophage cocktails (1,010 PFU of P5, P8, and P11 each) were administered orally. When stool samples were taken, the amount of *E. coli* was reduced (75). Mice are yet another common model used

in phage therapy research. *Salmonella enterica* serotype enteritidis infection control in mice using phage treatment. Five-week-old female BALB/c mice with a mean weight of 20 g were obtained and weighed in order to assess the impact of phage SE20 on salmonellosis (76). The salmonellosis model and animal testing were done. Twenty-four mice were divided into three groups; in group A, which served as the control, 200 L of PBS was gavage; in group B, 200 L of *S. enteritidis* (1.5 × 107 CFU/mL) was gavage; and in group C, 200 L of *S. enteritidis* (1.5 × 107 CFU/mL) was gavage on the first day, and a single dose of phage SE20 was gavage. Anti-acid was gavage into the animal's stomach to protect the phages there from harm. After six days of infection, four mice from each group were put to sleep. Histopathology of the liver and spleen. The liver and spleen were then taken out and preserved in 10% formalin for 24 h at 6°C in the refrigerator (76).

6. Bacteriophages products available in market

A growing number of businesses worldwide are developing and commercializing phage biocontrol products because of the biological properties of lytic bacteriophages. Phage biocontrol, however, has some drawbacks and restrictions (77). Over the past 12 years, the number of regulatory approvals granted for the use of bacteriophage preparations to improve food safety has steadily increased (Table 2). The Food and Drug Administration (FDA) approved - *L. monocytogenes*-specific cocktail ListShieldTM as a food enhancer in 2006 as the first bacteriophage preparation for direct use in food (the FDA labels any product as "not approved" alternatively, the term "approval" is often used to indicating that obtaining FDA clearance means using the products for their intended applications) (81). Later that year, the FDA approved Listex TM (now Phage Guard Listex TM), Listeria guidelines, as a Generally Recognized as Safe (GRAS) substance. The FDA recently granted GRAS status to several phage products, including SalmoFreshTM and Phage Guard STM. As a result, applying for GRAS status now seems to be the standard for phage products for post-harvest food processing. Since wild-type (i.e., not genetically modified) soluble bacteriophages are completely natural and already present in food, the GRAS designation seems to be an appropriate regulatory pathway for these agents. Phage preparations can be used in the production of meat, poultry, and egg products. It is legal to use phages on food (e.g. *Salmonella* targeting phage) and livestock prior to slaughter (82). USDA has included information on several phage preparations in its published guidelines. Although these directives are made using specific phage preparations, in general, any phage product that meets the requirements of the directives may be considered legal. Several health organizations around the world have approved the use of phage products in food, following the example of US regulators (79, 80).

Phage products are non-genetically modified organisms (non-GMO), GMO-free phages that stay in a solution that is biocontrol chemicals. In comparison to other safety intervention techniques, these phage products are inexpensive, costing only per pound of treated food. These commercially available phage products are regarded as eco-friendly and organic sciences (78). Phage products such as these typically contain naturally occurring, GMO-free phages suspended without harsh chemicals commonly used in biocontrol. A phage product costs between 1–4 cents per pound of treated food, compared to other safety intervention techniques that typically cost between 10 and 30 cents per pound. The dramatic price difference between phage biocontrol products and existing interventions is another major advantage (83). It is essential to keep in mind that this represents the cost of a constructed item to treat a single pathogen. Using multiple products increases the overall cost of phage biological control. More and more companies are developing and marketing phage biocontrol methods that will increase their usefulness in the food industry (86).

7. Economic impacts of the use of bacteriophages

A major problem in food production facilities development such as microbiological bacilli on equipment surfaces. Bacterial biofilms are defined as aggregates of cells attached to biotic or abiotic surfaces and enclosed in an extracellular polymeric matrix consisting of self-assembly (EPS) (23). Biofilm-forming bacteria have a high level of resistance to harmful environmental factors, antibiotics, and cleaning agents. The majority of pathogenic bacteria in the fresh produce industry, including *L. monocytogenes, Salmonella, E. coli, and Yersinia,* can attach to plant tissues where they can grow and form biofilms. Because of the inherent structure of vegetables, these microorganisms

TABLE 2 Types of bacteriophage products available in the market.

Agency name	Product	Bacteria	References
Intralytix Inc.	ListShield™	*L. monocytogenes*	(78)
FINK TEC GMBH	EcoShield™	*E. coli*	(79, 80)
Intralytix, Inc.	SalmoFresh™	*Salmonella* spp.	(81)
Micreos Food Safety	Phage Guard S™	*Salmonella* spp.	(82)
Micreos Food Safety	Phage Guard Listex™	*L. monocytogenes*	(83)
Intralytix, Inc.	Shiga Shield™ (ShigActive™)	*Shigella* spp.	(84)
Phagelux	Agri Phage™	*Xanthomonas campestris* pv. Vesicatoria *Pseudomonas syringae* pv. tomato	(83)
Phagelux	SalmoPro®	*Salmonella* spp.	(82)
Passport Food Safety Solutions	Finalyse®	*E. coli*	(85)

have a harder time accessing sanitizers (87). In order to remove the biofilm on plant tissues, preparations that are safe for humans must be developed. Bacteriophages offer hope for developing human-safe sanitizers (88). *Campylobacter jejuni* is one of the pathogenic bacteria that can form biofilms on materials frequently used in industries (i.e. polyvinyl chloride and stainless steel). On glass Petri plates, *C. jejuni* biofilm formation was prevented using the lytic bacteriophages CP8 and CP30 that were isolated from chicken excrement (89). In comparison to a control biofilm that had not been treated with bacteriophage, The viable count was found to be reduced by 1.0–3.0 log CFU/cm2 24 h after infection following phage treatment of each biofilm, according to the research depending on the strain that forms a biofilm, there were differences in the degree of reduction and the potential for phage resistance (90). When compared to *C. jejuni* strain NCTC 11168, *C. jejuni* strain PT14 was distinguished by its ability to produce a much larger amount of biofilm on glass. No resistant *C. jejuni* cells were found in the PT14 biofilm treated by phages among the bacteria that made it through the bacteriophage treatment. Eighty-four percent of the *C. jejuni* 11,168 cells showed resistance to phage CP8 and 90 % showed resistance to phage CP30 (91). No resistant *C. jejuni* cells were found in the PT14 biofilm treated by phages among the bacteria that made it through the bacteriophage treatment. Eighty-four percent of the *C. jejuni* 11,168 cells showed resistance to phage CP8 and 90% showed resistance to phage CP30 (68). Endolysins are an enzyme made by bacteriophages that may be used in sanitization. Bacteriophages produce endolysins at the end of their lytic cycle, which allows the release of progeny virions by breaking down peptidoglycan found in the cell wall. Gram-positive bacteria can also be exogenously injected with them to kill them; Gram-negative bacteria have an outer membrane that shields them from endolysin activity. Based on where they cleave the peptidoglycan, lysins are divided into five groups: glycosaminidases, lytic transglycosylases, muramidase, amidases, and endopeptidases (92). Staphylococcal counts in the polystyrene-adhered biofilm were found to be reduced by 1.0–3.0 log units by endolysin LysH5, a product of the *S. aureus* phage vB SauS-phiIPLA88. Oliveira et al. (93) claim that an additional factor that acts on the bacterial envelope is necessary to disestablish the outer membrane in order to achieve good efficacy with endolysins against bacteria. It has also demonstrated that LysK endolysin and DA7 depolymerase work together to inhibit staphylococcus biofilm. These two enzyme mixtures in nano- and micromolar concentrations can remove biofilm from polystyrene and glass surfaces even at very low concentrations (89).

8. Challenges to the use of bacteriophages

To cope with the emergence of multi- and pan-drug resistant bacterial lines, new antimicrobial agents and therapeutic techniques are wished. As we input the submit-antibiotic generation, bacteriophages are considered the most promising way to the cutting-edge scientific disaster, with several benefits over traditional antimicrobials. It is unusual that phage therapy (PT) has produced big progress over a long time, however, gaps need to still be recognized and filled, the phage-based preparations ought to be secure and powerful to be used in hen production, chicken medication, and the rooster enterprise. Further to the dosage and management technique (consisting of education of fashionable formulations), the timing of administration of phage-primarily based merchandize in addition to concomitant preparations (together with competitive exclusion) or vaccination is essential. Bacteriophages can survive longer on meat product surfaces when refrigerated. To achieve positive outcomes for human health and well-being, action must be taken, and methods and practices must be modified (94). Designed for use in the poultry industry, phage-based formulations must be safe and effective. In addition to dose and method of administration (including preparation of standardized formulations), staging of drugs, as well as the timing of concomitant drugs (e.g., competitive exclusion) or vaccination, is also important. Individual bacteriophages and environmental factors can affect the persistence of bacteriophages in food (e.g., temperature) (95).

The industry that produces food has to continuously contribute to attempts to stop infectious diseases and the problems with antibiotic resistance in human infections that come from food animals. Microbial safety issues continue to be a problem despite numerous technological advancements in methods for the detection and elimination of foodborne pathogens at each stage of the food production process, in good manufacturing practices, quality control and hygiene, changes in animal husbandry, and in agronomic procedures (96). There is a need for the development of alternative antibacterial approaches at the production level to maintain safety standards, control foodborne pathogens, and limit their detrimental effects on the food industry and on human health. In addition, the restricted use of some antibiotics during food animal production, coupled with the lack of development of new antimicrobials, has put additional strain on the food production sector (97). Bacteriophages (phages) are advantageous candidates for use in both the detection and control of pathogens at each stage of the food production process from farm to fork due to their natural specificity to infect and kill their target bacteria, as well as the fact that they are ubiquitous in the environment and harmless to humans and animals (98). To combat some of the most common foodborne pathogens, such *as Escherichia coli, Salmonella* serovars, and *Listeria monocytogenes*, a variety of phage-based products have recently entered the commercial market (99).

9. Prospects

Recent decades have seen an increase in phages being used as biocontrol tools as a result of AMR bacteria emerging and antibiotics being limited in use in livestock and crops (100). In the fight against bacteria, phages remain a fascinating and natural alternative As we discussed in previous sections, phages have numerous applications and advantages concerning food safety. There are some issues that must be resolved before these findings can be widely applied, despite their encouraging results (101). As well as being assets, phage specificity, resistance to resistance, and self-dosing capacity can also be liabilities. The phage specificity of these antimicrobials significantly limits their effectiveness in biocontrol. Bacterial capsules and cell walls contain receptors responsible for a majority of host tropism. When dealing with pathogenic bacteria, the task of building phage banks or biobanks can be stressful and time-consuming. In this situation, direct hunting is likely to be more efficient and cost-effective. In a fascinating development, biobanks may make phages readily available for lysing

bacteria from various species. It is, however, necessary to perform programs to pick out the potential phages quickly (102, 103). Despite being rare, phage matching can be easily carried out with automated equipment, even though finding the precise phage can take some time. A phage biocontrol treatment can be successfully achieved as a customized treatment by hunting for phages, as well as knowing the bacterial host (88). Moreover, phages can be used as broad-range products when they are combined with broad-range phages. It may also be beneficial to broaden the host range by phage training (experimental evolution) or to develop chimeric phages able to recognize multiple strains or species, though this may negatively affect commensals. Food safety benefits from disinfectants that reduce bacterial burdens, and phage-derived enzymes, which can break down bacterial biofilms, may also offer promising solutions. It has been shown that phages produce hydrolytic proteins that are capable of actively destroying polysaccharide-based bacterial matrices and dislodging biofilms (88).

Another disadvantage is the inherently unstable nature of some phages. It may be necessary to take specific measures to keep phage-based products stable and, therefore, infective. To control phage release and deliver phages more precisely, nanoparticles can be embedded with phages. As a result of this method, commercial products may be stable over long periods and in a variety of environmental conditions (104). Besides freeze-drying, other preservation techniques might also be a viable option for long-term phage storage; they are significantly less expensive, making them an appealing choice for the industry. As phages lose their ability to maintain infectivity after processing, encapsulation may be the best method for protecting food (105, 106). It is also necessary to comply with legal requirements before using a phage application. It is, however, important to note that because of phages' inherent evolvability, their diversity and morphologies, as well as their inherent evolution and ability to self-replicate in bacterial hosts, regulatory agencies face a challenge, highlighting the importance of applying the same regulations and procedures to all phage-derived products. As can be seen, phage regulation is a complex issue that will delay phage use for routine and commercial applications. The regulatory bodies should, however, propose phage biocontrol recommendations quickly as an alternative to antibiotics to combat the emergence of resistant bacteria (13).

10. Conclusion

Food safety and sustainability remain the most pressing global food industry challenges. The food manufacturing industry has continuously contributed to efforts to prevent infectious diseases and antibiotic resistance related to human pathogens from food animals. Several leading and emerging pathogens in food have been successfully controlled by bacteriophages, a class of natural antibacterial agents. Although bacteria phages (phages) are ubiquitous in the environment and harmless to humans and animals, they make excellent candidates for detecting and controlling pathogens from farm to fork. Their natural ability to infect and kill their target bacteria makes them a powerful tool for both detection and control. Food contamination can be managed by biological procedures such as bacteriophage biocontrol rather than using chemical preservatives as they become less available. For phage propagation, it is advantageous to select nonvirulent and genetically well-characterized bacteria. The establishment of safe large-scale production processes will be necessary for bacteriophages to be used as bio preservatives in the future. However, in food systems, phages' antimicrobial activity could be greatly reduced as compared to laboratory conditions.

Author contributions

SZ conceived the idea, wrote the original manuscript and KA revised the manuscript drew the figures and provide financial support.

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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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Comparative analysis of gut fungal composition and structure of the yaks under different feeding models

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The yaks that inhabit the Tibetan plateau are a rare breed that is closely related to local economic development and human civilization. This ancient breed may have evolved a unique gut microbiota due to the hypoxic high-altitude environment. The gut microbiota is susceptible to external factors, but research regarding the effects of different feeding models on the gut fungal community in yaks remains scarce. In this study, we compared and analyzed the composition and variability of the gut fungal community among wild yaks (WYG), house-feeding domestic yaks (HFG), and grazing domestic yaks (GYG). The results revealed that Basidiomycota and Ascomycota were the most preponderant phyla in the gut fungal community, regardless of feeding models. Although the types of dominant fungal phyla did not change, their abundances did. Intergroup analysis of fungal diversity showed that the Shannon and Simpson indices of WYG and GYG were significantly higher than those of HFG. Fungal taxonomic analysis showed that there were 20 genera (*Sclerostagonospora* and *Didymella*) that were significantly different between WYG and GYG, and 16 genera (*Thelebolus* and *Cystobasidium*) that were significantly different between the WYG and HFG. Furthermore, the proportions of 14 genera (*Claussenomyces* and *Papiliotrema*) significantly decreased, whereas the proportions of eight genera (*Stropharia* and *Lichtheimia*) significantly increased in HFG as compared to GYG. Taken together, this study indicated that the gut fungal composition and structure differ significantly between yaks raised in different breeding groups.

KEYWORDS

yak, wild, Qinghai-Tibet plateau, gut microbiota, gut fungal community

Introduction

It is widely known that the gut microbiota is a complex micro-ecosystem involving a large number of different types of microorganisms, including bacteria, fungi, and viruses (1–3). Studies have shown that the gut microbiota can provide the host with nutrients and beneficial metabolites, such as amino acids, vitamins, and short-chain fatty acids, by fermenting sugars and carbohydrates (4–6). These metabolites play important roles in host health, immunity, intestinal homeostasis, and intestinal

barrier (7, 8). Similar to the gut bacterial community, the gut fungal community is also an important component of the gut microbiota, which plays vital roles in host health by improving gut functions (9–12). Early investigations indicated that the gut fungal community could induce significant shifts in the gut bacterial structure and shape the gut microbiota during early life (13). The gut fungal community may also play a role in the maturation of the host immune system by interacting with the gut bacterial community to produce strong local and systemic immune responses (13, 14). Furthermore, some fungi are considered to be intestinal probiotics due to their potential role in alleviating inflammation, inhibiting pathogenic bacteria, degrading cellulose, and regulating digestion (15–17). For instance, administration of *Candida kefyr* has been demonstrated to alleviate gastrointestinal inflammation by changing the gut microbiota (18). Additionally, some fungi can synthesize and release neurotransmitters such as norepinephrine and histamine (19, 20). However, gut microbial homeostasis is easily affected by external factors such as age, sex, diet, and disease (21, 22). Moreover, recent studies have indicated that feeding methods, altitude, and habitat environment are also important driving forces for the development of gut microbiota (23, 24).

Yak is a rare species of cattle of the Tibetan plateau (above 3,000 m), which is characterized by adapting to high, cold, and oxygen-deficient environments (25). Statistically, approximately 90% of the world's yaks live in the Sichuan, Qinghai, Tibet, and Gansu provinces of China (26, 27). Yaks are also an important source of milk and meat products for local herdsmen and play an important role in economic development. Given the importance of yaks on the Tibetan plateau, any factors that threaten the health and development of this breed may lead to enormous economic losses. Previous studies indicated that cold and hypoxic environments could cause changes in the gut microbial structure (28–31). Therefore, the altitude hypoxia environments of the Tibetan Plateau may induce the accumulation of special gut microbiota in yaks compared with animals living in plains. Indeed, several studies have reported the unique composition and diversity of the gut microbiota in yaks (32, 33).

In the past, yaks were mainly raised in the open and were easily affected by the external environment. For instance, changeable weather and nutritional deficiencies can cause low production efficiency and a high disease rate of yaks (34, 35). To improve the productivity of yaks, a combination of free grazing and barn feeding is also implemented in some areas. Furthermore, there are still some wild yaks in the same area of the Tibetan Plateau. Although these yaks are the same species, they may have evolved specific microbial communities to adapt to different farming methods. A previous study has demonstrated significant differences in the gut bacterial community of yaks under different feeding models (36, 37). However, until now, little research has been conducted on the gut fungal community of yaks. Therefore, the aim of our study was to evaluate the composition and variability of the gut fungal community of yaks under different feeding models.

Materials and methods

Sample acquisition

In this study, fecal samples of grazing domestic yaks (GYG) and wild yaks (WYG) were obtained from Shuanghu County and Chang Tang Nature Reserve, China. The average altitude of this area exceeds 5,000 meters and is characterized by high temperatures, low precipitation, and strong wind speed in July and August. Moreover, the zonal vegetation includes *Austrostipa pubescens* as the dominant vegetation. We also collected fecal samples from house-fed domestic yaks (HFG), which were mainly fed green hay, to explore the changes in the gut fungal community under different feeding models. All samples were collected in July 2022, and each group contained five animals. Fecal samples from yaks of different feeding models were collected using dung samplers. Freshly rectal feces were selected and sub-sampled (approximately 100 g) from the central portion to minimize contamination from bedding and flooring. Subsequently, fecal samples from yaks of different feeding models were labeled (GYG: GYG1, GYG2, GYG3, GYG4, and GYG5; WYG: WYG1, WYG2, WYG3, WYG4, and WYG5; HFG: HFG1, HFG2, HFG3, HFG4, and HFG5) and placed at −80°C for further analysis.

DNA extraction and high-throughput sequencing

Fungal DNA was extracted from homogenized intestinal contents using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), based on the manufacturer's protocol. Subsequently, we amplified the ITS2 regions using primers (ITS5F: GGAAG TAAAAGTCGTAACAAGG and ITS2R: GCTGCGTTCTTCATCGA TGC) and synthesized them as per conserved regions. PCR amplification procedures were determined according to previous research (38–40). After purification and fluorescence quantification of the amplified products, the sequencing library was constructed using the TruSeq Nano DNA LT Library Prep Kit (Illumina, CA, USA). The initial libraries were subjected to sequence end repair, enrichment, and purification to improve their quality. The final libraries that passed the quality assessment were used to perform paired-end sequencing using a MiSeq sequencing machine. Raw data from the amplicon sequencing were further processed and modified using QIIME software (Qiime1.9.1) due to the presence of questionable sequences such as unqualified, short, mismatched, and chimera sequences. After quality inspection and filtering, the effective reads were applied for OTU clustering based on 97% similarity. Fungal OTU representative sequences were taxonomically classified using RDP Classifier v.2.2 based on the UNITE database (39, 41). Meanwhile, the Venn diagram was also generated to observe the common and unique OTUs among the groups. To study the variation in gut microbial diversity and abundance, we calculated multiple alpha diversity indices based on the number of OTUs in each sample using QIIME software (Qiime1.9.1). Moreover,

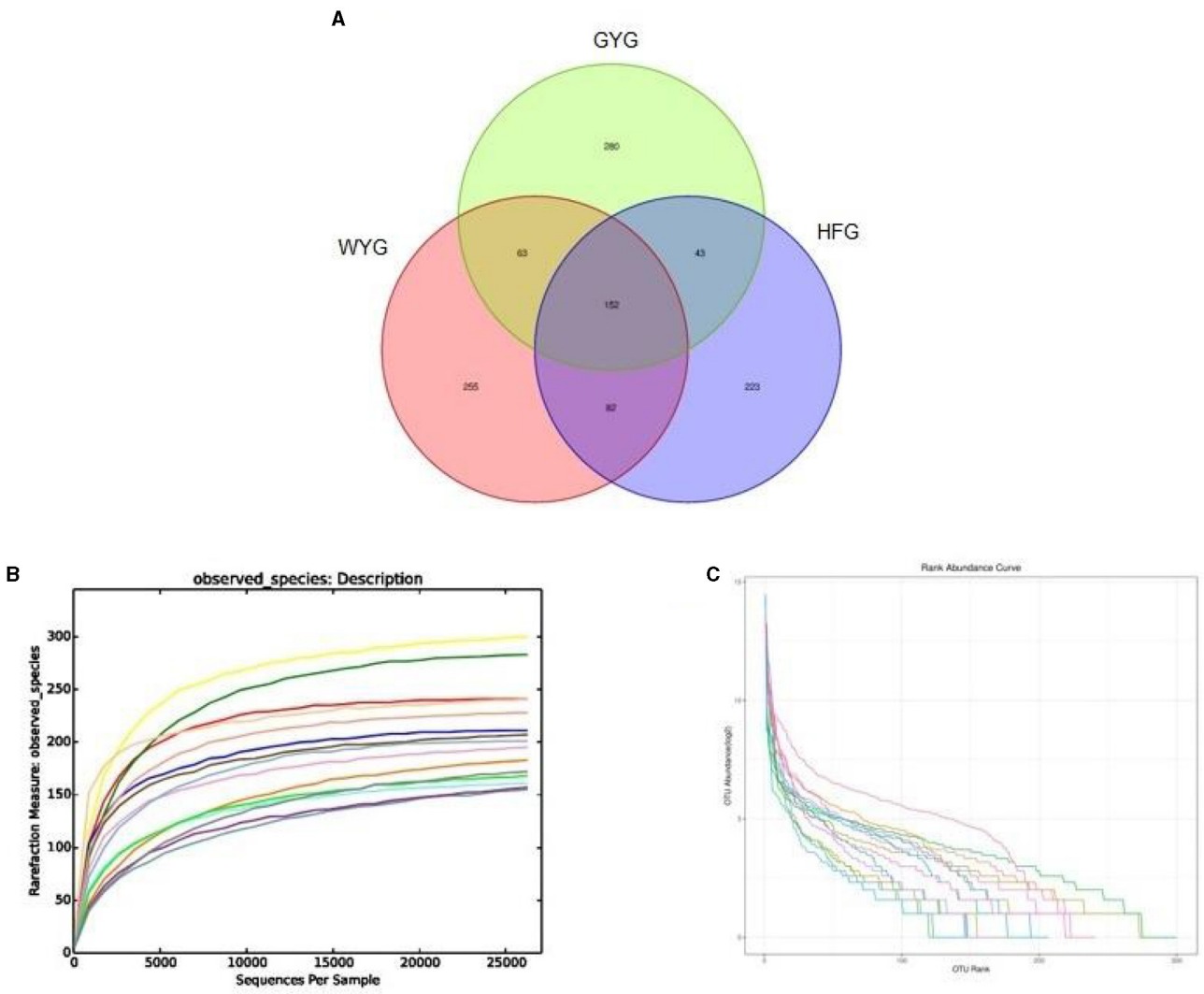

FIGURE 1
Sequencing data analysis and OTU distribution. Each colored curve in the rarefaction and rank abundance curves represents one sample. **(A)** The number of OTUs is indicated by different colored areas, and the middle area indicates the number of shared OTUs. **(B)** Rarefaction curve. **(C)** Rank abundance curve.

PCoA plots reflecting beta diversity were also generated using QIIME software (Qiime1.9.1) to further compare and analyze the differences in gut microbial principal components. Statistical analysis of the data was conducted using R (v3.0.3) and GraphPad Prism (version 8.0c). LEfSe and meta-statistics analyses were employed to detect differential fungal taxa. The p-values (means ± SD) of <0.05 were considered statistically significant.

Results

Sequencing data analysis

In this research, high-throughput sequencing generated 576,964 (WYG = 182,234, HFG = 193,448, and GYG = 201,282) valid sequences from the three groups with an average of 38,464 (ranging from 30,248 to 45,539) reads per sample (Appendix A). The high-quality sequences were clustered, and a total of 1,098 OTUs were identified, ranging from 500 to 552 OTUs per group (Figure 1A). Additionally, the numbers of unique OTUs in the WYG, HFG, and GYG were 255, 223, and 280, respectively. Notably, we also found 152 core OTUs in the three groups, which were not affected by the feeding models. To assess sequencing depth and evenness, we also generated rarefaction and species rank curves. The results indicated that the curves of all samples exhibited a saturation trend, suggesting the adequacy and reliability of the sequencing (Figures 1B, C).

Comparative analysis of the gut fungal community in yaks with different feeding models

To explore the effects of different feeding models on the gut fungal alpha diversity of yaks, we calculated four indices, including

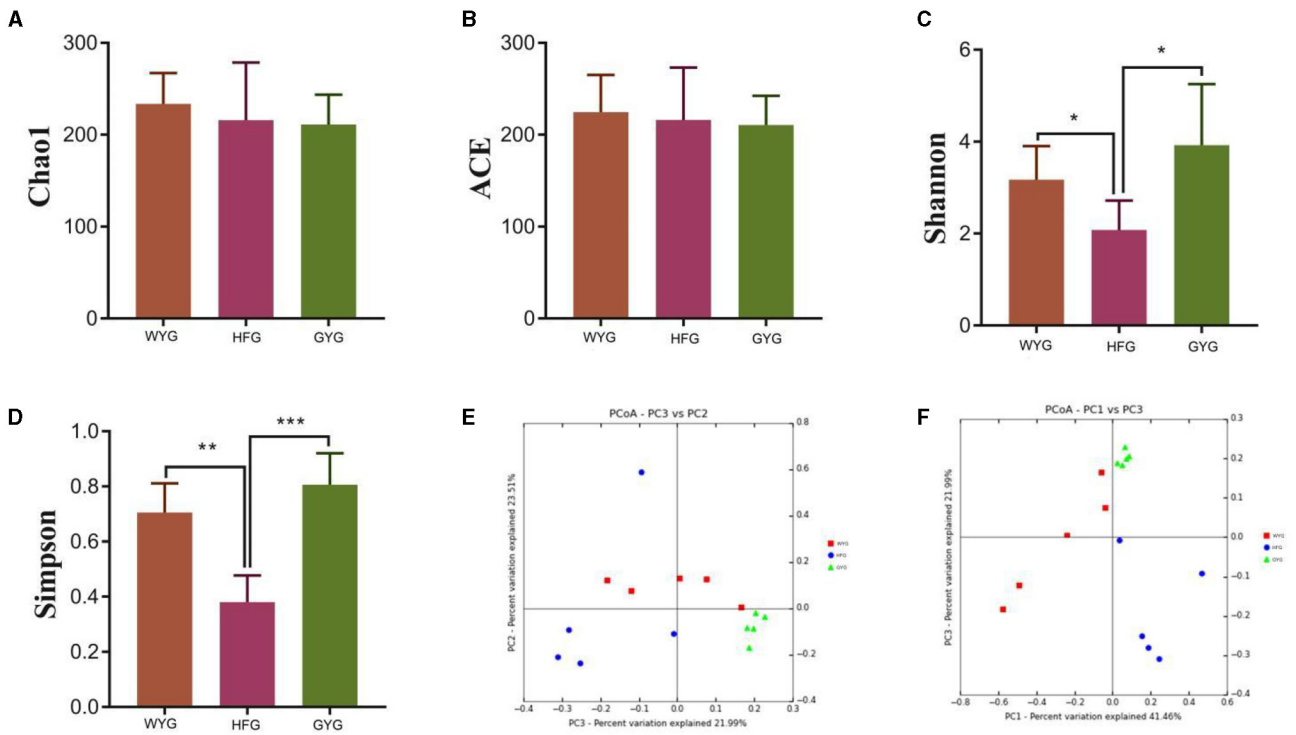

FIGURE 2
Comparative analysis of gut fungal diversity in yaks under different feeding models. The four indices including Chao1, ACE, Shannon, and Simpson were used to assess alpha diversity. **(A)** Chao1 index. **(B)** ACE index. **(C)** Shannon index. **(D)** Simpson index. **(E, F)** Gut fungal beta diversity was assessed by PCoA plots. Data are presented as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Chao1, ACE, Simpson, and Shannon indices (Figures 2A–D). The Chao1 and ACE indices of the gut fungal community, from high to low, are WYG, HFG, and GYG. Moreover, the GYG had the highest Simpson and Shannon indices, followed by WYG and HFG. There were statistically distinct differences in Simpson (0.71 ± 0.11 vs. 0.38 ± 0.097, $P < 0.01$) and Shannon (3.17 ± 0.73 vs. 2.08 ± 0.64, $P < 0.05$) indices, whereas the Chao1 (233.69 ± 33.47 vs. 215.94 ± 62.69, $P > 0.05$) and ACE (224.71 ± 40.33 vs. 216.11 ± 57.06, $P > 0.05$) indices were not significantly different between the WYG and HFG. Similarly, we also found that the Simpson (0.80 ± 0.11 vs. 0.38 ± 0.097, $P < 0.001$) and Shannon (3.92 ± 1.33 vs. 2.08 ± 0.64, $P < 0.05$) indices of the GYG were significantly higher than those of the HFG, while there was no difference in the Chao1 (211.04 ± 32.59 vs. 215.94 ± 62.69, $P > 0.05$) and ACE (210.71 ± 31.78 vs. 216.11 ± 57.06, $P > 0.05$) indices. The comparative analysis between WYG and GYG indicated that there were no significant differences in the four indices. These results indicated that the gut fungal diversity in WYG and GYG was significantly higher than that in HFG, while there was no difference in the gut fungal abundance among WYG, HFG, and GYG. The results of the beta analysis showed that the samples of different groups were separated from each other, indicating significant differences in the principal components of the gut fungal community (Figures 2E, F).

Composition and differences of the gut fungal community in yaks with different feeding models

The phyla Ascomycota (69.90, 92.02, and 90.18%) and Basidiomycota (28.33, 5.47, and 5.86%) were abundant in the WYG, HFG, and GYG, accounting for more than 95% of the total fungal composition, respectively (Figure 3A). Moreover, other phyla, such as Mucoromycota (0.058, 0.57, and 0.11%), Rozellomycota (0.078%, 0.39%, and 0.19%), Mortierellomycota (0.058, 0.26, and 0.21%), Anthophyta (0.011, 0.13, and 0.082%), GS19 (0.14, 0.012, and 0.067%), Olpidiomycota (0.00, 0.086, and 0.02%), Neocallimastigomycota (0.017, 0.0068, and 0.069%), Cercozoa (0.012, 0.00, and 0.059%), Blastocladiomycota (0.00, 0.00, and 0.044%), and Rotifera (0.022, 0.0053, and 0.012%), were detected in WYG, HFG, and GYG in low abundances. *Thelebolus* (54.27%), *Naganishia* (25.43%), and *Cutaneotrichosporon* (1.29%) were the most predominant genera in the WYG (Figure 3B). Moreover, the dominant genera found in the HFG were *Thelebolus* (84.52%), *Naganishia* (3.08%), *Cutaneotrichosporon* (1.11%), and *Lecanicillium* (1.03%). The fungal genera with an abundance of more than 1% in the GYG were *Thelebolus* (31.22%), *Naganishia* (1.71%), *Candida* (1.65%), *Acremonium* (1.72%), and *Vishniacozyma* (1.00%). Furthermore, the gut fungal compositions

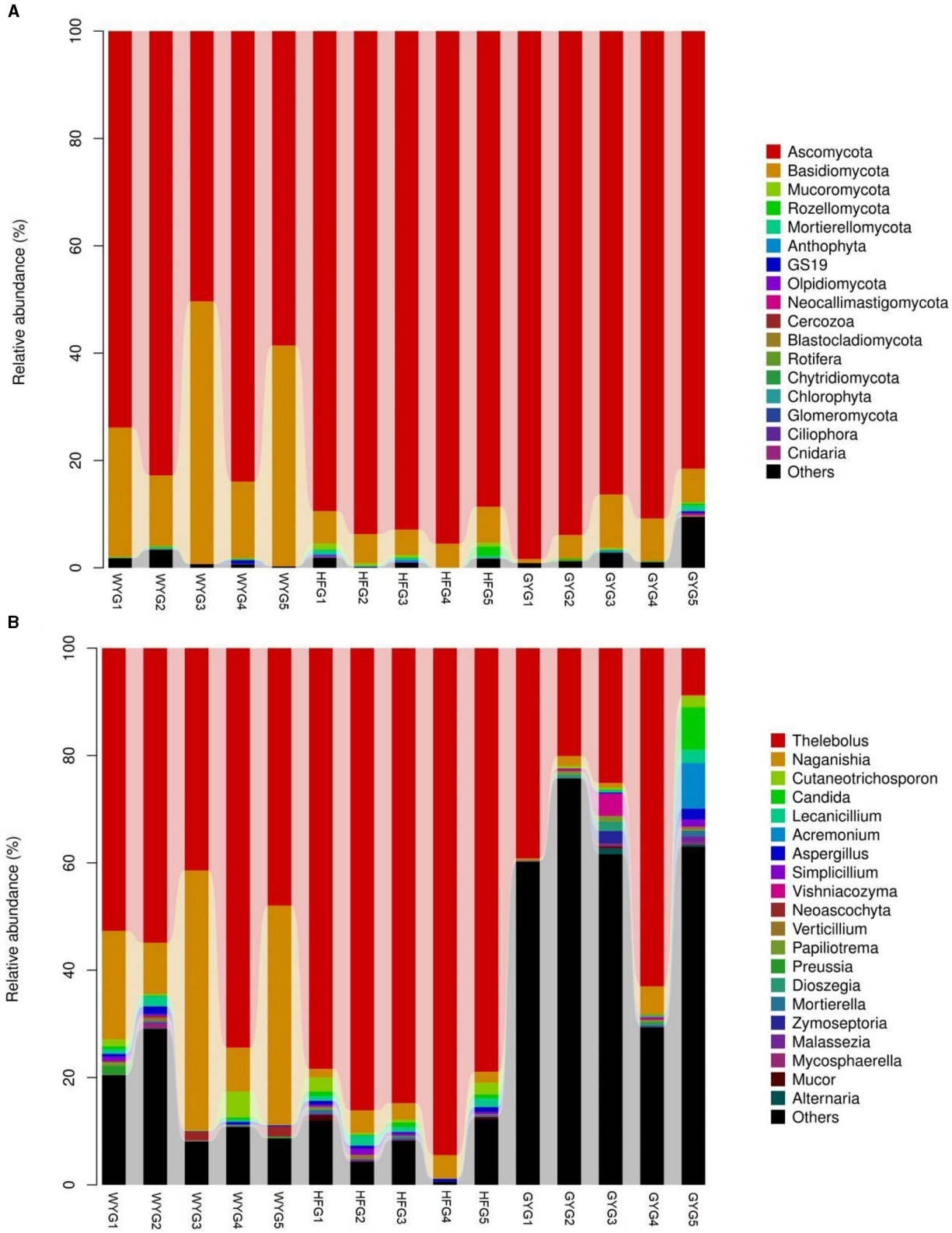

FIGURE 3
The abundance of dominant fungal phyla and genera in the gut fungal community of yaks under different feeding models. **(A)** The abundance of dominant fungal phyla. **(B)** The abundance of dominant fungal genera. The abundance of different fungal phyla or genera is represented by different colors and the height of the histogram.

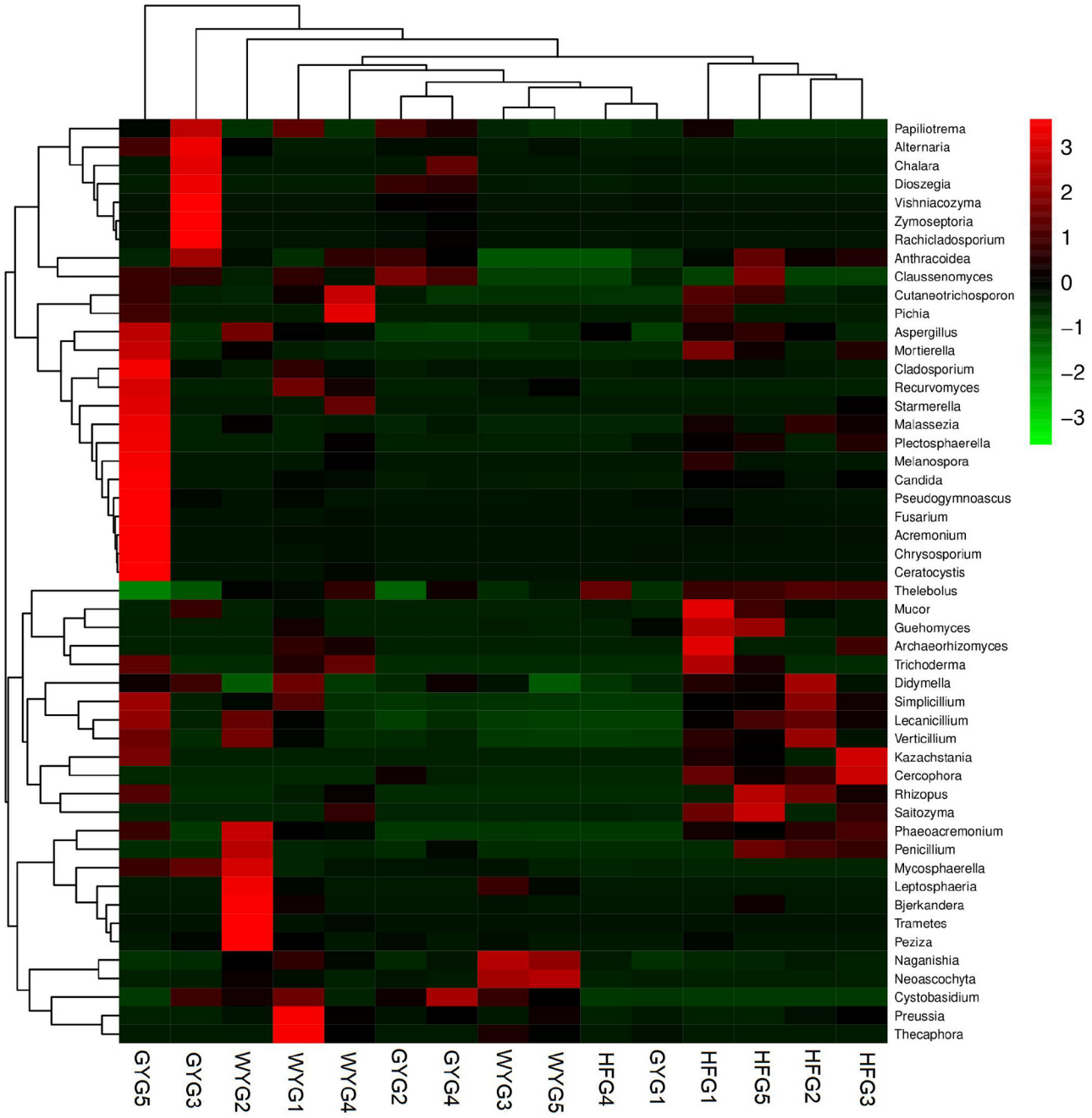

FIGURE 4
Heatmap of the abundant fungal genera in the WYG, HFG, and GYG. The abundance of different fungal genera is represented by different colors.

and changes in WYG, HFG, and GYG could also be observed through a visualized clustering heatmap (Figure 4).

We also conducted meta-statistics analysis to identify differences in the gut fungal community at different taxonomic levels. At the phyla level, the HFG indicated significantly higher levels of Ascomycota and Mucoromycota, while the WYG enriched for Basidiomycota (Table 1). A comparison of the GYG and WYG showed an obvious increase in the levels of Ascomycota and Cercozoa and a distinct reduction in the level of Basidiomycota. Additionally, the abundance of Cercozoa in GYG was significantly more dominant than that in HFG, whereas that of Mucoromycota was lower. Compared with the HFG, the fungal community in the WYG displayed an obvious increase in the relative abundances of *Cystobasidium*, *Naganishia*, *Sclerostagonospora*, *Physalospora*, *Neoascochyta*, *Recurvomyces*, *Schizonella*, *Orpinomyces*, *Herpotrichia*, *Sphaerulina,* and *Podospora*, while *Thelebolus*, *Cercophora*, *Stropharia*, *Lichtheimia,* and *Rhizopus* decreased dramatically. The GYG indicated dramatically higher proportions of *Didymella*, *Claussenomyces*, *Anthracoidea*, *Neoascochyta*, *Papiliotrema*, *Mastigosporium*, *Dioszegia*, *Heyderia*, *Ramularia*,

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TABLE 1 Statistical analysis of differential fungi between different groups.

Taxa	WYG (%)	HFG (%)	GYG (%)
Basidiomycota	28.61[a]	5.47[b]	5.86[b]
Ascomycota	69.90[b]	92.02[a]	90.18[a]
Mucoromycota	0.059[b]	0.58[a]	0.11[b]
Thelebolus	54.27[b]	84.52[a]	31.22[ab]
Cystobasidium	0.12[ac]	0.00080[b]	0.22[a]
Naganishia	25.43[a]	3.08[b]	1.71[b]
Cercophora	0.00[b]	0.14[a]	0.016[ab]
Stropharia	0.00[b]	0.014[a]	0.00[b]
Sclerostagonospora	0.095[a]	0.00[b]	0.00[b]
Lichtheimia	0.00[b]	0.065[a]	0.00[b]
Physalospora	0.077[a]	0.00[c]	0.015[b]
Neoascochyta	0.91[a]	0.00[c]	0.10[b]
Recurvomyces	0.077[a]	0.00[b]	0.071[ab]
Schizonella	0.019[a]	0.00[b]	0.0099[ab]
Rhizopus	0.019[b]	0.12[a]	0.032[ab]
Orpinomyces	0.021[a]	0.00[b]	0.059[ab]
Herpotrichia	0.0049[a]	0.00[b]	0.00[b]
Sphaerulina	0.0043[a]	0.00[b]	0.012[a]
Podospora	0.0098[a]	0.00[b]	0.055[ab]
Cercozoa	0.012[b]	0.00[b]	0.059[a]
Didymella	0.085[b]	0.14[ab]	0.25[a]
Claussenomyces	0.050[b]	0.046[b]	0.33[a]
Kurtzmanomyces	0.013[a]	0.022[b]	0.00[b]
Anthracoidea	0.074[b]	0.12[b]	0.37[a]
Papiliotrema	0.14[b]	0.056[b]	1.00[a]
Mastigosporium	0.00[b]	0.00[b]	0.022[a]
Dioszegia	0.0099[b]	0.00[b]	1.34[a]
Thermoascus	0.049[a]	0.039[ab]	0.0014[b]
Heyderia	0.00[b]	0.00[b]	0.041[a]
Ramularia	0.00[b]	0.00[b]	0.28[a]
Cladonia	0.00[b]	0.00[b]	0.024[a]
Urocystis	0.0075[b]	0.00[b]	0.11[a]
Panaeolus	0.0041[a]	0.00[b]	0.00[b]
Archaeorhizomyces	0.044[a]	0.10[ab]	0.00[b]
Parapenidiella	0.00[b]	0.00[b]	0.17[a]
Saitozyma	0.022[ab]	0.13[a]	0.00[b]
Mycosphaerella	0.20[ab]	0.00[b]	0.40[a]
Penicillium	0.073[ab]	0.11[a]	0.013[b]
Pyrenochaetopsis	0.056[ab]	0.058[a]	0.00[b]
Thermomyces	0.0038[ab]	0.094[a]	0.00[b]
Humicola	0.012[ab]	0.00[b]	0.19[a]
Knufia	0.0083[ab]	0.027[a]	0.00[b]
Melanocarpus	0.00[b]	0.044[a]	0.00[b]

Different letters in the same row indicate significant differences from each other.

Cladonia, *Urocystis,* and *Parapenidiella*, whereas the WYG was dramatically enriched for *Naganishia*, *Sclerostagonospora*, *Kurtzmanomyces*, *Thermoascus*, *Physalospora*, *Herpotrichia*, *Panaeolus,* and *Archaeorhizomyces*. Moreover, the abundances of *Cystobasidium*, *Claussenomyces*, *Papiliotrema*, *Mycosphaerella*, *Neoascochyta*, *Mastigosporium*, *Dioszegia*, *Urocystis*, *Humicola*, *Physalospora*, *Heyderia*, *Sphaerulina*, *Ramularia,* and *Cladonia* in the GYG were significantly preponderant compared to the HFG, while the abundances of *Stropharia*, *Lichtheimia*, *Saitozyma*, *Penicillium*, *Pyrenochaetopsis*, *Thermomyces*, *Knufia,* and *Melanocarpus* were lower. Given that this discriminant analysis did not distinguish the predominant taxon, LEfSe was used to generate a cladogram to identify the specific bacteria associated with different feeding models (Figure 5). In addition to the above-mentioned differential taxa, the GYG also showed significantly higher abundances of *Humicola*, *Vishniacozyma*, *Rachicladosporium,* and *Alternaria* as compared to the WYG and HFG.

Discussion

The wild yak is an endangered breed that has been listed as a first-class protected wild animal in China. Compared with WYG, domestic yaks can be artificially intervened and raised. Currently, there are two main feeding modes for domestic yaks: free range and artificial breeding. Growing studies have indicated that the gut fungal community plays a role in host health, metabolism, and the immune system (7, 11, 42). Recent studies on the gut fungal community have also revealed its important driving roles in the development of diarrhea, inflammatory bowel disease, and liver cirrhosis (3, 43). Currently, studies of the gut fungal community have involved many species, such as mice, pigs, and dairy cattle, and revealed their importance in host health and development (19, 39, 44). However, research on the characteristics and differences of the gut fungal community in yaks under different feeding models is still insufficient to date. Analyzing the gut microbiota of yaks under different feeding models may contribute to revealing the differences in various characteristics of these yaks. In this study, we dissected the compositions and differences of the gut fungal community among WYG, HFG, and GYG.

Microbes that inhabit the intestine can interact in symbiotic or antagonistic relationships, which are an important basis for the maintenance of gut microbial homeostasis (45, 46). Gut microbial homeostasis is the precondition for maintaining host metabolism, digestion, and absorption (47, 48). Inversely, the disruption of gut microbial homeostasis may affect intestinal barrier function and immune function, seriously threatening host health (49, 50). Gut microbial homeostasis could be assessed by diversity indices such as Chao1, ACE, Shannon, and Simpson. Typically, the diversity index representing gut microbial homeostasis is in a relatively stable state due to the plasticity of the gut microbiota (51, 52). However, multiple factors, such as exercise, diet, age, and environmental pollutants, can affect the composition and structure of the gut microbiota, thereby affecting gut microbial homeostasis (53–55). In this study, we observed that the gut fungal Shannon and Simpson indices of WYG and GYG were higher than those of HFG, indicating that WYG and GYG

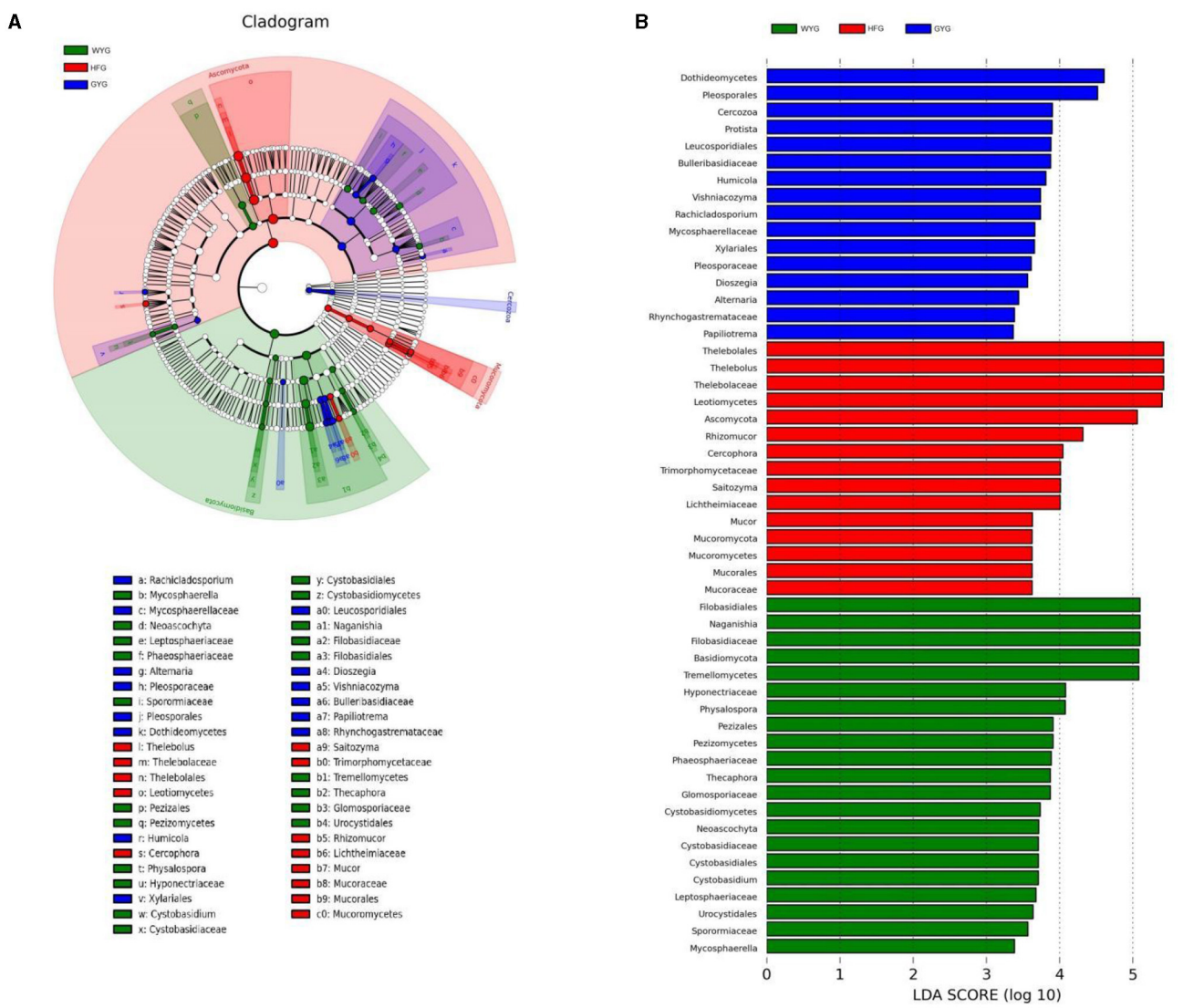

FIGURE 5
Detection of differentially abundant taxon using LEfSe and LDA scores. **(A)** Differential fungi and phylogenies are represented by a cladogram. **(B)** Differential fungi of yaks under different feeding models are represented by LDA scores.

have higher microbial diversity. Early surveys indicated that the higher diversity represents stronger gut microbial plasticity, which contributes to improving the gut microbiota's response to external stress, thereby maintaining gut microbial homeostasis (56, 57). Furthermore, increased gut microbial diversity also contributes to the maintenance and improvement of intestinal functions such as energy utilization and nutrient intake (58, 59). Studies have shown that primitive yaks existed in the Pleistocene more than 3 million years ago and were widely distributed in northeast Eurasia. Later, primitive yaks moved south to the Tibetan Plateau of China due to crustal movement and climate change, adapted to the Alpine climate, and continued to evolve into modern yaks. WYG inhabits plateau meadows, shrubs, and deserts at an altitude of 4,000 to 5,000 meters, which have strong adaptability to the environmental conditions of alpine grasslands. Environmental factors, such as altitude hypoxia and low temperatures, are important players in the evolution of gut microbiota (60, 61). Compared with domestic yaks, WYGs inhabit higher altitudes and harsher living environments. Therefore, WYG may have evolved a more complex gut microbial structure to adapt to complex environments. Compared with HFG, WYG, and GYG will constantly migrate in search of food and water supplies, which inevitably increases the amount of exercise for yaks. Numerous studies have indicated that the frequency of exercise is closely related to gut microbial composition and structure (62, 63). For instance, physical exercise could significantly alter the composition and diversity of the gut microbiota in mice (54). Compared with HFG, WYG, and GYG have more complicated and irregular diets. In addition, some plants in the wild environment may be polluted or contain pathogenic microorganisms, which also contribute to the evolution of a more complex gut microbial community. Therefore, we speculated that exercise, complex diet, and harsh environment may be some of the reasons for the more diverse gut microbiota of WYG and GYG.

The gut fungal community, as a vital constituent of the gut microbiota, is critical for host health (64, 65). The gut fungal community has been shown to be involved in the development of intestinal barrier function and intestinal inflammation (14, 66). Furthermore, recent investigations of the gut fungal community have also provided evidence that it is closely associated with diarrhea (3, 34). However, the importance and role of the gut fungal community in the host have long been overlooked because of their low proportion in the gut microbiota. To further explore the effects of different feeding models on the yaks, we also assessed the composition and variability of the gut fungal community in these populations. The results demonstrated that the phyla Ascomycota and Basidiomycota were abundantly present in the wild yak, HFG, and GYG, indicating that the different feeding models could not affect the species of the main dominant phyla. Moreover, Ascomycota and Basidiomycota have also been demonstrated to be major dominant fungal phyla in other animals, such as giraffes, sheep, and cows, suggesting their importance in the ruminant fungal community and intestinal function (3). Notably, although the species of the main dominant fungal phyla did not change, their abundances did. For instance, the abundance of Basidiomycota was significantly higher in WYG than in GYG and HFG. Basidiomycota is the highest phylum of fungi with more than 20,000 species, characterized by wide distribution, large number, and variety (67). Among these three types of yaks, WYG possessed the most complicated habitat environment and diet structure, which may change the gut microbial composition of yaks. Meanwhile, this may also be one of the reasons for the increase in Basidiomycota in WYG. In this study, we also observed significant changes in the abundance of some fungal genera between different feeding models. Previous investigations showed that shifts in some specific microbial communities could affect host phenotypes and intestinal functions (34, 68). The ecological environment and dietary structure are important driving factors for gut microbial succession, and the gut microbiota will change appropriately under various external stimuli to adapt to the habitat environment.

Conclusions

This research explores the dynamic changes of the gut fungal community in yaks under different feeding models. The results indicated that feeding models could significantly alter the gut fungal composition and structure of yaks, including significant changes in some dominant phyla (Ascomycota and Basidiomycota) and fungal genera (*Thelebolus*, *Naganishia,* and *Vishniacozyma*). Moreover, WYG and GYG had a higher gut fungal diversity compared to HFG. This investigation elucidated the characteristics of the gut fungal community in WYG, HFG, and GYG and filled a gap in the effects of different feeding models on the gut fungal community. Meanwhile, this research also indicated that different feeding models are important drivers of changes in the gut fungal community. However, this study has some limitations that need to be acknowledged, such as the small sample size and lack of information on age and dietary habits.

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 at: https://www.ncbi.nlm.nih.gov/, PRJNA948534.

Ethics statement

The study was conducted under the guidance and approval of the Animal Welfare and Ethics Committee of Institute of Animal Husbandry and Veterinary Medicine, Tibet Academy of Agriculture and Animal Husbandry Sciences.

Author contributions

YZ and WB provided the research idea. YC, GS, XL, ZL, LS, and ZS contributed reagents, materials, and analytical tools. YZ wrote the manuscript. MS, YZ, and WB revised the manuscript. All authors participated in the writing and review 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.

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Appendix

APPENDIX A Fungal sequence information for each sample.

Sample	Effective reads
WYG1	30,248.00
WYG2	31,083.00
WYG3	42,954.00
WYG4	35,967.00
WYG5	41,982.00
HFG1	38,727.00
HFG2	37,160.00
HFG3	41,853.00
HFG4	39,301.00
HFG5	36,407.00
GYG1	45,539.00
GYG2	42,840.00
GYG3	36,021.00
GYG4	32,081.00
GYG5	44,801.00

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Revealing novel *cytb* and *nad*5 genes-based population diversity and benzimidazole resistance in *Echinococcus granulosus* of bovine origin

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Cystic echinococcosis (CE) is a neglected zoonotic disease caused by *Echinococcus granulosus* (*sensu stricto*). The parasite affects a wide range of livestock and wild animals. In this study, the population diversity of the *Echinococcus* species was investigated based on mitochondrial cytochrome b (*cytb*) and NADH dehydrogenase subunit 5 (*nad*5) genes. In addition to this, β-tubulin gene isoforms of *Echinococcus granulosus* were amplified to determine the resistance against benzimidazoles. For this purpose, 40 cyst samples from cattle (n=20) and buffaloes (n=20) were collected from the main abattoir of Sialkot. DNA extraction was performed using Qiagen Blood and Tissue Kits. Amplification was performed through PCR. Each amplicon was confirmed by GelRed™ stained agarose gel (2%). Samples were sequenced in a DNA analyzer and viewed for any misread nucleotide by using MEGA (v.11). Corrections in nucleotide sequence and multiple sequence alignment were made through the same software. NCBI-BLAST was used for sample specific sequences to identify them as belonging to a particular species. Diversity indices were estimated using DnaSP (v.6) while phylogenetic analysis was inferred using the Bayesian method using MrBayes (v.1.1). β-tubulin gene isoforms sequence analysis was performed to find out the candidate gene causing benzimidazole resistance. All 40 isolates were found positive for *E. granulosus.* BLAST-based searches of sequences of each isolate for each gene (*nad*5 and *cytb*) confirmed their maximum similarity with the G1 genotype. Overall, high haplotype diversity (Hd *nad5*=1.00; Hd *cytb*=0.833) and low nucleotide diversity (π *nad5*=0.00560; π=*cytb*=0.00763) was identified based on diversity indices. For both the genes, non-significant values of Tajima's D (*nad*5=−0.81734; *cytb*=−0.80861) and Fu's Fs (*nad*5=−1.012; *cytb*=0.731) indicate

recent population expansion. Bayesian phylogeny-based results of *nad5* and *cytb* sequences confirmed their genotypic status as distinct from other *Echinococcus* species. This study shed light on the status of benzimidazole resistance in *Echinococcus granulosus* for the very first time from Pakistan. The findings of this study will significantly add in the information available on genetic diversity of *Echinoccous granulosus* based on *cytb* and *nad5* genes sequences.

KEYWORDS

Echinococcus granulosus*, genetic diversity, phylogeny, benzimidazole resistance, *cytb*, *nad5

1. Introduction

Pakistan is an agriculture-dependent country heavily relying on the livestock sector which contributes 60.03% in agriculture and plays an important role in uplifting the national economy by accounting for 11.53% of the national gross domestic product (GDP) (1). A recent livestock census revealed that there are at least 51.5 million and 42.4 million heads of cattle and buffalo in the country, respectively (1). Several worldwide reports suggest that parasitic diseases cause significant economic losses to the livestock industry and severely affect weight gain, feed conversion efficacy, and the health of the animals. Among these parasitic diseases, potential harms contributed by *Echinococcus* infection are being neglected; therefore, cystic echinococcosis (CE) was listed in a subcategory of selected neglected tropical diseases (NTDs) to be addressed by the World Health Organization's action plan to control NTDs (2, 3). The reported prevalence in Pakistan of the *Echinococcus* species in cattle, buffalo, sheep, goat, and camel is 5, 7, 7.5, 5, and 17%, respectively (4).

The *Echinococcus granulosus sensu* lato complex includes at least five cryptic species and some distinct genotypes namely *E. granulosus sensu stricto* (G1, G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6-8, G10), and *E. felidis* (5–7). Cystic echinococcosis (CE) is transmitted between carnivores and herbivore/omnivore species which serve as definitive and intermediate hosts, respectively. *Echinococcus granulosus sensu stricto* (s.s.) possesses a wide intermediate host range (including bovine) which bear parasite larval stages in different visceral organs (8). This neglected zoonosis is a disease of public health concern and has serious economic setbacks. *E. granulosus* poses significant threats to human and animal health besides significant economic losses (9). Cosmopolitan distribution of this ailment had led to losses of USD 3 billion annually (10).

Resistance to benzimidazole drugs, an inherited genetic trait in parasites, has been observed worldwide and it appears to be enhancing (11). It is associated with nucleotide substitution in the β-tubulin gene, which indicates conservative point mutations. Anthelmintic resistance allele identification is critical for understanding the mechanisms involved and the epidemiology of anthelmintic resistance. The lack of data on these aspects of disease put livestock and human populations at high risk and may result in a high burden of disease in the future.

Owing to the limited data on the population structure of CE especially based on *nad*5 and *cyt*b genes, and with no research on benzimidazole resistance having been conducted in Pakistan, the present study was designed to determine genetic diversity and benzimidazole resistance in *Echinococcus granulosus* recovered from cattle and buffalo in Pakistan.

2. Materials and methods

2.1. Sample collection, processing, and DNA extraction

This study was carried out in the Sialkot district of the Punjab province of Pakistan. Twenty cyst samples were taken from cattle and another 20 from buffalo from the main abattoir under the jurisdiction of the municipal corporation of Sialkot from male as well as female animals during January–April, 2022. After slaughtering, carcasses were examined thoroughly for the presence of *Echinococcus* cysts particularly in the liver, lungs, and kidneys through visual inspection and palpation. Collected samples were delivered to the Department of Clinical Medicine and Surgery, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan, under proper refrigerated conditions and processed aseptically. Ethical approval/consent from the animal owners was not required as no living animal was included in the study and the samples for this study were taken from the condemned carcasses of slaughtered animals.

After samples were delivered to the laboratory, each cyst was handled carefully. Each cyst was washed multiple times with a normal saline solution to decrease the chances of contamination. Then each cyst was washed with 70% ethanol. Cyst contents including fluid and protoscoleces were collected using sterile syringes and placed in conical 50 mL tubes (SPL Life Science Co., Ltd.). Then, an incision was made on the cyst wall and the remaining protoscoleces and cyst fluid were collected. The germinal membrane was taken from the cyst not apparently having protoscoleces at the bottom. Cyst contents were shifted to the sterile test tubes for centrifugation at 3,000 rpm at room temperature for 10 min. The supernatant was removed and only 1.5 mL of the precipitate from the bottom of the test tube was collected in non-pyrogenic, 1.5 mL microcentrifuge tubes (Kirgen, Solutions for Science). DNA was extracted from the germinal layer and/or collected protoscoleces using Qiagen Blood and Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. DNA amplification and sequencing

Partial segments of *nad*5 and *cyt*b genes were amplified to investigate population diversity. In addition to this, β-tubulin 1 (β-*tub*1), β-tubulin 2 (β-*tub*2), and β-tubulin 3 (β-*tub*3) gene isoforms amplification was carried out to determine benzimidazole resistance. The stock solution of the primers was made by adding the mentioned volume of the double deionized water whereas the working solutions

TABLE 1 The sequence of primers used in this study to amplify full-length *cytb*, *nad5*, and β-tubulin gene isoforms of *Echinococcus granulosus*.

Primer name	Sequence (5′ - 3′)	Reference
nad5 Forward	GTTGTTGAAGTTGATTGTTTTGTTTG	(12)
nad5 Reverse	GGAACACCGGACAAACCAAGAA	
cytb Forward	GTCAGATGTCTTATTGGGCTGC	(13)
cytb Reverse	TCTGGGTGACACCCACCTAAATA	
β-*tub1* Forward	GGATTGCTCTCAGCTTTGAAAACTA	(14)
β-*tub1* Reverse	CACGGTACTGTTGACTGCCACGACT	
β-*tub2* Forward	CCTCCAGGGCTTCCAGCTCACCCAC	
β-*tub2* Reverse	GAAGTGCAGGCGCGGGAATGGAACC	
β-*tub3* Forward	TTTAGCAGGTGACCAGCCCTTCTAA	
β-*tub3* Reverse	GAGGTTCGACTGGCGAGGGGCGCAA	

of the forward as well as reverse primers were prepared by adding 10 µL of the stock solution to the 90 µL of the ultra-pure water in a 1.5 mL microcentrifuge tube. The forward and reverse sequences of the primers are mentioned in Table 1. The reaction mixture in the PCR tube was comprised of forward and reverse primers (10 pmol), Premix (12.5 µL), sample DNA (0.5 µL), and ultrapure PCR water to make up the final volume of 25 µL. The PCR conditions for amplification of *nad5*, *cytb*, *tub1*, *tub2*, and *tub3* genes are given in Table 2.

2.3. Gel electrophoresis

TAE buffer (1%) was prepared by adding 20 mL of the TAE buffer (50X) to 980 mL of distilled water. Agarose gel (2% w/v) was prepared by adding 1.2 gm of the agarose powder (Agarose, Tsingke Biotechnology Company, Beijing) to the 60 mL of TAE buffer (1%) in a flask. It was allowed to melt in the refrigerator for 2–3 min until it became transparent. Three microliters of the GelRed™ (Biotium, Fremont, United States) were added for the purpose of staining. The gel was poured into the gel casting tray and allowed to solidify. The gel was then placed in the gel electrophoresis apparatus and 5 µL of each amplicon were loaded. A DNA ladder with a length of 2,000 base pairs were run to determine the size of each amplicon.

The gel was run in a gel electrophoresis apparatus (Bio-Red) at 110 V for 20 min. Amplicon (5 µL) in each well of the gel were observed under a UV illuminator. Amplicons were sent to Lanzhou Veterinary Research Institute, China, for sequencing and further analyzes.

2.4. Molecular analysis

Sequences were viewed for any misread nucleotide by using MEGA software (v11.0). Corrections in nucleotide sequence and multiple sequence alignment were made through the same software. NCBI-BLAST was used for sample specific sequences to identify them as belonging to a particular species. Sequences were downloaded from the NCBI in FASTA format in order to make the phylogenetic tree. *Taenia hydatigena* was used to make an outgroup. Diversity indices were estimated using DnaSP (v.6) software while phylogenetic analysis was inferred using the Bayesian method using MrBayes (v.1.1) software.

3. Results

All 40 samples were found positive for *E. granulosus* when they were subjected to amplification through PCR using *nad5* and *cytb* partial genes. The *nad5* and *cytb* partial genes generated PCR products of about 680 bp and 580 bp, respectively. β-tubulin gene isoforms (β-*tub1*, β-*tub2*, and -*tub3*) produced PCR products of 440 bp as shown in Figure 1.

All PCR products for both of the partial genes were sent for sequencing. After trimming, alignment, and correction of misread nucleotides, the final length of sequences was 625 bp (*nad5*) and 393 bp (*cytb*) with a total length of 1,018 bp. The aligned and edited sequences created a consensus sequence which was compared to sequences archived in the NCBI database using NCBI BLAST-N.[1] The sequences were deposited in the GenBank with the accession numbers (ON241337-ON241340) and (ON921008-ON921011) for both *nad5* and *cytb* genes. In this study, only one genotype (G1) of *E. granulosus* was identified.

All the samples were collected from the lungs and livers of cattle and buffaloes; although other organs were also observed for the presence of cysts, samples were only found in those two kinds of organs (lung and liver). It was identified that livers (70%) were more commonly infected compared with lungs (30%). The majority of the samples were infertile. Regarding fertile cysts, 50 % of cysts were of liver origin whereas the remaining fertile samples were collected from the lungs. Comparative data on cyst location and cyst fertility is given in Table 3.

Sequences of nucleotides and their respective encoded amino acids were examined for mutations. A total of seven and six nucleotide mutations were found in *nad5* and *cytb* genes, respectively. These resulted in amino acid changes at six and five positions in *nad5* and *cytb* genes, respectively.

Nucleotide variation positions of *nad5* and *cytb* genes and their respective amino acids changes are mentioned in Tables 4–7. Positions at which no variation was identified are marked with dot marks whereas a letter indicates a variant at this position. Overall, nucleotide mutations in *nad5* and *cytb* genes were found at seven and six different positions, respectively. Amino acids substitution in *nad5* and *cytb* genes were identified at six and five different sites, respectively.

3.1. Nucleotide polymorphism and population indices

The diversity of nucleotides and neutrality indices for *E. granulosus* population were calculated on the basis of *nad5* and *cytb* partial genes. We observed high haplotype diversity (Hd) and low diversity of nucleotides (π) for *E. granulosus* for both genes in large ruminants (cattle and buffalo). Overall, haplotype diversity (Hd) and nucleotides diversity (π) values were as follow: *nad5* (Hd = 1.00, $\pi = 0.00560$) and *cytb* = (Hd = 0.833, $\pi = 0.00763$). The Fu's Fs values were non-significant

1 http://blast.ncbi.nlm.nih.gov

($p > 0.05$) for the *nad*5 gene and *cyt*b genes. The values of Tajima's D were insignificant and negative for the whole population (Table 8).

3.2. Phylogenetic analysis

The *nad*5 main haplotype showed 99% homology with the Turkish isolates from cattle, Italian isolates from sheep, Australian isolates from the dingo, Indian isolates from buffaloes, Mexican isolates from pigs, and Chinese isolates from the yak. Likewise, the *cyt*b main haplotype showed 99% similarity with the Indian isolates from buffaloes, Mexican isolates from pigs, Turkish isolates from sheep, Iranian isolates from cattle, and Argentinian isolates from sheep. Two phylogenic trees were constructed based on *nad*5 and *cyt*b partial genes by combining our submitted sequences with the deposited sequences from other regions in the GenBank. All of the *E. granulosus* (s.s.) isolates from Pakistan were grouped in the same clusters with the relevant reference strain sequences from GenBank, according to a Bayesian phylogeny relying on data of *nad*5–*cyt*b gene sequences. The genotype/species status as distinct from other *Echinococcus* species was validated by Bayesian phylogenetic inference. Pakistani isolates were grouped together and formed a separate cluster while the isolates of other countries formed another cluster (Figure 2). *Taenia hydatigena* (NC012896) was used as an outgroup.

3.3. β-tubulin gene isoforms analysis

After the alignment of the three β-tubulin gene isoforms through MEGA (v. 11.0) software, sequences were compared with the reference sequences from GenBank of *E. multilocularis* in order to identify amino acid substitutions. The accession number of the reference sequences for β-tubulin isoforms I, II, and III were FJ997216, CAB91640, and CAB91642, respectively. The mutation was identified in β-tubulin gene isoform I (position 167), isoform II (position 165 and 167), and isoform III (position 200) as shown in Figures 3–5, respectively.

4. Discussion

Infectious diseases including parasitic infestations are important health problems in both animals and humans, which cause economic losses and severe illness (15–20). Parasites are responsible for causing diseases that lead to heavy economical losses in terms of decreased productivity and illness (21–24).

CE is a neglected livestock and public health issue in the world. A high prevalence of this disease can be seen in some areas of China, Central Asia, Peru, and Africa (25). The prevalence is high in those rural/peri-urban areas where aged livestock animals are preferred for slaughtering (26). The reasons behind the presence of disease include lack of awareness and poor hygiene practices (27). However, researchers are trying to change the situation by diagnosing the disease using advanced assays and identifying the extent of drug resistance with the aid of molecular tools.

Since CE is caused by different *Echinococcus* genotypes (G1, G3), therefore, molecular investigations help in better characterization of these genotypes. Identification of the genotype responsible for causing the disease aids in devising preventive and control strategies in a particular geographical area. A limited number of studies on

TABLE 2 PCR reaction conditions to amplify partial *cytb*, *nad5*, β-*tub1*, β-*tub2*, and β-*tub3* genes of *Echinococcus granulosus*.

Steps	Cycles	*cytb*	*Nad5*	*Tub1*	*Tub2*	*Tub3*
Initial denaturation	1	7 min at 95°C				
Denaturation	35	15 s at 95°C	15 s at 95°C	30 s at 95°C	30 s at 95°C	30 s at 95°C
Annealing		40 s at 55°C	30 s at 54°C	30 s at 60°C	30 s at 63°C	30 s at 63°C
Extension step		90 s at 72°C	60 s at 72°C	60 s at 72°C	60 s at 72°C	60 s at 72°C
Final extension	1	7 min at 72°C				

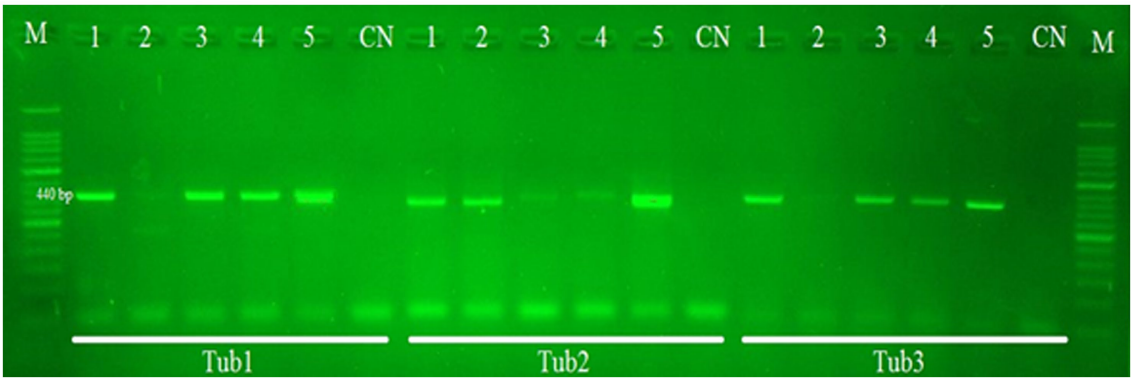

FIGURE 1
PCR gel results of *Echinococcus tub*1, *tub*2, and *tub*3 partial gene (440bp) amplification. Lane M contains the DNA marker (50bp). Lane CN indicates the negative control. Lanes 1–5 indicate positive as well as negative samples from cattle and buffalo.

TABLE 3 Data on cyst fertility and organ location in cattle and buffaloes.

Cyst location	Cyst fertility	No. of cyst in cattle	No. of cyst in buffaloes
Liver	Infertile	11	15
Liver	Fertile	1	1
Lung	Infertile	6	4
Lung	Fertile	2	0
Total		20	20

TABLE 4 *Echinococcus granulosus* mitochondrial *nad*5 gene nucleotide sequence mutation sites.

	2	8	19	83	257	336	572
Seq1	C	T	T	A	C	G	T
Seq2	.	.	C	.	.	.	.
Seq3	T	C	.	.	T	C	C
Seq4	.	.	.	G	.	.	.

TABLE 5 *Echinococcus granulosus* mitochondrial *nad*5 gene substitution in amino acids among haplotypes from bovines.

	1	3	7	28	86	191
Seq1	S	V	S	N	P	I
Seq2	.	.	P	.	.	.
Seq3	F	A	.	.	L	T
Seq4	.	.	.	S	.	.

TABLE 6 *Echinococcus granulosus* mitochondrial *cytb* gene nucleotide sequence mutation sites.

	14	19	98	100	107	114
Seq1	G	G	C	A	C	A
Seq2	.	.	.	G	.	.
Seq3	.	.	.	.	.	.
Seq4	A	A	A	.	A	C

TABLE 7 *Echinococcus granulosus* mitochondrial *cytb* gene substitution in amino acids among haplotypes from bovines.

	5	7	33	34	36
Seq1	R	E	A	N	A
Seq2	.	.	.	D	.
Seq3	.	.	.	.	.
Seq4	Q	N	E	.	E

E. granulosus have been done in Pakistan up to now which indicates that the disease is underestimated in this region.

Still, government abattoirs continue to play an important role in slaughtering animals in several developing countries including Pakistan, offering economy of scale by putting together livestock owners, processors, and purchasers. Surprisingly, the majority of CE prevalence investigations have been done at urban or peri-urban

TABLE 8 Diversity and neutrality indices for *Echinococcus granulosus* (s.s.) populations from Pakistan based on *nad*5 and *cytb* genes.

Indices	*nad*5 (625bp)	*cytb* (393bp)
No. of isolates	40	40
No. of mutations	7	6
Parsimony informative sites	0	1
No. of haplotypes	4	3
Haplotype diversity (Hd)	1.000	0.833
Nucleotide diversity (π)	0.00560	0.00763
Tajima's D (*p*-value)	−0.81734	−0.80861
Fu's Fs (*P*-value)	−1.012	0.731

government abattoirs, implying that such facilities might also exacerbate disease transmission (28). The factors augmenting the disease transmission comprise lack of cooperation by the butchers, poor inspection methods of the veterinary staff, and improper disposal of the infected viscera (29). The current analysis included samples from the main abattoir in Sialkot where people bring cattle and buffalo from different rural, urban, and peri-urban areas with different geographical conditions. This city has a significant population of stray dogs around abattoirs and butcher shops which act as definitive hosts in CE dissemination. Poor hygienic conditions and waste management make the environment favorable for disease transmission to livestock as well as humans. Dogs become infected by eating the viscera (liver and lungs) of diseased animals dumped by the butchers around the slaughterhouse. In this way, the life cycle of the parasite is completed. The same observations were also highlighted previously (30).

Travel to or from prevalent areas and domestic or occupation-based exposure to canids are the risk factors of CE (31, 32). Humans and animals are infection through parasite eggs. The areas around the abattoirs and butcher shops are the main risk factor. The lifestyles of people in rural areas increases the chances of disease transmission where humans and animals live in close vicinity by sharing residence boundaries. Sanitary conditions are very poor not only in rural areas but also in urban areas. All these risk factors contribute to the spread of disease between humans and animals. Therefore, strategies should be devised to control these factors of disease transmission.

Mitochondrial DNA has a significant role in studying intraspecific differences and population genetics due to maternal inheritance, conserved nature, lack of reassortment, high mutation, and evolutionary rate (33–35). Nuclear genes are not preferred due to their different recombination at each generation. In this study, we revealed the population structure of *E. granulosus* (G1/G3) cyst samples collected from cattle and buffalo from the main abattoir in the Sialkot district, Pakistan, based on the partial sequences of two mitochondrial genes, *nad*5 and *cyt*b. The correct identification and characterization of genotypes G1 and G3 is of great epidemiological concern because of the dominance of these two genotypes in animals and humans all over the world.

Although many mitochondrial DNA markers were devised for the molecular identification of *E. granulosus* (36–42), it is controversial to differentiate the genotypes G1 and G3. The *nad*5 gene has been considered more reliable in differentiating differences between G1 and G3 genotypes because it possesses six important positions in the comparatively small mitochondrial DNA fragment of 680 bp (12). The

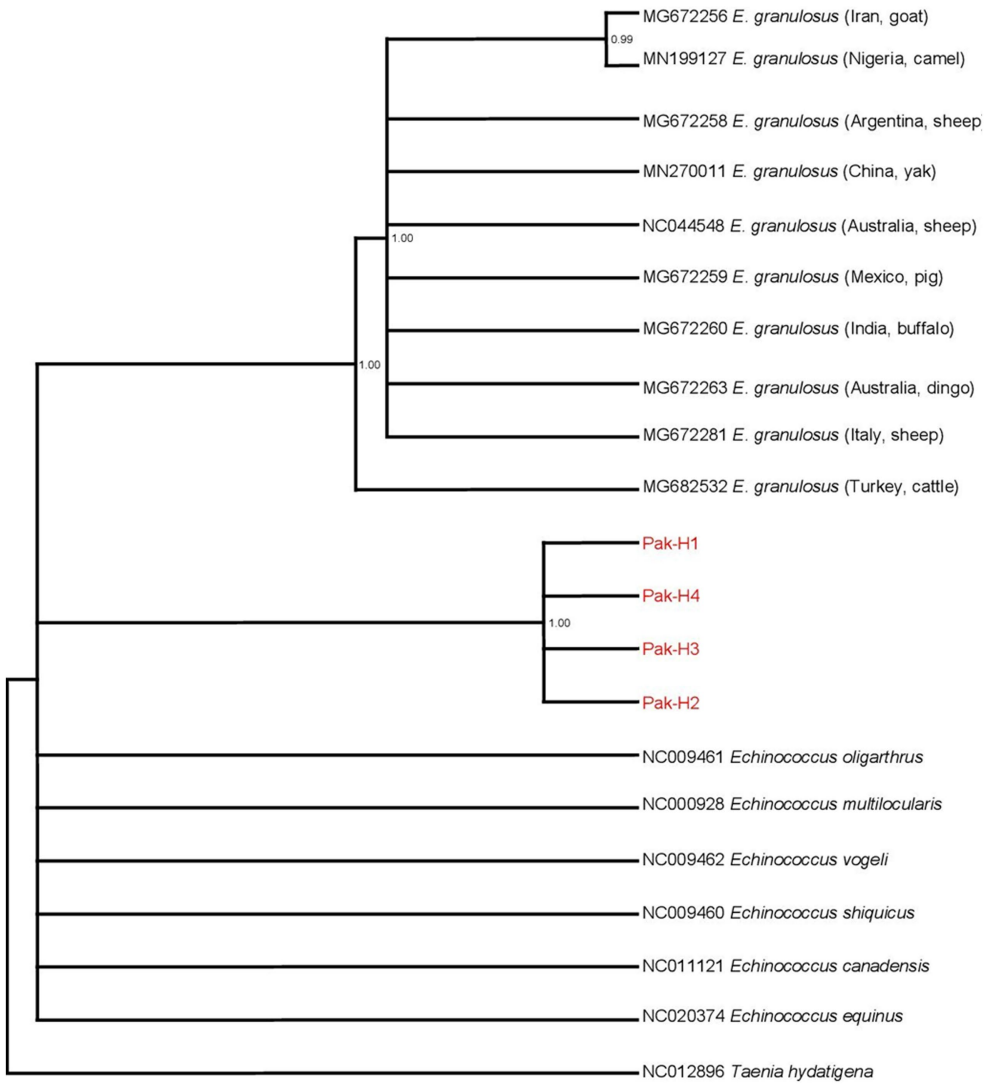

FIGURE 2
Bayesian phylogeny of Pakistani *E. granulosus* isolates inferred from the *nad*5 and *cytb*. *Taenia hydatigena* was used as an outgroup. Red font indicates *E. granulosus* haplotypes isolated in this study. Posterior probability values are depicted at the nodes.

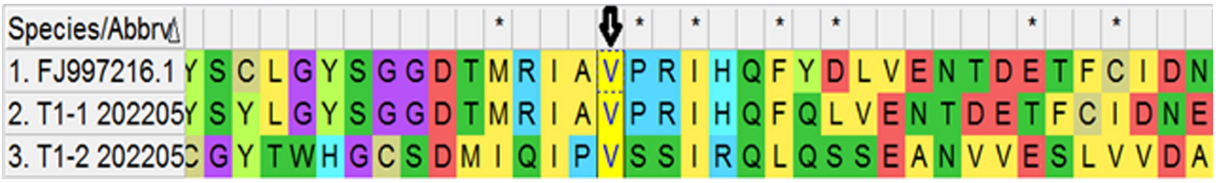

FIGURE 3
Predicted amino acid sequence of two conformers of Tubulin I. Arrows indicate the valine (V) at position 167.

*cyt*b gene fragment contains three informative positions. Therefore, we used *nad5* along with the *cyt*b gene in order to explain in a better way the population structure of the *E. granulosus* s.s. complex. Moreover, Kinkar et al. (12) also explained that considering single nucleotide polymorphism in genotype is not sufficient for the discrimination of genotypes G1 and G3 and multiple positions are important to consider. In the previous studies in Pakistan, the population structure of *E. granulosus* in cattle and buffalo was assessed using other genes like *cox*1, *nad*1, 12S *rRNA*, and *rrnL* (ribosomal RNA larger subunit) (4, 43–46), but *nad*5 and *cyt*b genes were not used. In our study, we used short DNA fragments with various informative sites as using larger mitochondrial DNA fragments is not effective or appropriate when the quality of isolated DNA is poor. It is also in accordance with the study by Kinkar et al. (12), who preferred

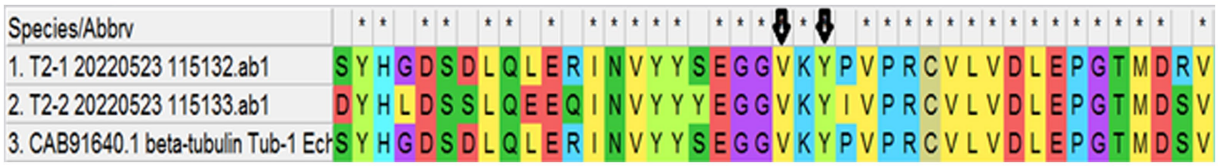

FIGURE 4
Predicted amino acid sequence of two conformers of Tubulin II. Arrows indicate the valine (V) and tyrosine at position 165 and 167.

FIGURE 5
Predicted amino acid sequence of two conformers of Tubulin III. Arrow indicates the valine (V) at position.

using small mitochondrial DNA fragments with multiple positions. Furthermore, Kinkar et al. (12) suggested that it is better to use the *nad*5 gene along with other markers, which also supports our study since we have used *nad*5 with the *cyt*b gene marker.

Regassa et al. (47) reported that *E. granulosus* mostly infects the liver, lungs, kidneys, heart, and spleen of cattle and buffalo. In this study, all the organs of large ruminants were carefully inspected but only the liver and lungs were found infected with CE which is in accordance with the study conducted by Alvi et al. (48). It was identified that livers (70%) were more commonly infected compared with lungs (30%), which was also verified by Varcasia et al. (49). In contrast to these results, Regassa et al. (47) declared that most samples were of lung origin due to dilatation of lung capillaries in cattle of older age. This might allow the oncosphere to directly pass into the lungs. It could also be carried via thoracic ducts to the lungs and heart and, in this way, the lungs of cattle are more prone to infection compared with the liver.

In this study, it was reported that the lungs and liver have an equal chance of possessing fertile cysts because 50% of cysts were of liver origin whereas the remaining fertile samples were collected from the lungs. In contrast to these results, Regassa et al. (47) reported that the lungs were more commonly identified with fertile cysts compared with the liver.

In this study, genotype G1 was found in all isolates and was confirmed as the most dominant genotype, which is in accordance with the reports from other countries: China (96%), Iran (87%), Brazil (11%), Italy (72%), Turkey, (66%), and Pakistan (44%) (4, 50–54). Moreover, in contrast to current results, genotype G3 was identified as the most prevalent strain as reported by Sharma et al. (55) and Pednekar et al. (56). Thus, it can be concluded that G1 has emerged as the most dominant genotype in East Asia.

In the present study, low nucleotide and high haplotype diversity in the case of both genes revealed the expansion of a small parasite population as previously reported by Sharma et al. (55). A variety of statistical approaches were devised to evaluate the nucleotide neutrality index and population growth (57). The *cyt*b main haplotype showed 99% similarity with the Turkish isolates from sheep, Iranian isolates from cattle, and Argentinian isolates from sheep (12, 58). This finding provides support to the theory of diffusion of CE which postulates that the *E. granulosus* s.s. emerged in the Middle East due to the domestication and farming of sheep and then spread to European, African, American, and Asian countries through the human-related movement of animals (intermediate host) via livestock trading activities (39, 59).

In the current investigation, four samples on the basis of the *nad*5 partial mitochondrial gene yielded four haplotypes, whereas four isolates of the *cyt*b partial gene yielded three haplotypes and were dissimilar to the six haplotypes yielded from 16 isolates collected in a previous study conducted by Alvi et al. (48) and nine haplotypes obtained from 121 isolates as reported by Mehmood et al. (46) using the *nad*5 partial gene. Overall, our findings suggest the occurrence of intraspecies diversity inside *E. granulosus* (s.s.) complex and a growing population in the study region. Variations in haplotype distribution among different populations of *E. granulosus* in Faisalabad and other regions of Pakistan have been reported (48). This might be due to the use of partial sequences (60), investigations on different mitochondrial genes, or the differences in the rate of evolution and mutation of different mitochondrial genes (61). The bovine isolates were identified as *E. granulosus* (G1) by the phylogenetic analysis of the sequenced genes, which grouped closely with the species of *E. granulosus* complex (G1-G10) sequences recovered from the GenBank. All Pakistani isolates formed a separate distinct cluster within the phylogenetic tree, indicating that these haplotypes are different from those of other countries.

In the current study, we used Tajima's D and Fu's Fs values to find the trend in population growth. Tajima's D is a test that compares the overall allelic probability of segregating nucleotides (62). A negative value of the test suggests a tendency toward an excess of uncommon alleles, which is an indication of the recent growing population. The alleles/haplotype distribution is the basis of Fu's Fs test, and negative results can suggest an overage of alleles, as is being predicted from recent population expansion (63). In the present study, Tajima's D values were negative and insignificant in the neutrality test based on both genes, showing an increase in low-frequency polymorphism and

indicating a significant population expansion. For combined *nad*5 + *cyt*b sequences, Fu's Fs test values were negative, also indicating a recent population expansion.

The development of resistance in the parasites against different chemical drugs has led to making more efforts to explore alternative or complementary medicines in order to overcome the emerging problem of drug resistance development (64–67). Benzimidazoles are anti-helminth drugs, extensively used all over the world against the helminths of ruminants as well as humans (68). The occurrence of resistance would be assessed phenotypically by considering the poor efficacy of the veterinary medications used to combat parasitic illnesses. This is frequently addressed by increasing the antiparasitic dose rate. Moreover, greater withdrawal times following anthelmintic administration would be required in such instances, but they would not always be maintained, resulting in higher drug residues in human-consumed products which could be a health issue. Anthelmintic resistance allele identification is critical for understanding the mechanisms involved and the epidemiology of anthelmintic resistance. The lack of data on these aspects of disease may put livestock and human populations at high risk and may result in a high burden of disease in the future.

The process of benzimidazole resistance in cattle and buffalo helminths, including *E. granulosus*, has been explored, suggesting that the difference in three different amino acids of the B-tubulin isotype II is involved. Amino acid residue at position 200, 167, or 165 of the gene sequences can result in the development of resistance. Previous studies suggested that phenylalanine (F) amino acid at position 167 and 200 of β-tubulin isoforms indicates benzimidazole sensitivity, whereas the presence of other amino acids such as valine (V) and tyrosine (Y) indicate resistance to benzimidazoles in parasites such as *Haemonchus contortus* and *Teladorsagia circumincta* (69, 70).

A review of the literature suggests that mutations in any one of the three codons (200, 167, and 198) can cause resistance to benzimidazoles, and genotypes analysis of β-tubulin genes of individual helminths revealed that combined genetic changes could not take place in the same allele of β-tubulin isotype-1 protein. This demonstrates that several mutations in the same gene/allele can result in death of the parasite (71, 72). In the current study, benzimidazole resistance was identified in β-tubulin isoforms I at position F165V, isoform II at position F165V and F167Y, and isoform III at position F200V, whereas Pan et al. (10) reported resistance against benzimidazoles in only β-tubulin isoform II at position F165V and F167Y. The results revealed that β-tubulin gene isoforms I, II, and III are responsible for benzimidazole resistance in the selected study area. Efforts should be oriented toward discovering nanoparticle-based antiparasitic drugs as these have shown promising *in-vitro* results (73, 74).

The presence of the zoonotic G1 genotype in cattle and buffalo is of great public health importance, particularly in those areas where the meat of large ruminants is being consumed. Therefore, proper hygienic measures including the disposal of infected viscera should be implemented. This is the first study from Pakistan reporting benzimidazole resistance in *E. granulosus* isolates. The recent findings imply that resistant parasites have been selected during the repeated treatment with benzimidazole and the resistant parasite load will increase if control measures are not optimized. Moreover, investigations should also be carried out in other areas of Pakistan as well to find out the infective parasitic species and status of benzimidazole resistance using the same genes. However, the current study was carried out in one district (Sialkot) and only 40 isolates from cattle and buffaloes were processed for investigations, which are potential limitations of the study.

5. Conclusion

The study demonstrates the preponderance of the G1 genotype in bovines slaughtered in the study area. More epidemiological research should be conducted in Pakistan's various climate zones using additional *Echinococcus* isolates from definitive hosts as well as all intermediate hosts. The molecular analysis of the *nad*5 and *cytb* genes showed a high degree of genetic variation among the Pakistani *E. granulosus* population. These findings on the genetic variation of *E. granulosus* will constitute useful baseline information for future studies on the prevalence and population structure of *E. granulosus* throughout the world based on *nad*5 gene sequences. The study also provided the first molecular evidence of benzimidazole resistance from Pakistan in *E. granulosus* isolates using β-tubulin isoforms that will also help to devise strategies for preventing the spread of benzimidazole resistance globally.

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 Ethical approval/consent from the animal owners was not required for the study as no living animal was included in the study and the samples for this study were taken from the condemned carcasses of the slaughtered animals.

Author contributions

MA, HBY, and MuS conceptualized the study. MA, MS, RA, RB, and MG designed the methodology. MA, RA, LL, YYL, and MSS carried out the validation. MA, RA, and SH wrote the original draft. AA, MI, MUI, HBY, BQF, and WZJ reviewed and edited the manuscript. MA, RA, MuS, and IA revised the manuscript. HBY and WZJ acquired the funding. All authors have read and agreed to the published 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.

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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.2023.1191271/full#supplementary-material

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Chromogenic culture media complements diagnostic cytology in the visual identification of pathogenic skin bacteria in dogs and cats

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In dogs and cats, bacterial skin infections (pyoderma and otitis externa) are a common cause for visiting the veterinary clinic. The most frequent skin pathogens are *Staphylococcus pseudintermedius*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, often requiring different therapeutic antibiotic protocols. Unfavorably, existing diagnostics based on cytology cannot reveal bacterial species but only bacterial shapes such as cocci or rods. This microscopic limitation could be overcome by clinical translation of affordable chromogenic media, which enable species identification based on bacterial colonies growing in different colors and sizes. In this study, we determined how well inexperienced general veterinary clinicians identified bacterial pathogens from the skin and ears on two commercial (Chromatic™ MH and Flexicult® Vet) and one custom-made Mueller Hinton agar-based chromogenic medium. For this purpose, four veterinarians evaluated 100 unique samples representing 10 bacterial species. On average, clinicians correctly identified between 72.1 and 86.3% of bacterial species. Colony colors developed quickly on the Chromatic™ MH medium, leading to the highest 81.6% identification accuracy after 24 h incubation. However, Flexicult® Vet exhibited the highest accuracy of 86.3% after prolonged 48h incubation. Evaluators easily recognized bacteria displaying uniquely colored colonies like green-brown *Pseudomonas aeruginosa*, blue *Enterococcus faecalis,* orange-brown *Proteus* spp., and red *Escherichia coli*. Oppositely, staphylococci shared uncharacteristically pale pink colonies causing misidentifications among the genus, deteriorating overall accuracy by around 10 percentage points (from 90.9%). Another reason for identification errors was the evaluators' inexperience, reflected in not recognizing colony size differences. For example, although *Streptococcus canis* exhibited the tiniest colonies, the species was frequently mistaken for other cocci. Finally, around 10% of errors were negligence-related slips due to unconsidered sample history. To conclude, the introduction of chromogenic media into veterinary clinics can significantly complement diagnostics in skin inflammations by identifying pathogen species in around 80% of cases. The extra information may help in therapeutic dilemmas on antibiotics and standard antimicrobial susceptibility testing. Additional personnel training and evaluation help by visuals, flowcharts, checklists, and, if necessary, microbiologists could further improve identification accuracy.

KEYWORDS

chromogenic medium, chromogenic agar, skin pathogens, pyoderma, otitis externa, point-of-care assay, bacterial identification, veterinary dermatology

1. Introduction

Skin diseases in dogs and cats are one of the main reasons for visiting a veterinarian. Bacterial or fungal infection of the skin (pyoderma) and external ear canal (otitis externa), parasitic infestation, and neoplasia account for the majority of dermatological cases (1, 2). *Staphylococcus pseudintermedius* (*S. pseudintermedius*) causes most pyodermas in dogs (3–5) and *S. aureus* in cats (6–9). Other less common infection-causing bacteria are *Staphylococcus felis* (*S. felis*) (in cats), *Staphylococcus schleiferi* (*S. schleiferi*), *Escherichia coli* (*E. coli*), *Enterococcus faecalis* (*E. faecalis*), *Klebsiella* spp., *Proteus* spp., *Pseudomonas aeruginosa* (*P. aeruginosa*), *Corynebacterium* spp., and *Streptococcus canis* (*S. canis*) (4, 7–11). The scientific and medical community pays special attention to methicillin-resistant *S. pseudintermedius* (MRSP) and *S. aureus* (MRSA) due to their potential impact on the health of animals and people (12). More troublesome, Blondeau et al. (13) demonstrated that *S. pseudintermedius* directly transmitted from a family dog caused a urinary tract infection in a human. Finally, bacteria such as *P. aeruginosa* may produce biofilms leading to very challenging treatment of (often persistent) otitis externa (14).

Diagnosing skin and ear infections relies on cost-effective and fast cytology (15). A clinician swabs the inflamed spot and stains the deposited material on the microscopic slide, generally with commercial Romanowsky stains like Diff-Quik™ (RAL Diff-Quik™, CellaVision, Lund, Sweden). Observation by microscope can reveal inflammatory response cells (e.g., neutrophils) and pathogens like bacteria or yeasts (e.g., *Malassezia pachydermatis*). A significant cytological shortcoming is that bacterial identification is limited to their shape, i.e., round cocci (*Staphylococcus* spp., *Enterococcus* spp.) and elongated rods (*E. coli*, *P. aeruginosa*). Therefore, cytology does not significantly contribute to our awareness of potentially present hazardous strains of bacteria (1, 16).

The limited availability of affordable and straightforward diagnostic tools probably contributes to the fact that most antibiotics are still empirically prescribed (17). Moreover, standard microbiological analysis with bacterial culture and antimicrobial susceptibility testing (AST) is rarely employed. Culture and AST are mostly reserved for complicated and persistent inflammations (17, 18), although being recommended for diagnosing pyodermas that require systemic antibiotic therapy (1, 12, 15, 19). The standard microbiological analysis exhibits a few days-long (>3) turnaround time, and its costs often exceed the expenses for antibiotic therapy (10, 12, 17).

Consequently, a few affordable and faster point-of-care (POC) tests appeared to complement cytology and offer bacterial identification and AST. These tests are expected to improve veterinary adherence to the consensus antibiotic guidelines and increase the use of standard microbiological analysis (17). Two well-known POC tests are chromogenic culture media-based Flexicult® Vet (Figure 1, SSI Diagnostica, Hillerød, Denmark) and Speed Biogram (Virbac, Carros, France). Identification and AST are based on the color and presence of bacterial growth (20–22).

However, chromogenic culture media accuracies seemed inconsistent since the reported Flexicult® Vet values for urinary pathogen identification and AST ranged between 39 and 100% (20, 21, 23, 24). Enrolled experts (microbiologists and microbiological technicians) outperformed veterinary clinicians in bacterial identification by 16 (68.7 and 84.6%) (21) and 47 percentage points (53 and 100%) (20), indicating the correlation between medium performance and evaluator's experience. Another essential factor in POC test performance could be the selection of enzyme substrates, releasing specific signalophors after the enzymatic reaction (25). In these media, chromophores ensure that bacterial colonies (or agar) exhibit particular colors (Figure 1). Undoubtedly, the differentiation of several bacteria on the same plate often requires a combination of substrates optimized for the anticipated pathogens.

Existing studies primarily focused on identifying urinary bacteria by a single, mostly experienced evaluator using a specific chromogenic culture medium. Oppositely, we wanted to determine how well general clinical veterinarians identified bacterial pathogens from the skin and ears on several chromogenic media. Two enrolled agars were available on the market and adjusted for general or urinary pathogens. The last medium was custom-made with commercially available enzyme substrates, focusing on identifying typical skin pathogens like distinguishing *Staphylococcus* species between themselves and from other bacteria. Successful identification of bacterial species on affordable chromogenic culture media could complement cytology, helping the clinician to select proper antibiotics, conduct in-clinic agar-based AST, or decide whether further diagnostic tests are needed.

2. Materials and methods

The ethics committee (Ministry of Agriculture, Forestry and Food, Republic of Slovenia) granted the ethical acceptability of this study (No. U34405-246/2022/18, Administration for Food Safety, Veterinary Sector, and Plant Protection, Republic of Slovenia).

2.1. Bacterial samples

We collected 36 bacterial isolates from pyoderma and otitis externa in dogs and cats visiting our veterinary clinic (Table 1). Bacterial species were determined by a certified microbiological laboratory (University of Ljubljana, Ljubljana, Slovenia) utilizing matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Microflex LT system, Bruker Daltonics, Bremen, Germany). The accumulated isolates representing 10 bacterial species served to prepare 100 agar plates in different bacterial concentrations between 200 and 2000 colony-forming units (CFU) per plate. The number of prepared samples per species was

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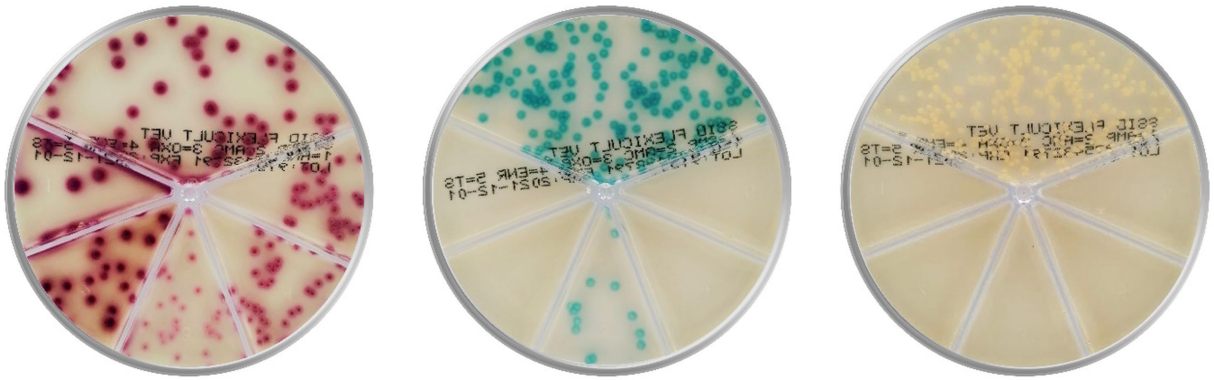

FIGURE 1
Culture of *Escherichia coli* (on the left), *Enterococcus faecalis* (in the middle), and *Staphylococcus aureus* (on the right) on the popular chromogenic media Flexicult® Vet (SSI Diagnostica, Hillerød, Denmark), offering primary bacterial identification and antimicrobial susceptibility testing in canine and feline urinary tract infections (Agar plate images are acquired in the PetriView BOX, Vets4science, Celje, Slovenia).

TABLE 1 The number of clinically collected bacterial strains and prepared samples (i.e., agar plates).

Bacterial species	No. of unique strains	No. of prepared samples
Staphylococcus pseudintermedius	13	25
Staphylococcus aureus	2	8
Staphylococcus felis	3	10
Staphylococcus schleiferi	1	5
Streptococcus canis	2	9
Escherichia coli	4	10
Enterococcus faecalis	4	10
Pseudomonas aeruginosa	3	10
Proteus spp.	2	8
Corynebacterium auriscanis	2	5
Total	36	100

based on the typical pathogen prevalence while ensuring that at least five samples represented each species (Table 1).

2.2. Chromogenic media

We studied three Mueller Hinton agar-based chromogenic media. Two enrolled agars were found on the market, while the third medium was custom-made with commercially available enzyme substrates.

LFCA: Liofilchem Chromatic™ MH (Liofilchem, Roseto degli Abruzzi, Italy). We prepared the plates from the supplied powder according to the instructions.

FLEX: Flexicult® Vet Scandinavia (SSI Diagnostica, Hillerød, Denmark). We bought agar plates as ready-to-use.

CAVD: Custom-made chromogenic medium based on the Mueller Hinton II agar (Merck Millipore, Tullagreen, Ireland). After autoclaving for 15 min at 121°C, the molten agar was mixed by magnetic stirring and cooled to 50°C in a water bath. Then, 100 mM stock solutions of chromogenic substrates in dimethyl sulfoxide (DMSO) and 0.5 M stock solutions of isopropyl-beta-D-thiogalactopyranoside (IPTG) and 1-O-Methyl-beta-D-glucuronic acid, sodium salt in ultrapure water were prepared and sterilized by filtration. IPTG is an inducer for beta-galactosidase and 1-O-Methyl-beta-D-glucuronic acid is an inducer for β-glucuronidase. After adding chromogenic substrate and inducer stock solutions (1 to 4 ml per liter, depending on the final concentration), the molten agar was poured into sterile single-use plastic Petri dishes and left to solidify. Plates were stored in a refrigerator in plastic bags to prevent drying. The final concentrations of chromogenic substrates were: 0.15 mM Aldol® 467 beta-D-galactopyranoside (product number A-4676, Biosynth AG, Staad, Switzerland), 0.14 mM 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronic acid, sodium salt trihydrate (product number B-7400, Biosynth AG), 0.12 mM 5-Bromo-6-chloro-3-indoxyl phosphate, disodium salt trihydrate (product number B-7453, Biosynth AG), 0.2 mM 5-Bromo-4-chloro-3-indoxyl-beta-D-glucopyranoside (product number B-7250, Biosynth AG), 0.5 mM IPTG, 0.5 mM 1-O-Methyl-beta-D-glucuronic acid, sodium salt (product number M-3600, Biosynth AG), and 0.35% DMSO.

2.3. Sample preparation

We inoculated Mueller Hinton liquid medium (Biokar, Allonne, France) with known bacterial species (Table 1). After 24 h of incubation at 37°C, we prepared dilutions of bacterial suspension in distilled water to cultivate between 200 and 2000 distinct CFU on each plate. Then, we transferred 100 µl of the suspension onto the LFCA and CAVD agars and smeared it in three directions with a sterile inoculation loop. According to the manual, 1 ml of suspension was added to the entire surface of Flexicult® Vet plates, and the excess fluid was decanted. In total, we inoculated 300 plates (100 per medium), which were incubated for 48 h at 37°C.

2.4. Evaluations

After 24 and 48 h of incubation, agar plates were evaluated visually by three small and one large animal clinical veterinarians, all without

experience in microbiological diagnostics. Due to the numerous plates (300) and evaluations (1800), we divided the study into four sessions with 30–90 plates per session. A function *randperm* (Matlab R2016a, MathWorks, Natick, USA) ensured a random sample (count) selection. Three evaluators participated in each evaluation session, performing 432 (Evaluator 1), 600 (Evaluator 2), 408 (Evaluator 3), and 360 evaluations (Evaluator 4). Evaluators examined all samples on one medium before addressing the next one. The medium order was random and unique for each session (function *randperm*, Matlab, MathWorks). As an evaluation result, the evaluator selected one of the 10 possible bacteria species for each agar plate. From the following analysis, we removed 12 evaluations since three agars with *S. canis* (two LFCAs at 24 h and one CAVD at 24 and 48 h) did not exhibit any growth.

Before evaluations, veterinarians were briefly introduced to each medium's characteristics, the appearance of colors specific to each bacterium, and the morphological characteristics of the colonies (Figure 2). Evaluators had instant digital access to a manual with bacterial images on all three media during the whole duration of the study. Additionally, we supplemented each sample with information on the sample history: *animal* (dog or cat), *sample location* (skin or ear), and *microscopic pathogen shape* (cocci or rods). Due to the collected samples and for the sake of study simplicity, we informed evaluators that *S. felis* (cats) and *S. pseudintermedius* (dogs) samples are animal-specific.

2.5. Statistics

We calculated accuracies, agreements, sensitivities, and specificities in the programming language Matlab (MathWorks). We defined accuracy as the ratio between correct bacteria identifications against the gold standard (MALDI-TOF) and all samples. Agreements with the gold standard and between evaluators (inter-rater) were estimated by Krippendorff's Alpha (α_K). We evaluated the significant differences between the agreements with a non-parametric Mann–Whitney U test. The sensitivity for each pathogen was calculated as a ratio between true positives (correctly identified) and the number of correct identification at the gold standard. Oppositely, specificity was a ratio between true and all negatives (i.e., identifications of other bacterial species at the gold standard). In the manuscript, *error* (also *misidentification*) stands for all wrong bacterial species identifications, including false positives and false negatives.

3. Results

All three enrolled chromogenic media performed similarly, exhibiting the mean identification accuracy (a percentage of correctly identified bacteria) between 72.1 and 86.3% (Table 2). Prolonged incubation improved bacterial identification, and consequently, the mean accuracy increased by 5.7 percentage points, from 76.4% (24 h) to 82.1% (48 h). The difference was statistically significant ($p = 0.049$, Mann–Whitney *U* test, function *signrank*, Matlab). Only LFCA agar, studied by evaluator 3, performed worse with additional incubation time (90.9 vs. 82.8%). Evaluators 2 and 3 reached high, ~90.0% accuracy using LFCA and FLEX media. On the other hand, evaluator 4 consistently delivered the worst performance, once recognizing only 62.1% bacterial species (LFCA medium).

Staphylococci exhibit similar colors on chromogenic media; thus, we also studied accuracies when all *Staphylococcus* samples were grouped in the same species category. In this case, identification accuracy improved, on average, by 8.8–10.5 percentage points (Table 2).

Similar to the accuracies, agreement in bacterial identification between chromogenic agars and the gold standard (i.e., MALDI-TOF) improved with prolonged incubation, i.e., Krippendorff's Alpha (α_K) increased from 0.73 to 0.79 (Table 3, $p = 0.092$, Mann–Whitney *U* test). Surprisingly, inter-rater reliability (IRR, i.e., agreement between evaluators) was in the same range as the agreement with the gold standard.

Evaluators ideally identified bacterial species that grew in unique colors (Table 4), reaching almost perfect (~100%) sensitivities and

FIGURE 2
Prevalent canine and feline skin bacteria (1) *Staphylococcus pseudintermedius*, (2) *Staphylococcus aureus*, (3) *Escherichia coli*, (4) *Enterococcus faecalis*, (5) *Pseudomonas aeruginosa*, and (6) *Proteus* spp. on three chromogenic media: Liofilchem Chromatic™ MH (LFCA), Flexicult® Vet Scandinavia (FLEX), and a custom-made chromogenic agar (CAVD; Agar images were acquired in the PetriView BOX, Vets4science, Celje, Slovenia).

TABLE 2 Mean accuracies in bacterial identification (%) by all (Accuracy) or a single evaluator after 24 and 48h incubation on average (All) or per specific agar: Liofilchem Chromatic™ MH (LFCA), Flexicult® Vet Scandinavia (FLEX), and custom-made agar (CAVD).

	24h incubation				48h incubation			
	All	LFCA	FLEX	CAVD	All	LFCA	FLEX	CAVD
Accuracy	76.4 ± 9.9	81.6 ± 12.8	75.7 ± 10.6	72.1 ± 7.0	82.1 ± 4.8	82.7 ± 8.5	86.3 ± 3.5	77.1 ± 3.5
Evaluator 1	81.0	86.1	81.9	75.0	81.5	84.7	84.7	75.0
Evaluator 2	75.1	83.7	71.0	70.7	86.3	90.0	90.0	78.8
Evaluator 3	85.6	90.9	86.8	79.1	82.8	80.9	86.8	80.6
Evaluator 4	62.7	62.1	63.3	62.7	74.9	70.0	81.7	72.9
Staph. spp. grouped	86.9 ± 9.8	90.1 ± 9.4	85.7 ± 13.4	84.9 ± 6.7	90.9 ± 3.5	88.0 ± 5.3	94.7 ± 2.0	89.9 ± 4.3

Accuracies are also listed when all *Staphylococcus* species are grouped in the same species category (*Staph.* spp. grouped). Values listed after "±" mark standard deviation.

TABLE 3 Mean agreement (Krippendorff's Alpha α_K) and (±) standard deviation between the gold standard (MALDI-TOF) and evaluators, and between evaluators (Inter-rater reliability) in bacterial identification after 24 and 48h incubation on average (All) or per specific agar: Liofilchem Chromatic™ MH (LFCA), Flexicult® Vet Scandinavia (FLEX), and custom-made agar (CAVD).

	24h incubation				48h incubation			
	All	LFCA	FLEX	CAVD	All	LFCA	FLEX	CAVD
Agreement (α_K)	0.73 ± 0.11	0.79 ± 0.15	0.72 ± 0.12	0.67 ± 0.08	0.79 ± 0.05	0.80 ± 0.10	0.84 ± 0.04	0.73 ± 0.04
Inter-rater reliability	0.69 ± 0.12	0.72 ± 0.08	0.64 ± 0.20	0.71 ± 0.18	0.80 ± 0.06	0.74 ± 0.08	0.84 ± 0.05	0.82 ± 0.11
IRR(best) / IRR(worst)	0.81 / 0.49	0.81 / 0.59	0.88 / 0.42	0.96 / 0.43	0.91 / 0.75	0.85 / 0.64	0.88 / 0.74	1.00 / 0.71

IRR (best) and IRR (worst) mark the best and worst agreement between two evaluators.

specificities for bluish *E. faecalis*, orange-brown *Proteus* spp., green-brown *P. aeruginosa* and red *E. coli*. Oppositely, differentiation between *Staphylococcus* species was challenging due to similar shades of bacterial colonies. For instance, less common *S. schleiferi* was rarely recognized (sensitivity of ~9%). Evaluators satisfactorily identified only *S. pseudintermedius* and *S. felis* (sensitivity and specificity between 81 and 100%).

For staphylococci and *S. canis*, misidentifications mostly happened within the mentioned bacteria (Table 5). Other notable errors included typically intense yellow-exhibiting *S. aureus*, which sometimes turned bluish after 48 h incubation on LFCA agar (Figure 3) and was consequently identified as *E. faecalis*. Furthermore, *E. faecalis*, after 24 h incubation on LFCA, was occasionally mistaken for *S. canis,* although these bacteria do not exhibit the same colony size (Figure 4). Finally, there were a few misidentifications between *S. pseudintermedius* and *E. coli* due to similar pink-red colony shades on LFCA and FLEX media. There were no errors on CAVD agar since *E. coli* colonies were green (Figure 2). Based on the errors in human healthcare and laboratory medicine (26, 27), we classified errors when interpreting chromogenic agars into three categories related to chromogenic medium or evaluator (knowledge- and negligence-based; Table 6).

4. Discussion

Skin infections are one of the most common reasons for prescribing antibiotics in veterinary medicine (1). Thus, it is essential to confirm whether the infection is bacterial, what contributes to the diagnostic dilemma of when to prescribe antibiotics (and which), or to wait for antimicrobial susceptibility testing (AST) results (1, 3). Currently, certified laboratories perform most microbiological tests due to the specific knowledge and experience needed. Despite high accuracy, the traditional laboratory-based culture and AST often lack convenience for veterinary clinics regarding turnaround time and costs. For that reason, straightforward point-of-care (POC) tests based on chromogenic media could be a good alternative due to their affordability, speed (~24 h), and in-clinic use if ensuring microbiological safety (10, 12). Chromogenic culture media have been developed over the past 25 years to identify mostly pathogenic or resistant bacteria, like methicillin-resistant *S. aureus* (MRSA), in medicine and food safety (28).

The common chromogenic medium-based POC tests are meant to identify a wide range of pathogens in urinary tract infections (UTI) on a single agar plate. In humans, these tests exhibited high >90% sensitivity and specificity (29, 30). In veterinary studies, only experienced evaluators achieved similar accuracy (20, 21, 23, 24), while beginners correctly recognized 53% (20), 68.7% (21), and, in this study, ~80% samples. The reason for the improved accuracy of inexperienced evaluators in this study could lie in the enrolled pure (not mixed) cultures prepared by a microbiologist. Moreover, we studied pathogens from skin and ears (versus urinary tract in other studies), and the evaluators knew the possible bacterial species in advance, although a limited range of bacteria should not significantly impact overall accuracy due to the inclusion of the majority of skin pathogens (31). However, bacterial species' prevalence could significantly impact medium performance in practice. For example, veterinarians in areas with higher *S. schleiferi* frequency (32) would have more difficulty identifying a specific pathogen because *S. schleiferi* colonies seemed not to differ enough to be distinguished from other staphylococci (Tables 4, 5).

On all agars, evaluators quickly recognized bacteria growing in distinct colors like green-brown *P. aeruginosa*, blue *E. faecalis,* and orange-brown *Proteus* spp. (Table 4). Colors developed the quickest on the LFCA medium, leading to the highest identification accuracy after 24 h incubation, which did not improve significantly with

TABLE 4 Sensitivities and specificities (in %) in identifying separate bacterial species after 24 or 48h incubation, average on all (All) or specific agar: Liofilchem Chromatic™ MH (LFCA), Flexicult® Vet Scandinavia (FLEX), and custom-made agar (CAVD).

Bacteria	Sensitivity (%)								Specificity (%)							
	24h incubation				48h incubation				24h incubation				48h incubation			
	All	LFCA	FLEX	CAVD	All	LFCA	FLEX	CAVD	All	LFCA	FLEX	CAVD	All	LFCA	FLEX	CAVD
S. pseudintermedius	86.2	100	84.0	74.7	92.4	92.0	100	85.3	89.8	95.0	91.1	83.3	89.6	94.2	92.9	81.5
S. aureus	31.9	70.8	20.8	4.2	37.5	54.2	54.2	4.2	97.9	96.7	97.8	99.3	98.1	97.1	97.5	99.6
S. felis	81.1	66.7	80.0	96.7	87.8	86.7	80.0	96.7	96.1	97.0	95.9	95.5	97.3	99.3	96.7	95.9
S. schleiferi	8.9	20.0	6. 7	0	8.9	26.7	0	0	99.1	99.3	97.9	100	99.9	100	99.7	100
S. canis	61.1	71.4	63.0	50.0	65.4	81.5	74.1	37.5	93.4	95.2	92.7	92.3	96.5	96.0	97.4	96.0
E. coli	93.3	96.7	96.7	86.7	96.7	93.3	96.7	100	99.8	100	99.6	99.6	99.6	99.3	100	99.6
E. faecalis	83.3	60.0	100	90.0	88.9	66.7	100	100	98.9	98.5	98.2	100	98.1	95.6	99.6	99.3
P. aeruginosa	85.6	90.0	83.3	83.3	97.8	96.7	100	96.7	99.3	100	99.3	98.5	99.9	99.6	100	100
Proteus spp.	94.4	95.8	91.7	95.8	100	100	100	100	99.2	99.3	99.3	98.9	99.8	99.6	100	99.6
C. auriscanis	86.7	86.7	73.3	100	88.9	86.7	93.3	86.7	99.7	100	100	98.9	99.8	99.3	100	100

prolonged 48 h incubation. Rapid colony coloration after 24 h was especially evident for pinkish *S. pseudintermedius* (still being pale on the other two media), which was important to prevent confusion with more reddish *E. coli* colonies (see mistakes in FLEX agar, Table 5). Therefore, LFCA enabled excellent distinction of *S. pseudintermedius*, also from other staphylococci (Table 4). However, one strain of *S. aureus* exhibited bluish color on LFCA and was mistaken for *E. faecalis* (Figure 3). This misidentification happened even though the colonies' shape was not typical of *E. faecalis*, indicating that beginners seem to rely more on colony color than its morphology. Inexperience could also be a reason why evaluators recognized only ~60% *E. faecalis* samples even after prolonged incubation. Evaluators labeled most misidentified plates as *S. canis*, which colonies exhibited different shades and sizes (Figure 4).

Opposite from LFCA, FLEX agar improved its performance after prolonged 48 h incubation, achieving the highest mean identification accuracy of 86.3% (Table 2). Since Flexicult® Vet is meant for urinary tract infections (UTI), evaluators perfectly recognized typical urinary pathogens such as *E. coli, E. faecalis*, and *Proteus* spp. (Table 4). However, the FLEX was less optimal for skin pathogens, especially after 24 h of incubation. The slow color formation of staphylococcal colonies was probably the reason for suboptimal agar performance. The same problem was also recognized by Guardabassi et al. (20), who recommended incubating Flexicult® Vet longer (48 h instead of 24 h) for *S. pseudintermedius* to develop typical colony colors.

CAVD medium was excellent in identifying *E. coli* due to its distinctive green color (Figure 2). Oppositely, all staphylococci grew in similar colors, including otherwise yellowish *S. aureus*. This was probably the main reason for generally lower CAVD accuracy compared to other media (Table 2). However, CAVD performance was comparable to the other two agars when differentiation between staphylococci was excluded.

Occasional staphylococci species misidentifications do not pose a significant risk for clinical practice and canine patient care (3). Moreover, minimal inhibitory concentration (MIC) and zone diameter breakpoints are the same for AST in the staphylococci genus (33). However, it is important to identify potentially multiple resistant bacteria like methicillin-resistant *S. pseudintermedius* (MRSP) and *S. aureus* (MRSA). In these cases, AST is necessary due to the limited selection of suitable antibiotics like non-beta-lactam fluoroquinolones (enro- or marbofloxacin). Furthermore, resistant staphylococci carry additional hazards due to potential transmission to other animals and humans.

Bacterial intrinsic resistances leading to different antibiotic treatment protocols are another reason for the importance of correct species identification. For example, there were misidentifications between *S. pseudintermedius* and *P. aeruginosa*. The latter, usually growing in distinguishable light green color (Figure 2), often exhibits resistance to multiple classes of antimicrobials like beta-lactams, including penicillins (e.g., amoxicillin) and cephalosporines (cefalexin), fluoroquinolones (enrofloxacin), and aminoglycosides (tobramycin) (34). Furthermore, misidentifying *S. pseudintermedius* for *S. canis* could have led to a serious mistake in the presence of MRSP. Oppositely, *S. canis* demands only a straightforward protocol with penicillins (35), and labeling the species as MRSP would cause additional AST-related costs. We think that the inexperience of the evaluators was the main contributing factor to these errors because *S. canis* exhibits evidently smaller colonies on all three enrolled media. As we demonstrated in our previous study, the experience helped to

TABLE 5 The number of bacteria samples (%) mistaken for another bacteria species (per row) after 24 and 48h incubation for all (All) or per specific agar: Liofilchem Chromatic™ MH (LFCA), Flexicult® Vet Scandinavia (FLEX), a custom-made agar (CAVD).

		S. p.	*S. aureus*	*S. felis*	*S. schleiferi*	*S. canis*	*E. coli*	*E. faecalis*	*P.aeruginosa*	*Proteus* spp.	*C.auriscanis*
		24h, 48h	24h, 48h	24h, 48h	24h, 48h	24h, 48h	24h, 48h	24h, 48h	24h, 48h	24h, 48h	24h, 48h
S. pseudintermedius	All		9.7, 5.9		12.9, -	74.2, 76.5	3.2, 11.8		-, 5.9		
	LFCA		-, -		-, -	-, 50.0	-, 33.3		-, 16.7		
	FLEX		16.7, -		33.3, -	41.7, -	8.3, -		-, -		
	CAVD		**5.3**, 9.1		-, -	94.7, 90.9	-, -		-, -		
S. aureus	All	40.8, 28.9		40.8, 33.3		18.4, 13.3		-, 24.4			
	LFCA	28.6, -		57.1, 9.1		14.3, -		-, 90.9			
	FLEX	21.1, -		42.1, 54.6		36.8, 45.5		-, -			
	CAVD	60.9, 56.5		34.8, 34.8		4.4, 4.4		-, 4.4			
*S. felis**	All	23.5, 81.1	17.7, -		11,8, -	29,4, 18.2					
	LFCA	30.0, 100.0	20.0, -		20.0, -	-, -					
	FLEX	16.7, 67.7	-, -		-, -	83.3, 33.3					
	CAVD	-, 100.0	100, -		-, -	-,-					
S. schleiferi	All	48.8, 63.4	19.5, 19.5	22.0, 17.0		9.8, -					
	LFCA	16.7, 18.8	58.3, 72.7	25.0, 9.1		-, -					
	FLEX	50.0, 80.0	7.1, -	21.4, 20.0		21.4, -					
	CAVD	73.3, 80.0	-, -	20.0, 20.0		6.7, -					
*S. canis**	All	78.6, 74.1	10.7, 25.9					3.6, -			
	LFCA	50.0, 100	-, -					16.7, -			
	FLEX	70.0, -	30.0, 100					-, -			
	CAVD	100, 100	-, -					-, -			
E. coli	All	16.7, 66.7			-, 33.3			16.7, -	50.0, -	16.7, -	
	LFCA	-, 100			-, -			100, -	-, -	-, -	
	FLEX	100, -			-, 100			-, -	-, -	-, -	
	CAVD	-, -			-, -			-, -	75.0, -	25.0, -	
E. faecalis	All					86.7, 80.0					13.3, 20.0
	LFCA					100, 80.0					-, 20.0
	FLEX					-, -					-, -
	CAVD					33.3, -					66.7, -
P. aeruginosa	All	7.7, -		7.7, -	15.4, -		7.7, -	7.7, -		46.2, 100	7.7, -
	LFCA	33.3, -		-, -	-, -		-, -	-, -		66.7, 100	-, -
	FLEX	-, -		-, -	40.0, -		-, -	20.0, -		40.0, -	-, -
	CAVD	-, -		20.0, -	-, -		20.0,	-, -		40.0, 100	20.0, -
Proteus spp.	All			25.0, -					75.0, -		
	LFCA			100, -					-, -		
	FLEX			-, -					100, -		
	CAVD			-, -					100, -		
C. auriscanis	All						-, 20.0	100, 80.0			
	LFCA						-, -	100, 100			
	FLEX						-, -	100, 100			
	CAVD						-, 50.0	-, 50.0			

Example in bold: 5.3% of *Staphylococcus pseudintermedius* (*S. p.*) misidentifications were classified as *S. aureus* (CAVD, 24 h). **Due to a few empty plates, the sum per row is not 100%.*

consider all colony characteristics leading to better bacterial identification (21).

Our study additionally revealed several misidentifications between cocci and rods (Table 5), like *S. pseudintermedius* vs. *E. coli* and *E. faecalis* vs. *C. auriscanis*. Since information on bacterial shape was known in advance (from presumed cytology), these errors can only be attributed to the evaluator's negligence. Moreover, misidentifying feline *S. felis* for canine *S. pseudintermedius* was surprising because the origin of the analyzed isolates was known to evaluators, and we informed them that either species occurs only in one animal (i.e., we did not retrieve any *S. pseudintermedius* in cats). The negligence-related misidentifications above accounted for 8.9–11.7% of all mistakes (Tables 4, 5).

Most errors revealed in this study can be categorized (26, 27) and related to (i) chromogenic medium, (ii) evaluator (in)experience, and (iii) negligence (Table 6). We had initially expected that media-caused errors would be dominant, causing inter-rater variability to be smaller than disagreement with the gold standard (Table 3). Although evaluators relied strongly on colors and many errors happened due to similar colony shades (e.g., staphylococci), all agreements were comparable, indicating various errors evaluators had made. It seemed that each evaluator responded individually when presented with uncertainty. Unfortunately, the study design did not allow us to estimate the proportions of error related to medium design or evaluator (in)experience. Previous studies showed that, in some instances, an expert familiar with the particular medium perfectly identified bacterial species (20). Because presuming ideal bacterial prevalence for specific agar and perfection reached by all experts is not realistic (21), we suggest further agar improvements. On the one hand, advances in agar design can focus on maximizing

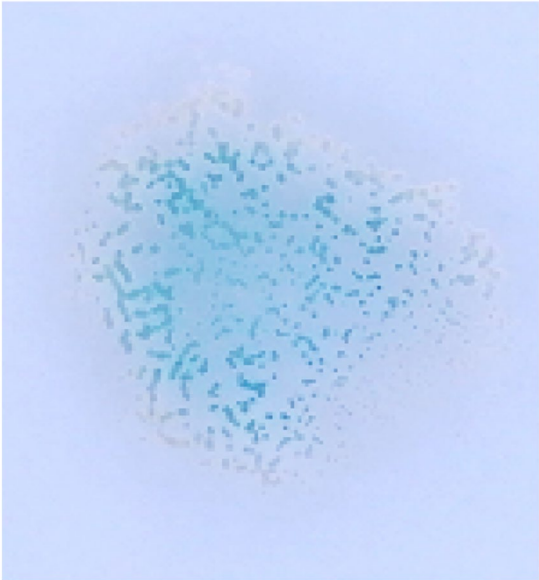

FIGURE 3
Staphylococcus aureus colonies, typically intense yellow, occasionally exhibited blue color after 48h incubation on an LFCA medium (Liofilchem Chromatic™ MH).

general performance in identifying veterinary bacteria, e.g., by optimizing the type and concentration of chromogenic enzyme substrates added. Alternatively, and more reasonably, media could be adjusted for a specific organ system like skin, focusing on better staphylococci differentiation.

Additional personnel training, evaluation flowchart, and imaging or other visual support could prevent identification errors in recognizing less noticeable or not color-related differences in bacterial colonies. Interactions with clinical microbiologists to discuss the bacterial growth on agar plates in real time would also help clinicians interpret unfamiliar cases and provide important educational processes. Furthermore, this study showed that around 10% of errors could not be explained by the medium design and evaluator experience. We classified them as negligence-related slips and lapses as a result of evaluator distraction or fatigue. Due to many agar plates (i.e., 30–90) being evaluated in a single session, the study definitely provoked a significant load on the enrolled evaluators, causing stress, distraction, and fatigue. These emotional and mental conditions are often present in bustling veterinary clinics driving veterinarians to perform various laboratory tests in a hurry or at the end of the shift. However, establishing suitable working conditions and checklists, reminding the evaluator to consider sample history, could significantly improve bacterial identification on the chromogenic media.

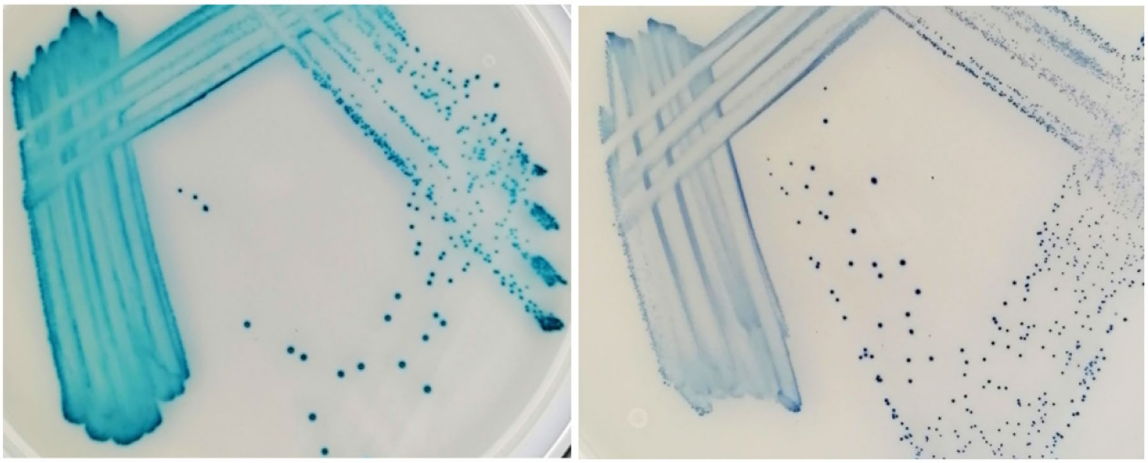

FIGURE 4
Enterococcus faecalis (left) and *Streptococcus canis* (right) after 48h incubation on the Liofilchem Chromatic™ MH medium plates (LFCA).

TABLE 6 Classification of error categories in bacterial identification on chromogenic media by inexperienced veterinary clinicians [based on errors in medicine and medical laboratories (26, 27)].

Error category	Description	Type	Cause	Preventability	Corrective action
Chromogenic medium	Colonies too similar (color and morphology)	Latent (equipment-based)	Test design	Low	• Improved or • Disease-adjusted chromogenic media
Evaluator (experience- and knowledge-based)	Differences in colonies not recognized	Active – execution (knowledge-based)	Lack of skills and training	Medium	• Training • Cognitive aids (manuals)
Evaluator (negligence-based)	Sample history not considered	Active – planning (slips/lapses)	Distraction, fatigue, carelessness	High	• Checklist • Improved working conditions

5. Conclusion

Translating chromogenic media into veterinary clinics can significantly advance current in-clinic diagnostics of canine and feline pyodermas and external otitises. Cytology reveals only the presence and shape of bacteria (cocci and rods), while chromogenic agars enable the identification of skin pathogens with around 80% accuracy. The extra information can help clinicians choose treatment protocols or opt for standard antimicrobial susceptibility testing (AST). Knowing bacterial species is particularly useful for initial, non-complicated infections (assuming local epidemiological data is known).

Various general chromogenic agars (including our CAVD) exhibit similar performance accuracies and seem suitable for introduction into clinics. On all media, inexperienced evaluators easily identified unique-colored colonies of bacteria like *P. aeruginosa*, *E. faecalis*, and *Proteus* spp. Challenges arose when the morphology (colony size) was essential or bacteria grew in similar shades (staphylococci). Around 10% of errors were slips related to not considering sample history. To improve bacterial identification, media could be improved to serve specific purposes or organ systems like distinguishing between skin staphylococci species. Additional personnel training, evaluation help by visuals, flowcharts, checklists, and, if necessary, microbiologists can all further improve identification accuracy.

Future research should add real, often mixed bacterial skin cultures prepared by clinicians directly from clinical samples. We shall also test if chromogenic media with added antibiotics could be applied for AST in skin infections. We do not think that in-clinic POC tests are meant to replace standard AST in microbiological laboratories but to fill a gap between currently dominant empirical antibiotic treatments and rarely applied standard AST.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the ethics committee, Ministry of Agriculture, Forestry and Food, Republic of Slovenia. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MA and BC ideated, designed, and performed the experiments. JI designed the CAVD agar and provided guidelines on its use in veterinary settings. BC performed the statistics. All authors contributed to the article and approved the submitted version.

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Conflict of interest

JI, GF and US are employed by Biosynth AG.

The authors conducted the experimental design, experiments, and data analysis independently. Biosynth AG (Staad, Switzerland) provided the chromogenic substrates and the inducer compounds for the CAVD agar preparation at no cost. Aldol® reagents are proprietary patent-protected products of Biosynth AG. Liofilchem Chromatic™ MH and Flexicult® Vet Scandinavia were purchased from the official distributors.

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Glossary

AST	Antimicrobial susceptibility testing
C.	*Corynebacterium*
CAVD	Custom-made chromogenic medium based on the Mueller Hinton II agar
CFU	Colony-forming units
E.	*Escherichia*
FLEX	Flexicult Vet Scandinavia
IRR	Inter-rater reliability
LFCA	Liofilchem Chromatic MH
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant Staphylococcus aureus
MRSP	Methicillin-resistant Staphylococcus pseudintermedius
P.	*Pseudomonas*
POC	Point-of-care tests
S.	*Staphylococcus*, or *Streptococcus* for *S. canis*
UTI	Urinary tract infections

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An inflammatory bowel disease-associated SNP increases local thyroglobulin expression to develop inflammation in miniature dachshunds

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Inflammatory colorectal polyp (ICRP) in miniature dachshunds (MDs) is a chronic inflammatory bowel disease (IBD) characterized by granulomatous inflammation that consists of neutrophil infiltration and goblet cell hyperplasia in the colon. Recently, we identified five MD-associated single-nucleotide polymorphisms (SNPs), namely *PLG*, *TCOF1*, *TG*, *COL9A2*, and *COL4A4*, by whole-exome sequencing. Here, we investigated whether *TG* c.4567C>T (p.R1523W) is associated with the ICRP pathology. We found that the frequency of the T/T SNP risk allele was significantly increased in MDs with ICRP. *In vitro* experiments showed that TG expression in non-immune cells was increased by inducing the IL-6 amplifier with IL-6 and TNF-α. On the other hand, a deficiency of *TG* suppressed the IL-6 amplifier. Moreover, recombinant TG treatment enhanced the activation of the IL-6 amplifier, suggesting that TG is both a positive regulator and a target of the IL-6 amplifier. We also found that *TG* expression together with two NF-κB targets, *IL6* and *CCL2*, was increased in colon samples isolated from MDs with the T/T risk allele compared to those with the C/C non-risk allele, but serum TG was not increased. Cumulatively, these results suggest that the T/T SNP is an expression quantitative trait locus (eQTL) of *TG* mRNA in the colon, and local *TG* expression triggered by this SNP increases the risk of ICRP in MDs *via* the IL-6 amplifier. Therefore, *TG* c.4567C>T is a diagnostic target for ICRP in MDs, and *TG*-mediated IL-6 amplifier activation in the colon is a possible therapeutic target for ICRP.

KEYWORDS

miniature dachshund, canine, inflammatory colorectal polyps, SNP, thyroglobulin, inflammation, IL-6 amplifier

1. Introduction

Inflammatory colorectal polyp (ICRP) in miniature dachshunds (MDs) is a chronic inflammatory disease confined to the colorectal region of dogs. It is characterized by multiple polypoid lesions or a single pedunculated granulomatous lesion growing from the colorectal mucosa, which has been reported only in Japan (1). Histopathologically, ICRP has been differentiated from other types of polyps by its traits of granulomatous inflammation with neovascularization, the infiltration of neutrophils, lymphocytes, and/or macrophages, goblet cell hyperplasia, exaggerated mucus production, and crypt dilation. Clinical signs of hematochezia, tenesmus, and mucoid feces have also been reported (1–3). ICRP responds well to immunosuppressive treatments such as leflunomide, prednisolone, and cyclosporine (4, 5). However, recurrences are common despite these treatments (2, 3), and further study of the ICRP pathogenesis is needed to develop a better cure. The inflammatory mechanism of ICRP has been reported to include the dysregulation of innate immunity, the upregulation of toll-like receptors (TLRs), and the overexpression of pro-inflammatory cytokines (6–10). Notably, MDs have a much higher tendency to develop ICRP, with an odds ratio of 24.63, compared to other dog breeds (1), signifying the importance of the genetic basis of this disease. The single-nucleotide polymorphisms (SNPs) of nucleotide-binding oligomerization domain 2 (*NOD2*) gene were investigated in the pathogenesis of ICRP but no major effect was found (11), leaving the detailed molecular mechanism of the inflammation unknown. Using whole-exome sequencing (WES), we found several genes related to inflammatory responses are MD-associated SNPs in ICRP (12).

Pioneer studies in chronic inflammation have shown that pro-inflammatory cytokine stimulation including IL-6 can enhance the expressions of inflammatory mediators in non-immune cells in the development of inflammatory diseases (13, 14). The simultaneous activation of NF-κB and STAT3 in non-immune cells has been shown to be the key molecular mechanism that enhances the activation of NF-κB. Although IL-6 is one of very few stimulators of STAT3, there are multiple stimulators of NF-κB, including TNF-α, IL-17A, growth factors, noradrenaline, and TLR ligands, during inflammation development (13–25). The combined effects of IL-6 and NF-κB on NF-κB activation were named the "IL-6 amplifier" (13). The IL-6 amplifier's role in the pathogenesis of inflammatory diseases has been well described in both mouse disease models and clinical samples of human patients (15, 17, 19, 26–38).

A genome-wide screening of the IL-6 amplifier identified approximately 500 target genes and 1,200 positive regulators (39). Recently, we used WES and found that some of these positive regulators, *PLG*, *TCOF1*, *TG*, *COL9A2*, and *COL4A4,* are MD-associated SNPs in ICRP (12). In this study, we verified that *TG* (thyroglobulin) is associated with the pathogenesis of ICRP. We found that MDs have a higher frequency of the *TG* risk allele T/T, which in turn increases *TG* expression in the colon, resulting in the activation of the IL-6 amplifier and chronic local inflammation. Furthermore, we found that TG deficiency suppressed and recombinant TG treatment enhanced the IL-6 amplifier activation in non-immune cells *in vitro*. Along with *TG*, the expression of other *NF-κB* targets, such as *IL6* and *CCL2*, was concurrently increased in the colons of MDs with the risk allele T/T, strengthening the conclusion that TG is a potential therapeutic target for ICRP. These data uncover a new function of TG related to chronic inflammation and the breed specificity of ICRP in MDs.

2. Materials and methods

2.1. Animals

The genotyping of selected SNPs (Table 1) was performed by Sanger sequencing on 155 MDs diagnosed with ICRP, 90 age-controlled MDs without ICRP, 36 juvenile MDs aged <1 year, and 40 dogs of other breeds. The details of the animals recruited and the samples used in this study are summarized in Supplementary Table 1. A DNA Blood & Tissue Kit (QIAGEN, Hilden, Germany) was used to derive genomic DNA (gDNA) from EDTA-archived blood, fresh colon tissue samples, or formalin-fixed paraffin-embedded colon tissue samples of the recruited dogs. Blood was collected by a veterinarian or a veterinary student supervised by a licensed veterinarian through a jugular venipuncture as part of routine blood workup for screening or diagnosis purposes. A signed consent form was obtained from the owners of all dogs for the tissue sample collection and use in this study.

2.2. Tissue sample collection

Endoscopic examinations were performed under general anesthesia for all dogs to retrieve the colon tissues. Each dog was administrated with midazolam (0.1 mg/kg) and butorphanol tartrate (0.2 mg/kg) intravenously as pre-medication and then with propofol (4 to 6 mg/kg) using the same route. Anesthesia was then maintained through the inhalation of isoflurane with oxygen, where additional butorphanol was administrated when necessary. Pulse oximetry readings, electrocardiograph, capnograph, arterial blood pressure, and rectal temperature were monitored throughout the anesthesia to ensure a smooth endoscopic procedure. Endoscopic procedures were completed within 2 h in all dogs, and all dogs recovered uneventfully. In MDs with ICRP, samples were collected as part of the diagnostic procedure, where polyp lesions deemed inflammatory on gross appearance were collected endoscopically with forceps or through electrosurgical snare polypectomy. Normal colorectal mucosae were collected endoscopically from the colorectal region adjacent to the diseased area based on gross appearance. At least six tissue samples were collected from both the polyp lesion site and normal colorectal mucosal region in MDs with ICRP. All biopsy specimens were assessed by a board-certified veterinary pathologist (YK) according to histopathological standards established by the World Small Animal Veterinary Association Gastrointestinal Standardization Group (40). The severity of ICRP inflammation in each case was staged according to a previous report (3). The information on the medication and histopathology for each group is shown in Supplementary Table 2. Each tissue sample was stored at −80°C for protein analysis or

TABLE 1 Chromosomal position and primers used for targeted genotyping.

Gene	Chromosome number	Position	Genbank accession number	Variant (mRNA)	Variant (protein)	Forward primer (5′-3′)	Reverse primer (5′-3′)
TG	13	29406010	NM_001048104	c.4567C>T	p.R1523W	GATGGGCGGTGAAGGGTTAA	GCACACAGCCCACAAGAAAG
TG-FFPE	13	29406010	NM_001048104	c.4567C>T	p.R1523W	ACCGACTGTCAGAGGAGTGA	TGACACTGGGAGTCGGTGA

TABLE 2 Primer sequences used for qPCR in H4 cells.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Human *IL6*	GGTACATCCTCGACGGCATCT	GTGCCTCTT TGCTGCTTTCAC
Human *GAPDH*	GAGTCAACGGATTTGGTCGT	CGCTCCTGGA AGATGGTG
Human *TG*	CCAGTGGCTTCTCTTCCTGACT	CCTTGGAGGAAGC GGATGGTTT

RNA*Later* RNA Stabilization Solution (Ambion Inc., Austin, TX, USA) for 24 h at 4°C to allow penetration and stabilization and then at −80°C for prolonged storage. Five specimens were collected for the qPCR analysis to perform a definitive diagnosis of ICRP.

2.3. Targeted genotyping

Polymerase chain reactions (PCRs) and Sanger sequencing were utilized to validate the genotype variants selected from WES data (12). The WES data have been deposited with links to BioProject accession number PRJDB16014 in the DDBJ BioProject database. Primer pairs used for the variant validation were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; Table 1). Amplicons post-PCR were purified using a commercial clean-up reagent, ExoSAP-IT Express (Thermo Fisher Scientific, Waltham, MA, USA), and sequencing was performed using the Sanger method utilizing BigDye Terminator v3.1 (Thermo Fisher Scientific) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). FinchTV (https://digitalworldbiology.com/FinchTV) was used to interrogate the reads generated, and the allele frequency was compared between groups using Fisher's exact test.

2.4. Cell lines and stimulation conditions

The H4 human cancer cell line was purchased from ATCC (Sumitomo Dainippon Pharma, Osaka, Japan). All cell lines were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA) enriched with 10% fetal bovine serum (Thermo Fisher Scientific) and treated with 1% penicillin and streptomycin at 37°C under 5% CO_2. For the cytokine stimulation, cells were seeded into 96-well plates (1×10^4 cells/well) and stimulated with human IL-6 (30 ng/ml; Toray Industries, Tokyo, Japan) plus human soluble IL-6 receptor (30 ng/ml; R&D Systems, Minneapolis, MN) and/or TNF- α (10 ng/ml; PeproTech, Tokyo, Japan) for 3 h after 2 h of serum starvation in Opti-MEM (Thermo Fisher Scientific, Waltham, MA). For the TG stimulation, the cytokine stimulation was modified to include cells stimulated with a 5x dilution of the cytokine with the serial addition of recombinant human TG (1 μg/ml, 5 μg/ml, and 10 μg/mL) as per the manufacturer's recommendation (Cloud-Clone Corp., USA). After stimulation, the cells were lysed, and the total RNA was retrieved for real-time PCR.

2.5. Quantitative real-time PCR in H4 cells

The Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) and THUNDERBIRD SYBR quantitative PCR (qPCR) Mix (TOYOBO Co. Ltd., Osaka, Japan) were used to quantify the levels of target mRNA and internal control mRNA (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*). Total RNA was prepared from cells using a SuperPrep Cell Lysis Kit for qPCR (TOYOBO). The conditions for real-time qPCR were 40 cycles at 94°C for 15 s followed by 40 cycles at 60°C for 60 s. Relative *IL6* mRNA expressions were normalized to the level of *GAPDH* mRNA expression. Primers and their sequences for the qPCR are described in Table 2. For the *in vitro* experiments, all the experiments were performed in triplicate but also performed twice to ensure replication.

2.6. Human small-interfering RNAs

Human small-interfering RNAs (siRNA) were transfected into H4 cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific). The sequences for the sense oligonucleotides of the knockdown constructs were human si-TG (1: CCU UAUGAGUUCUCACGGAtt and 2: GCUGCUACAUGG UAUUACUtt; Ambion Silencer Select siRNA, Thermo Fisher Scientific), human si-p65 (Ambion Silencer Select RELA siRNA, Thermo Fisher Scientific), and human siRNA negative control (Ambion Negative Control #1 siRNA, Thermo Fisher Scientific).

2.7. Enzyme-linked immunosorbent assay (ELISA) detection of serum *TG* levels

Serum samples from 22 MD-ICRP, 22 MD-Control, and 36 juvenile MDs were enrolled in this study. For the 44 adult MDs, blood samples were collected from April 2017 to April 2021 by a licensed veterinarian or a supervised veterinary student by venipuncture during the dogs' first visits to the Hokkaido University Veterinary Teaching Hospital (HUVTH) for routine diagnostics with written informed consent obtained from the owners. For the juvenile MDs, blood samples were collected by a licensed veterinarian (YBT) using a venipuncture. The juvenile MDs were owned by a small and medium enterprise pet store in Hokkaido, Japan; written informed consent was obtained from the enterprise's owner and veterinarian on duty, and sample collection was performed from November 2021 to February 2022. Serum samples separated after centrifugation were stored at −80°C until the TG analysis. Serum TG concentrations were measured as per protocol provided by the manufacturer using the Canine Thyroglobulin ELISA Kit (MyBioSource, San Diego, CA, USA, Catalog No: MBS2608140). The intra-assay coefficient of variability between sample replicates was <20%. The information on the regional/systemic inflammation and medication (e.g., corticosteroids) is given in Supplementary Table 3.

TABLE 3 Primer sequences used for qPCR in canine colonic mucosa.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
IL6	TTAAGTACATCCT CGGCAAAATCT	CAGTGCCTCTTTG CTGTCTTCA
SDHA	GCCTTGGATCTC TTGATGGA	TTCTTGGCTCTTA TGCGATG
CCL2	GAGTCACCAGCA GCAAGTGT	TGGGTTTGGCTTT TCTTGTC

2.8. Real-time quantitative PCR (qPCR) in canine colonic mucosa samples

For qPCR using canine colonic mucosa samples, seven MD-ICRP (three with T/T allele and four with C/C allele), which visited HUVTH between April 2017 and April 2021, were included; total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA); and genomic DNA was removed using an RNase-free DNase Set (Qiagen) following the manufacturer's instructions. cDNA synthesis was then performed using oligo (dT) and M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA) from 1 μg total RNA as per the manufacturer's recommendation. Real-time TaqMan qPCR was performed using a commercially available set of a pre-designed probe and primers (Applied Biosystems) for *TG* (product no: Cf02701382_m1) with an endogenous control for colonic samples using *SDHA* (product no: Cf02664981_m1) confirmed by a previous study (41). Real-time qPCR was performed using TaqMan PCR probe master mix (KAPA Biosystems) and at the following cycle conditions: 40 cycles at 94°C for 3 s and 40 cycles at 60°C for 30 s. The relative *TG* mRNA expression levels were normalized to the level of *SDHA* mRNA expression. THUNDERBIRD SYBR qPCR Mix (TOYOBO Co. Ltd., Osaka, Japan) was used to quantify the levels of the target mRNA (*IL6*, *CCL2*) and internal control mRNA (succinate dehydrogenase complex, subunit A; *SDHA*). The condition for real-time qPCR was 40 cycles at 94°C for 15 s followed by 40 cycles at 60°C for 60 s. Relative *IL6* and *CCL2* mRNA expressions were normalized to the level of *SDHA* mRNA expression. The primers and their sequences for the qPCR are described in Table 3 and reported previously (42, 43). For the qPCR using these samples, all the experiments were performed in duplicates and performed twice to ensure replication.

2.9. Statistical analysis

Two-tailed Student's *t*-test and Fisher's exact test were used to analyze differences between

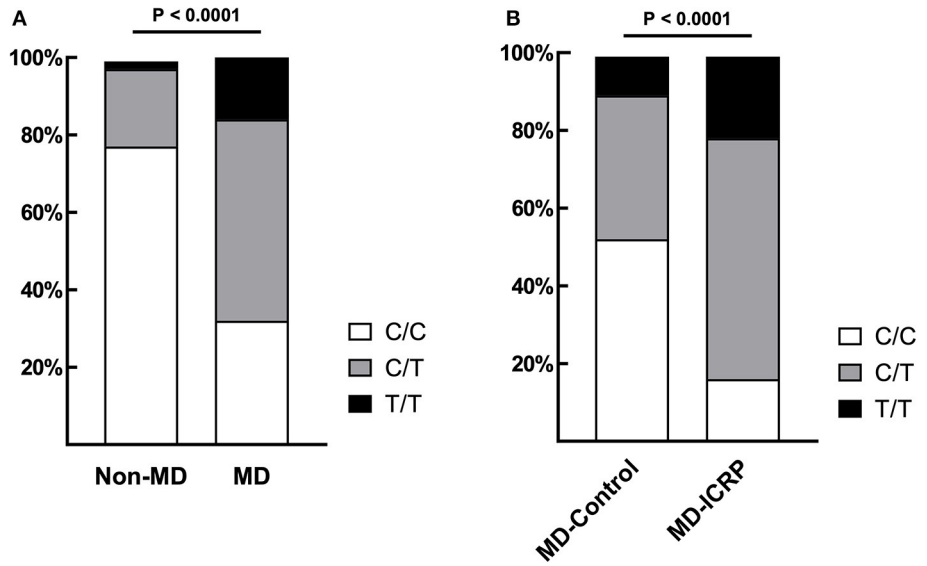

FIGURE 1
TG SNP is associated with ICRP in MDs. **(A)** The frequency of variant *TG* c.4567C>T (p.R1523W) was significantly higher in MDs ($n = 281$) when compared to other breeds ($n = 40$). $p < 0.0001$. **(B)** Among MDs, age-matched, case–controls showed that the same variant was significantly higher in MD-ICRP ($n = 155$) than MD-Control ($n = 90$). $p < 0.0001$.

the two groups. Values of $P < 0.05$ were considered significant.

3. Results

3.1. *TG* c.4567c>t (p.1523r>w) allele is increased in MDs with ICRP

The frequency of *TG* c.4567C>T (p.1523R>W) allele in MDs ($n = 281$) was 69.04% (48 homozygous and 246 heterozygous), which was significantly higher than in dogs of other breeds (22.5%; one homozygous and eight heterozygous, $n = 40$) ($P < 0.0001$, Figure 1A). Sequence chromatograms of the target variant are shown in Supplementary Figure 1A. Furthermore, the frequency of the T risk allele (84.52%) was significantly higher in MD-ICRP (median age = 10 years old; 34 homozygous, 97 heterozygous, $n = 155$) than in MD-controls (47.78%; median age = 12 years old; 9 homozygous, 34 heterozygous, $n = 90$) ($P < 0.0001$, Figure 1B). Thus, the *TG* c.4567C>T (p.1523R>W) allele was increased in MDs with ICRP. Finally, we found that one amino acid of the *TG* gene was conserved in dogs and other mammals (Supplementary Figures 1B, C).

3.2. *TG* is a positive regulator and target of the IL-6 amplifier activation

Because we found that TG is one positive regulator candidate of the IL-6 amplifier (39) and showed that *TG* c.4567C>T (p.1523R>W) allele is increased in MDs with ICRP, we hypothesized that TG is functionally involved in ICRP development via activation of the IL-6 amplifier. Consistently, we found that *TG* expression was increased during the activation of the IL-6 amplifier (Figures 2A, B), suggesting that *TG* is a target gene of the IL-6 amplifier. We then treated H4 cells, a human neuroglioma cell line, with siRNA of *TG* to investigate cytokine-mediated IL-6 amplifier (39) and found that *IL-6* expression and *TG* expression were reduced after cytokine stimulation (Figures 3A, B). Furthermore, recombinant human TG enhanced IL-6 amplifier activation in H4 cells (Figure 3C). These results suggested that TG is both a positive regulator and a target of the IL-6 amplifier.

3.3. *TG* is highly expressed and the IL-6 amplifier activation is enhanced in the colon of MD-ICRP with risk allele

We hypothesized that the TG SNP functions to increase *TG* expression either systematically or locally. To test this hypothesis, we investigated the serum concentrations of TG but found they were unchanged in MDs with or without ICRP and with or without the risk T/T SNP (Figures 4A–C). In addition, we found no difference in serum TG concentrations between dogs receiving systemic anti-inflammatory medication or across genotypes (Supplementary Figures 2A–C). We then investigated the expression levels of *TG* in the non-inflammatory colonic mucosa of MD-ICRP. We found that *TG* expression in samples with the risk T/T SNP was significantly higher than those with the non-risk C/C SNP (Figure 5A), suggesting that the TG SNP is an expression quantitative trait locus (eQTL) in the colon of MDs. We next investigated whether *TG* is induced by the IL-6 amplifier in the colon. Because the IL-6 amplifier reflects the activation of NF-κB in non-immune cells, we analyzed the expression of two NF-κB targets, *IL6* and *CCL2*, in the same colon samples. We found that

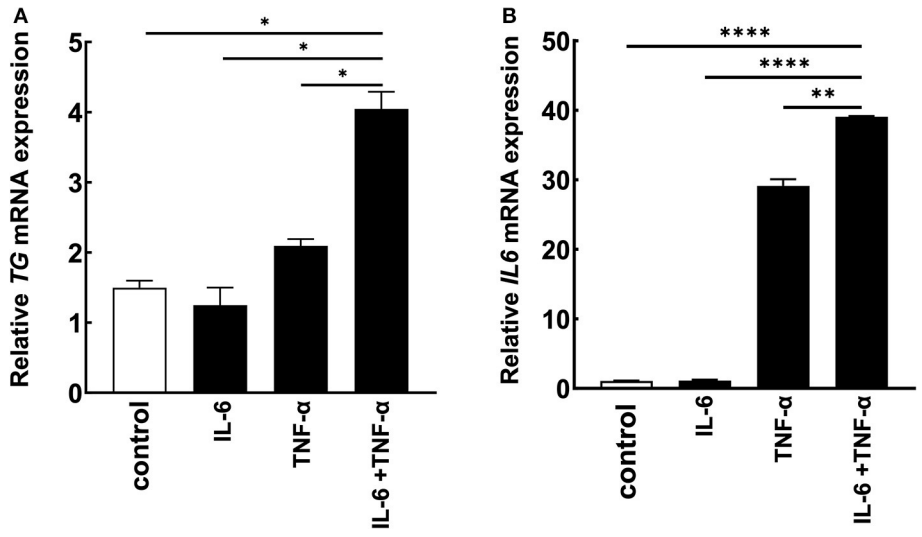

FIGURE 2
TG is a potential target of the IL-6 amplifier *in vitro*. **(A, B)** H4 cells were stimulated with cytokines, and *IL6* and *TG* levels were assessed. Means ± standard deviations are shown. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

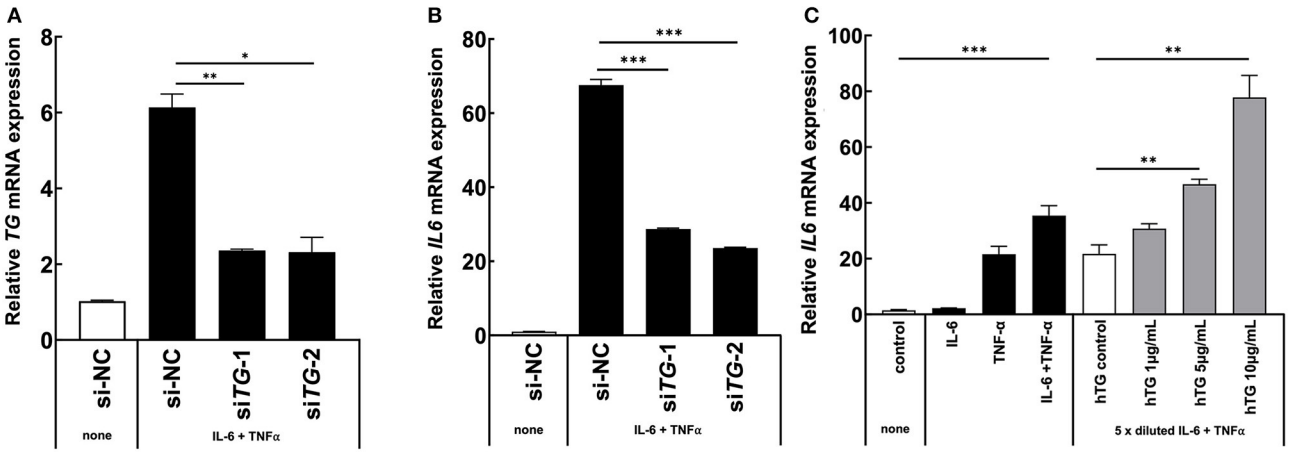

FIGURE 3
TG is critical for IL-6 amplifier activation *in vitro*. **(A, B)** H4 cells were transfected with two different siRNAs for all *TG* variants or a siRNA negative control. *IL6* levels and *TG* knockdown efficiency were assessed. Means ± standard deviations are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. **(C)** H4 cells were stimulated with cytokines and recombinant human TG. *IL6* levels were assessed. Means ± standard deviations are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

IL6 and *CCL2* were also enhanced in colon samples with the risk T/T SNP (Figures 5B, C). These results indicate that the risk T/T SNP of *TG* is an eQTL and enhances *TG* expression. They also indicate that TG is involved in the activation of the IL-6 amplifier in the colon of MD-ICRP. Therefore, the risk T/T SNP of *TG* may be critical in the pathogenesis of ICRP through the activation of the IL-6 amplifier.

4. Discussion

ICRP has been reported to be breed-associated (1, 44, 45). We recently identified several disease-associated SNPs, such as *PLG*, *TCOF1*, *TG*, *COL9A2*, and *COL4A4*, and found that PLG SNP is associated with IL-6 amplifier activation in the colon (12). In this study, we show that the risk SNP of *TG* is associated with the pathogenesis of ICRP in MDs and that the increased expression of *TG* is associated with the risk allele T/T in the non-inflammatory colonic mucosa, showing that TG possesses a biological role in the activation of NF-κB, which is a critical component of the IL-6 amplifier.

TG is commonly known as the precursor protein to thyroid hormones T3 and T4, which regulate multiple metabolic pathways in the mammalian body (46). Recent advances have shown that TG may function both inside and outside of the thyroid, which is no surprise as *TG* expression was detected in multiple non-thyroidal

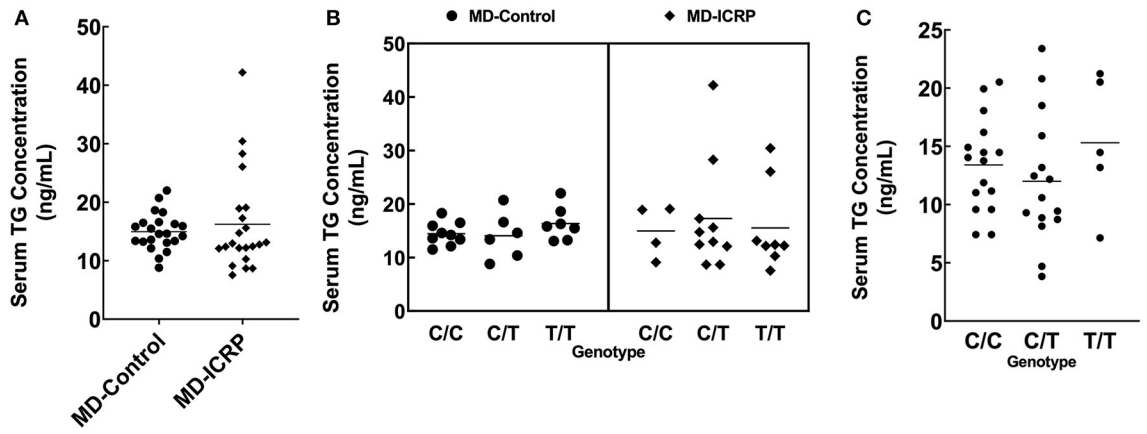

FIGURE 4
The *TG* SNP does not affect systemic TG levels. **(A)** Serum TG concentration between age-controlled MD-Control and MD-ICRP groups. **(B)** Serum TG concentration between genotypes in MD-Control and MD-ICRP groups. **(C)** Serum TG concentration between genotypes in MDs <1-year-old.

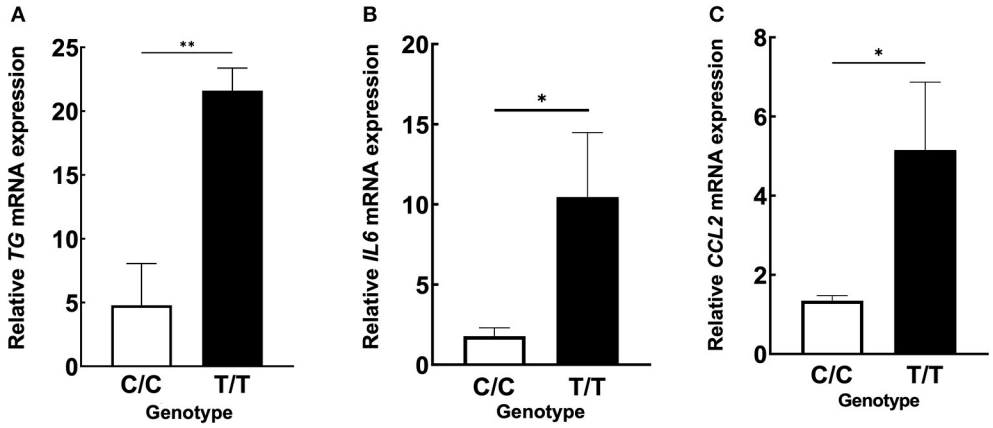

FIGURE 5
ICRP-MDs with risk alleles have a higher expression of *TG* and NF-κB-related chemokines in non-inflammatory colonic mucosa. **(A)** Relative *TG*, **(B)** *IL6*, and **(C)** *CCL2* mRNA expressions in canine non-inflammatory colonic mucosa adjacent to the ICRP lesion site and diagnosed as normal histopathologically in MDs with wild-type (C/C) and risk (T/T) alleles. Means ± standard error of means is shown. $*p < 0.05$, $**p < 0.005$.

cells such as human and mouse kidney cells (47). Additionally, *TG* mRNA is expressed in many organs including the testis, suprarenal gland, appendix, lung, and thymus, as well as the hypophysis, lymphocytes, and leukocytes (48–52). Here, we showed that *TG* mRNA is expressed in the colon, especially in MDs with the risk allele T/T of TG SNP. Considering that TG expression with the risk alleles in the colon correlated with the development of ICRPs in MDs, TG-mediated activation of the IL-6 amplifier in the intestine may contribute to the high concurrent occurrence of thyroidal disease and inflammatory bowel disease in human patients (53–56).

Because the local expression of *TG* was higher in the risk allele T/T group compared with the wild-type allele C/C group, we concluded that the function of the SNP is to induce a higher local expression of *TG* in the colon (Figure 5A) but not in the circulation. The localized increase in *TG* mRNA levels was accompanied by localized increases in *IL6* and *CCL2* expression in non-inflammatory colonic mucosa (Figures 5B, C), suggesting that the IL-6 amplifier is activated in the colon of MDs with the risk allele T/T. This result may explain why ICRP only occurs in the colorectal region, although more studies of the expression of TG and other NF-κB targets in other organs, including other regions of the gastrointestinal tract, with or without the risk allele T/T of TG SNP, are needed. Because we found that MDs without the risk allele T/T (C/T or C/C) were affected with ICRP, we hypothesize that one of the reasons is that ICRP in MDs is a polygenic disease and other mechanisms may also be involved in its pathogenesis, although the number of cases for the qPCR analysis in canine colonic mucosa is too small to verify this conclusion. In addition, we are not able to confidently state that there is no blood relationship between the dogs because we do not have the pedigree of each dog used in this study.

In summary, we found a novel SNP variant, *TG* c.4567C>T (p.R1523W), which is involved in the pathogenesis of ICRP in MDs. We also suggested that TG has a functional role in the development of ICRP in MDs through the IL-6 amplifier in colon cells and that the presence of the risk allele T/T correlates with a higher

expression of *TG* locally compared to allele C/C. Our results also suggest that the T/T SNP is an eQTL of *TG* mRNA in the colon and the local *TG* expression triggered by this SNP increases the risk of ICRP in MDs via activation of the IL-6 amplifier. Therefore, *TG* c.4567C>T is a potential diagnostic target for ICRP in MDs and *TG*-mediated IL-6 amplifier activation in the colon is a possible therapeutic target for ICRP.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found at: https://academic.oup.com/intimm/advance-article/doi/10.1093/intimm/dxad006/7080519.

Ethics statement

Ethical review and approval was not required for the animal study because samples retrieved from the animals were part of the diagnostic workup for the disease, including blood test, lower gastrointestinal endoscopy, and biopsy and histopathology submission. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MM designed the study and edited the manuscript. J-JJ and YT performed most of the experiments. RH, TY, and NN contributed to the experiments. HO provided the canine samples. KN, NS, and NY advised on the research design. YK confirmed the diagnosis of the canine pathology. YT, MT, and MM wrote the manuscript. MM and MT supervised the study. All authors have read and agreed to the published 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.

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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.2023.1192888/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) Sequence chromatograms of *TG* c.4567C>T (p.R1523W) in wild-type, heterozygote, and homozygote variant samples. **(B)** A schematic diagram of the exon of canine *TG* gene and thyroglobulin protein. SP, Signal protein; TGT1, Thyroglobulin Repeat Type 1. **(C)** Alignment of the amino acid sequence in the final thyroglobulin type-1 repeat domain of canine thyroglobulin against selected mammals using the UniProt repository (https://www.uniprot.org/). Asterisks (*): complete conservation. Colon (:): strongly similar. Period (.): weakly similar properties. R1523 in dogs is strongly similar to other mammals but is highly conserved among other mammals.

SUPPLEMENTARY FIGURE 2
The systemic anti-inflammatory drug does not affect systemic TG levels. **(A)** Serum TG concentration between MDs that are treated with or without anti-inflammatory drugs. **(B)** Serum TG concentration between MD-control and MD-ICRP groups that are treated with or without anti-inflammatory drugs. **(C)** Serum TG concentration between genotypes of MDs that are treated with or without anti-inflammatory drugs.

SUPPLEMENTARY TABLE 1
Details of MDs and other breeds included in this study.

SUPPLEMENTARY TABLE 2
Case details of MDs recruited for qPCR analysis.

SUPPLEMENTARY TABLE 3
Case details of MDs recruited for serum TG evaluation.

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Sero-epidemiological study of zoonotic bacterial abortifacient agents in small ruminants

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Abortion is one of the leading causes of economic losses in the livestock industry worldwide. *Chlamydia abortus*, *Coxiella burnetii*, and *Brucella* spp. are the leading cause of late fetal loss in small ruminants. This study determined the seroprevalence of these agents in small ruminants in district Jhang. A total of 385 serum samples were taken from the sheep and goats from different flocks with a history of abortion and subjected to i-ELISA. Further, samples were analysed for liver enzymes and total serum protein using a semi-automated chemistry analyzer. The result of indirect commercial ELISA showed 13.0, 4.2 and 11.2% prevalence for *Coxiella burnetii*, *Chlamydia abortus*, and *Brucella* spp., respectively. Values of different serum parameters (ALT, AST, and total protein) of seropositive animals were also determined. There was a significant rise in AST and ALT values of infected animals ($p \leq 0.05$). Total protein decreased for all three infections, but a significant drop was noted in *Brucella* positive sheep serum samples. Various risk factors were studied. Binary logistic regression proved a significant role of ticks for coxiellosis and brucellosis. Age, parity, and species did not impact the prevalence of diseases studied.

KEYWORDS

abortion, *Brucella*, *Coxiella*, *Chlamydia*, small ruminants

1. Introduction

Abortifacient agents are the essential factors for tremendous losses in the livestock industry. These losses are caused by infertility, stillbirths, repeated breeding, decreased milk production, meat loss by aborted fetuses, additional treatment management costs, and veterinary services (1, 2). Moreover, abortion is a significant threat to public health when caused by zoonotic organisms. The most important zoonotic microorganisms causing abortions in small ruminants are *Brucella* spp., *Chlamydia abortus (C. abortus)*, and *Coxiella burnetii (C. burnetii)* (3).

Coxiellosis is caused by *C. burnetii*, an obligate intracellular, Gram-negative, and pleomorphic bacterium ranging in size from 0.4–1 μm in length and 0.1–0.4 μm in width. This agent has very similar characteristics to bacteria of the group Rickettsia (4). Once a neglected

disease, it has attracted attention in human and veterinary medicine due to its zoonotic character and the economic losses caused. The organism can infect a wide range of animal species, but dairy animals, including cattle, buffaloes, sheep, and goats, are considered the main reservoirs of *C. burnetii* (5). It resides in the soil for a long period as it forms spore-like particles in harsh conditions and becomes resistant to high temperatures, ultraviolet radiation, and drying (6). Infection may occur after inhaling contaminated aerosols, uptake of contaminated feed, or ticks bites, while humans may also become infected after consuming contaminated raw milk food (5). Coxiellosis is usually an asymptomatic disease in many species. Clinical manifestations may involve abortion, stillbirth, premature delivery, and weak offspring in ruminants. Abortion in advanced pregnancy is the most important clinical observation of Q fever in sheep and goats (7). Q fever resembles atypical pneumonia in humans, a self-limiting flu-like disease that causes headaches, respiratory symptoms, and hepatitis (8). The chronic disease may result in endocarditis in humans (8, 9). The pathogen is shed in high numbers with secretions of infected animals into the environment at the time of parturition or abortion (10).

Chlamydia abortus causes abortion in sheep and goats worldwide and is known as Ovine Enzootic Abortion (OEA) or (EAE) Enzootic Abortion of Ewes (11, 12). *C. abortus* is a Gram-negative obligate intracellular bacterium. This disease causes acute placentitis and abortion in advanced pregnancy, particularly in the last 2–3 weeks. There may be noticed stillbirth or birth of weak lambs if abortion does not occur. Behavioral changes and vulvar discharge may be seen before abortion in some cases (13). Premature lambs often succumb within 24 h after birth (14). The vagina and placenta are covered with highly infectious pinkish and reddish-yellow colored exudates, respectively. Metritis, especially in goats, may also be found due to placenta retention due to secondary bacterial infection (15). Abortion, 2–3 weeks before the expected lambing time, could be the first clinical sign noticed. Uterine discharges contaminate the environment, and fetal fluids and organisms are shed with the infected placentae. Infection occurs via ingestion or inhalation of contaminated materials (16, 17).

Brucellosis in small ruminants is mainly caused by Gram-negative, non-motile, highly contagious, zoonotic bacteria of *Brucella melitensis*. High economic losses result from sterility, fetal death, or abortion (7). The pathogen is shed in large numbers in feces, urine, milk, and reproductive secretions by infected animals, spreading the disease to other animals and humans and posing a considerable threat to public health. The animals may get infected by ingesting or inhaling the contaminated feed and air. Wounds, bruises, or any discontinuity of the skin can also be a point of entry for the bacteria (18).

Abortifacient bacteria cause serious health problems in small ruminants by damaging vital organs such as the liver, kidney, and heart (19). The injury to these organs results in biochemical alterations, for instance, the necrosis of hepatocytes due to *Brucella* spp. results in the increase of certain liver enzymes such as ALT and AST and a decrease in proteins produced by the liver (20, 21); similarly, a study conducted in Iraq reported the biochemical changes due to chlamydiosis in ewes (22). However, the data is scanty about the biochemical alteration caused by abortifacient bacteria in small ruminants and needs further investigation.

In the current study, we investigated the prevalence of antibodies against *C. abortus*, *C. burnetii*, and *Brucella* spp. and identified risk factors along with the effects of these infections on liver enzymes and total serum protein.

2. Materials and methods

The study was conducted in District Jhang. Ethical approval was taken from the Ethical Review Committee (IRC) of UVAS. The study area comprises four tehsils (Jhang, Athara Hazari, Shorkot, and Ahmadpur Sial). The sample size essentially required to carry out the present study was determined by using the formula $n = 1.96^2\ P\text{exp}\ (1 - P\text{exp})/d^2$ with a 95% confidence interval, where n = required sample size, P = expected prevalence, and d = desired absolute precision (23). A prevalence of 50% was expected. A total of 385 serum samples were collected from animals with a history of abortion within the last month (30 days) randomly from all over the district. During sampling, data of the individual animals, i.e., species, parity, age, and presence of ticks, were collected using a questionnaire. The samples were collected during winter, from November to March, as most parturitions and abortions in small ruminants occur in these months. Out of 385 serum samples, 235 samples were taken from goats, and 150 samples were taken from sheep depending upon the availability of aborted animals of both species not previously vaccinated. Animals were divided into two age groups, 1–3 years and 3–6 years. Whole blood was collected from the jugular vein of sheep and goats with a history of abortion using gel clot vacutainers. After clotting at room temperature, the samples were centrifuged at 1,500 rpm for 10 min. Supernatants were stored in 1.5 mL aliquots at −80°C until use.

2.1. Serological analysis

Antibodies against *Brucella* spp. were detected using ID Screen® Brucellosis Serum Indirect Multi-species ELISA (IDvet, France) (24) and against *C. burnetii* using an indirect Enzyme-Linked -Immunosorbent Assay (ID Screen® Q Fever Indirect Multi-species, IDvet, France) (25). This kit is based on phase I and II *C. burnetii* antigens.

The serum antibodies against *C. abortus* were detected using the commercial ELISA kit (ID Screen® *Chlamydophila abortus* Indirect Multi-species, CHLMS-MS-2P / CHLMS-MS-5P) (26). The ID Screen *C. abortus* Indirect ELISA uses a synthetic antigen from a major outer-membrane protein (Momp) specific to *C. abortus*, which reduces the frequency of non-specific reactions. The kits were used according to the instructions of the manufacturers.

2.2. Biochemical analysis

A total of 86 serum samples from aborted (39 goats and 27 sheep) and clinically healthy animals (10 goats and 10 sheep) were analyzed for serum biochemistry (27). Total protein (TP) and serum enzymes ALT and AST were estimated on an automated chemistry analyzer (Optizen HF 1412, Korea) using commercial kits (Bioactiva diagnostica GmbH, Germany) according to the manufacturer's recommendations (28).

2.3. Statistical analysis

Data regarding antibody seroprevalence was analyzed using the chi-square test, while the impact of different risk factors, such as area, age, parity, etc., was estimated by binary logistic regression. Variations in the concentrations of serum enzymes and total protein were analyzed by one way ANOVA test. A value of p lower than 0.05 was considered significant.

3. Results

3.1. Seroprevalence of anti-*Coxiella burnetii*, *Chlamydia abortus* and *Brucella* spp. antibodies in sera of sheep and goats

The serological analysis revealed a comparable prevalence of antibodies against the three abortifacient agents, *C. burnetii*, *C. abortus*, and *Brucella* spp., in sheep and goats. No significant difference was found between the prevalence of these pathogens in sheep and goats (Table 1).

There is a total prevalence of 13.0% for anti-*Coxiella burnetii*, 4.2% for anti-*Chlamydia abortus*, and 11.2% for anti-*Brucella* spp. antibodies were found in small ruminants. Risk factors like parity and age of animals showed a no-significant relationship with the seroprevalence. Anti-*Coxiella burnetii* antibodies' prevalence was 12.6% in sheep and 13.1% in goats (Figure 1). In the case of anti-*Chlamydia abortus* antibodies, 3.3% prevalence was found in sheep and 4.6% in goats. The prevalence of anti-*Brucella* spp. antibodies were 16% in sheep and 8% in goats. No statistically significant relation was found for host prevalence or study area.

3.2. Risk factors

Species of animals (sheep and goats), age (two age groups: 1–3 years and 3–4 years), and parity were studied as possible risk factors. Statistical analysis (binary logistic regression) revealed that the species, age, and parity have no significant impact as risk factors (Figure 2). However, ticks play a significant role in the occurrence of anti-*C. burnetii* antibodies. Statistical analysis showed no significant relationship between anti-*Brucella* antibodies with ticks. The results are described in Table 2.

3.3. Serum biochemistry

The trends for increased or decreased serum enzymes (ALT and AST) and total protein with the prevalence of antibodies against the investigated pathogens were similar. A significant increase in ALT and AST was noted, while the total serum protein concentration was decreased. Detailed statistics are shown in Tables 3–5.

4. Discussion

Coxiellosis, brucellosis, and chlamydial infections are diseases of zoonotic importance and impose devastating economic losses to the livestock industry by decreased production and reduced reproduction (29, 30). Early diagnosis of diseases has a positive effect on the successful treatment of animals and may prevent the spread of disease to other animals and human beings (31, 32). Further, the economic losses to the livestock industry and costs for the public health sector may be prevented (33, 34). In Pakistan, data on the geographic distribution of Q fever based on epidemiological surveillance studies are limited for livestock and humans. In the current research, the prevalence of *C. burnetii* infection, i.e., the seroprevalence in small ruminants, has been investigated using indirect ELISA, the serodiagnostic technique is preferably used for screening as it is more sensitive and specific than any other serological technique (35–37) reported 100% sensitivity and specificity for the applied IDVET® Q fever Indirect ELISA. Thus, 13.0% sero-epidemiology of the pathogen in district Jhang is a significant finding concerning future disease management in animals and humans.

The current study investigated the relationship between the risk factor age with anti-*Coxiella burnetii* antibody prevalence in small ruminants. The statistical analysis revealed that age is not a significant risk factor. This result coincides with the results of the previous study (37). It can be assumed that the way of keeping the herds (mixed age group and species) results in chronic infection of the herd and

TABLE 1 Prevalence and anti-*Coxiella burnetii*, *Chlamydia abortus* and *Brucella* spp. antibodies in sheep and goats of Jhang, Pakistan.

Parameter		*Coxiella burnetii*			*Chlamydia abortus*			*Brucella* spp.		
		+ve (%)	Total	p value	+ve (%)	Total	p value	+ve (%)	Total	p value
Species	Sheep	19 (12.6)	150	0.38	5 (3.3)	150	0.6	24 (16)	150	0.19
	Goats	31 (13.1)	235		11 (4.6)	235		19 (8)	235	
	Total	50 (13.0)	385		16 (4.2)	385		43 (11.2)	385	
Parity	1st–3rd	29 (16.7)	174	0.124	7 (4.0)	174	0.5	15 (8.6)	174	0.45
	4th–6th	21 (10.0)	211		9 (4.3)	211		28 (13.2)	211	
	Total	50 (13.0)	385		16 (4.2)	385		43 (11.2)	385	
Age (years)	1–3	33 (15.1)	218	0.3	9 (4.1)	218	0.5	30 (13.8)	218	0.5
	4–6	17 (10.2)	167		7 (4.2)	167		13 (7.8)	167	
	Total	50 (13.0)	385		16 (4.2)	385		43 (11.2)	385	

The values in the parenthesis are indicating percentile prevalence.

infection pressure on the individual animal independent of its age. There was no significant difference concerning the prevalence of coxiellosis found among different four tehsils of District Jhang. This indicates that the disease is prevalent and endemic in District Jhang. Our study shows no significant difference in the prevalence of coxiellosis in sheep and goats, indicating that both species are equally susceptible to the disease and share the same epidemiological environment. These results are similar to a previous study conducted in Punjab, Pakistan (18). Notably, ewes and goats are more prevalent during the first, second, and third pregnancy than those with higher parities. The relationship between the prevalence of disease with the parity of animals has also been evaluated in the current study. Animals with the first three parities indicated a higher disease prevalence than those with higher parities (38). It can be assumed that older females may have developed a certain degree of immunity that hinders re-infection.

Biochemical analysis indicated the stoking rise of the amount of the ALT and AST serum enzymes. This may be due to the chronic infection, e.g., ongoing inflammation of placental tissue. Chronic infection of the liver by *C. burnetii* is also reported by various studies. This chronic infection is responsible for the increase of ALT and AST and the decrease in the total protein concentration in the serum. Our results coincide with the findings of (39). There are several reports of hepatitis associated with *C. burnetii* infection in humans as well (40, 41).

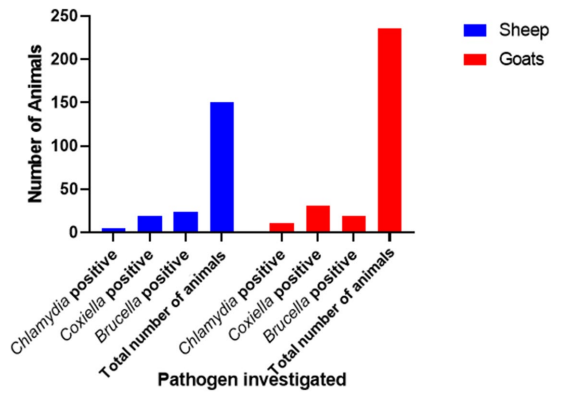

FIGURE 1
Prevalence of anti-*Chlamydia*, *Coxiella*, and *Brucella* spp. antibodies in small ruminants in Jhang, Pakistan.

TABLE 2 Binary logistic regression statistics of risk factors.

Risk factor	95% C-I	Odd ratio	value of *p*
Anti-*Coxiella burnetii* antibodies			
Species	0.65–1.64	1.431	0.373
Age	0.30–2.41	0.863	0.778
Parity	0.19–1.39	0.518	0.194
Ticks	3.97–21.3	9.213	0.000
Anti-*Chlamydia abortus* antibodies			
Species	0.743–6.640	2.221	0.153
Age	0.239–4.132	0.995	0.994
Parity	0.291–5.11	1.220	0.784
Anti-*Brucella* antibodies			
Species	0.205–3.858	0.889	0.876
Age	0.027–3.078	0.288	0.303
Parity	0.151–11.282	1.306	0.808
Ticks	0.657–13.189	2.945	0.157

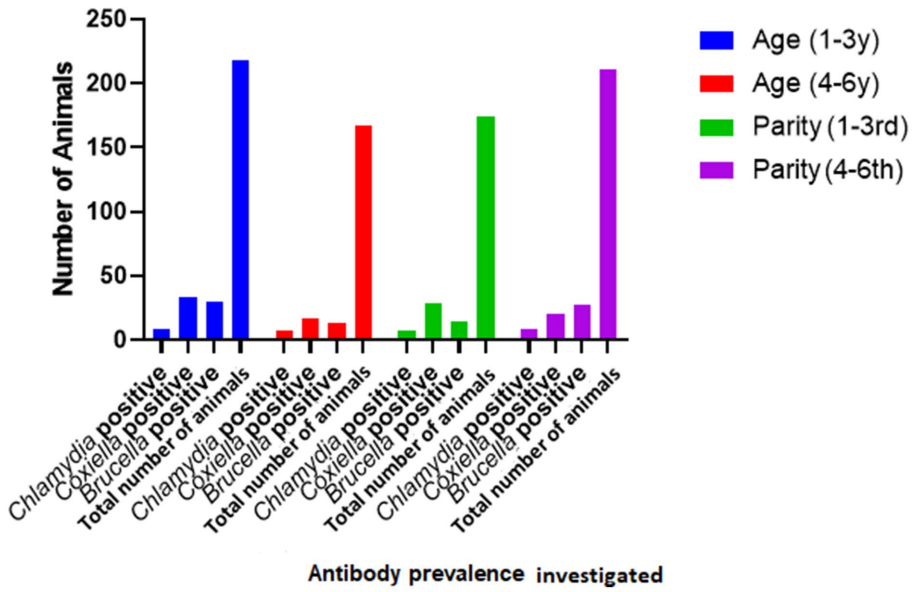

FIGURE 2
Relationship of seroprevalence with age and parity.

TABLE 3 Relations of anti *C. abortus, C. burnetii* and *Brucella* spp. antibodies prevalence to serum enzyme ALT and AST in sheep.

	ALT			AST	
	Number	Mean ± S.D	Value of *p*	Mean ± S.D	Value of *p*
Chlamydia positive	10	42.3 ± 9.9[b]	0.000	134.1 ± 46.0[b]	0.00
Coxiella positive	7	49.1 ± 4.7[b]	0.000	100.8 ± 12.6[a]	0.88
Brucella spp. positive	10	57.4 ± 7.3[b]	0.001	90.2 ± 12.6[a]	0.33
Healthy animals	10	29.5 ± 5.7[a]		59.5 ± 9.0[a]	

The values of ALT and AST with different superscripts in each column show significant difference among infected and healthy animals.

TABLE 4 Relations of anti *C. abortus, C. burnetii* and *Brucella* spp. antibodies prevalence to ALT and AST in Goats.

	ALT			AST	
	Number	Mean ± S.D	Value of *p*	Mean ± S.D	Value of *p*
Chlamydia positive	11	40.0 ± 8.9[b]	0.003	92.54 ± 3[b]	0.001
Coxiella positive	18	45.6 ± 9.7[b]	0.001	87.83 ± 2[b]	0.002
Brucella spp. positive	10	69.4 ± 6.3[b]	0.000	100.80 ± 8[b]	0.001
Healthy animals	10	10.9 ± 1.7[a]		64 ± .2[a]	

The values of ALT and AST with different superscripts in each column show significant difference among infected and healthy animals.

TABLE 5 Relations of anti *C. abortus, C. burnetii* and *Brucella* spp. antibodies prevalence to total protein in sheep and goats.

	Sheep				Goat			
	Number	Mean ± S.D	CI 95%	Value of *p*	Number	Mean ± S.D	CI 95%	Value of *p*
Chlamydia positive	10	5.46 ± 1.54[a]	4.35–6.56	0.946	11	6.0 ± 1.0[a]	4.14–8.6	0.50
Coxiella positive	7	4.67 ± 0.72[a]	3.9–5.34	0.284	18	6.6 ± .84[a]	5.10–8.6	0.20
Brucella spp. positive	10	4.07 ± 1.79[b]	2.7–5.35	0.027	10	4.4 ± .55[a]	3.0–5.5	0.92
Healthy animals	10	5.73 ± 0.87[a]	5.1–6.35		10	4.9 ± .31[a]	4.21–5.6	

The values of total protein with different superscripts in each column show significant difference among infected and healthy animals.

Our study also studied different risk factors for infection with *C. abortus*, such as species, age, and parties. A higher sero-prevalence was recorded in goats when compared to sheep. Five out of 150 sheep (3.3%), while 11 out of 235 caprine samples tested positive (4.6%). This higher prevalence in goats may be a sampling bias. However, statistical analysis showed that the host species is indeed no significant risk factor ($p > 0.05$), as already described in a recent study (42). Though statistically non-siginifcant ($p > 0.05$), however, it has been found that animals of the 1-3 year age group were more often found positive than those of the age group older than 3 years. This finding agrees with the findings of a previous study (43). Possible reasons for these findings have already been described above.

Brucellosis is also prevalent in Pakistan, causing significant losses for animal owners and posing a severe risk to human health (18). Serology is the preferred choice for diagnosing brucellosis to reduce the high risk of infection for laboratory personnel during cultivation (44). ELISA offers easy handling,high sensitivity and specificity. Our study revealed 11.2% prevalence in small ruminants from District Jhang. Serum parameters such as AST, ALT, and total protein were studied in infected animals. There was a significant increase in the values of AST and ALT in the infected animals. Mean values of AST and ALT were higher in the animals infected with *Brucella*. These results are in agreement with a previous study (45). The reasons for our findings have been discussed already.

Hence blood parameters need further investigation. In this study, ALT and AST values were increased in sheep and goats, as found in a previous study (22). In contrast, Kushwaha et al. (46) found a significant decrease in ALT and AST. The total protein showed a non-significant decrease in sheep and goats. The decrease in total protein may be caused by the damage of the endothelium of the liver due to chronic intracellular infection leading to decreased production of liver proteins (47). The excretion of proteins in urine may be an indicator of renal infection. Chronic liver infection may also cause extensive damage to the liver cells, which release ALT. Consequently, an increase in ALT activity is measured in the serum (48). Although generally thought to be specific to the liver, ALT is also found in the kidneys and, in much smaller quantities, in heart and skeletal muscle cells. In acute hepatocellular injury, serum AST levels usually rise immediately, reaching higher levels than ALT within 24–48 h. If ongoing chronic damage occurs, ALT levels will become higher than

AST levels because of their higher plasma half-life. Finally, ALT levels are more commonly elevated than AST levels (49). In human medicine, elevated levels of serum liver enzymes have been observed in hepatitis due to brucellosis (50).

5. Conclusion

Brucellosis, and coxiellosis have reltively higher prevalence than Chalmydiosis in the study area. This is the first study reporting the prevalence of *C. abortus* in small ruminants in Punjab province of Pakistan. This study further concludes that ticks are important risk factors for the occurance of coxiellosis and brucellosis. These infections may also affect the liver function as indicated by a rise in the serum levels of liver enzymes and decrease in the total protein concentration. Additional studies are required to estimate the prevalence of *C. abortus* in small ruminants in other areas of the country.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Ethical Review Committee, University of Veterinary and Animal Sciences Lahore (Sub Campus Jhang). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MZ, SA, IA, and AR planned and executed the study. MR, AN, MK, and AK contributed in the methodology and data analysis. KM-S, MA, SE-u-H, and HN performed data analysis, drafting and editing manuscript. 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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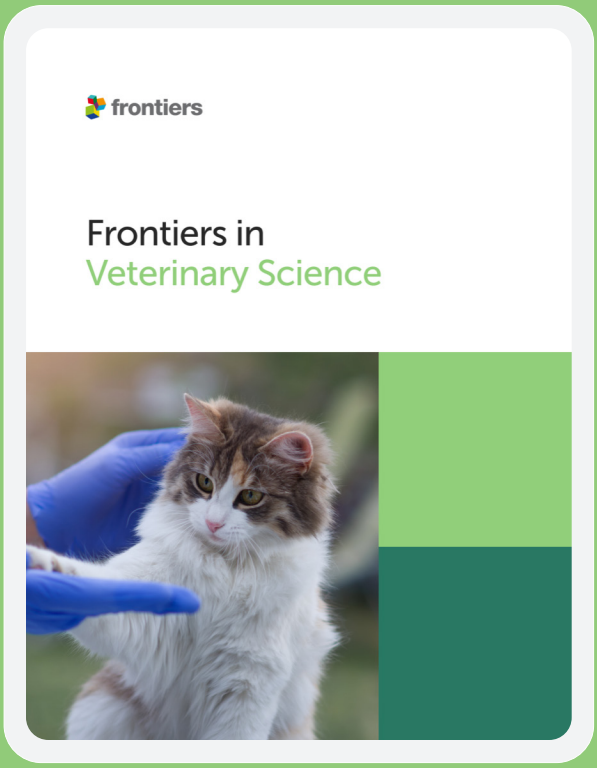

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