

Applications of Metabolomics in the Diagnosis of Animal Protozoan Diseases and Antiprotozoal Drug Resistance: A Review

Moges Maru^{*1}, Zewdu Seyoum Tarekegn¹, Mengesha Ayehu Getnet¹, Mastewal Birhan¹, Nega Yismaw Alemu³, Mersha Chanie Kebede¹, Abebe Tesfaye², Mohammed Yesuf Adem¹, Mersha Teka¹, Achenef Melaku Beyene¹

¹Department of Veterinary Pathobiology, College of Veterinary Medicine and Animal Sciences, University of Gondar, Ethiopia

²Department of Veterinary Biomedical Sciences, College of Veterinary Medicine and Animal Sciences, University of Gondar, Ethiopia

³Livestock and fishery development Bureau Bahir Dar, Ethiopia

*Corresponding author Email: malemayehu19@yahoo.com

SUMMARY

This review highlights the expanding role of metabolomics in understanding animal protozoan diseases and detecting antiprotozoal drug resistance. Articles published in English were searched and identified through databases, including PubMed, Scopus, Web of Science, and Google Scholar, using combinations of keywords related to metabolomics, metabolomic profiling, protozoan diseases, biomarkers, metabolic pathways, and antiprotozoal drug resistance from 02 November 2025 to 28 December 2025. Protozoan parasites including *Trypanosoma*, *Leishmania*, *Babesia*, *Theileria*, *Eimeria*, *Cryptosporidium*, and *Toxoplasma* induce significant metabolic disturbances detectable through advanced platforms such as NMR, LC–MS, and GC–MS. Metabolomics enables early diagnosis by identifying distinct biochemical fingerprints in biofluids (blood, saliva, urine, feces, and breath), differentiating infected from healthy animals while providing insight into host–parasite interactions, immune responses, and disease progression. Blood protozoan infections are associated with disruptions in amino acid, lipid, energy, and oxidative stress pathways, including ionone-related markers in trypanosomiasis, altered aldehydes and hydrocarbons in leishmaniasis, and amino acid and bile acid imbalances in babesiosis and theileriosis. Comparable metabolic alterations occur in intestinal and tissue infections, where changes in short-chain fatty acids, glycerophospholipids, long-chain fatty acids, and oxidative stress markers reflect gut dysbiosis, immune activation, and parasite nutrient exploitation. Metabolomics also facilitates detection of drug resistance by revealing metabolic reprogramming in resistant strains, including altered glycolysis, glutathione metabolism, and amino acid pathways, as well as sterol biosynthesis and oxidative stress adaptations. However, most reported biomarkers remain at the discovery or candidate stage and should not be considered clinically validated diagnostic tests or universal markers of drug resistance without independent validation. High analytical costs, pre-analytical and biological variation, incomplete metabolite annotation, small study populations, and limited external validation remain important barriers to routine veterinary application.

Keywords: Metabolomics; Protozoan diseases; Biomarkers; Drug resistance; Host–parasite interactions; Veterinary diagnostics

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INTRODUCTION

A wide range of domestic and wild animal species are susceptible to infection with parasitic protozoan, a major group of infectious diseases affecting global animal health (Fereig et al., 2023; Alimam et al., 2022; MacGregor et al., 2021; Abdelbaky et al., 2021). These infections include vector-borne diseases such as African trypanosomiasis and leishmaniasis spread by flies, and babesiosis and theileriosis carried by ticks, which can cause abnormal hematological findings, severe clinical illness, and death. They also include non-vector-borne infections such as toxoplasmosis, neosporosis, coccidiosis, and cryptosporidiosis, which affect diverse animal hosts. (Abdelbaky et al., 2021; Parija, 2016). In tropical and subtropical production systems, protozoan infections can cause substantial economic losses through mortality, reduced milk and meat production, reproductive disorders, impaired growth, and costs associated with prevention and treatment (Fereig et al., 2023). Beyond productivity impacts, many protozoan diseases such as babesiosis (*Babesia microti*, *B. divergens*, *B. duncani*, and *B. venatorum* between human and wild and domesticated animals) (Kim and Lee, 2021), trypanosomiasis (mostly by *Trypanosoma cruzi*, and *T. Brucei rhodesiense*) (Barrett et al., 2003), toxoplasmosis (*Toxoplasma gondii*) (Dubey et al., 2012), and cryptosporidiosis (*Cryptosporidium parvum*) (Pal et al., 2016) are zoonotic, posing public health risks that are intensified by asymptomatic carriers, close contact between domestic and wild animals, and increased wildlife–livestock interactions that sustain parasite transmission in natural ecosystems (Hunter-Barnett and Viney, 2024).

Diagnostic advances now combine conventional microscopy with molecular and metabolomic based tools, improving early detection, species identification and understanding of disease dynamics (De Waal, 2012; Page et al., 2013). Metabolomics, the large-scale study of small-molecule metabolites in biological systems, has emerged as a key analytical approach in understanding the complex biochemical processes underlying animal health and disease (Nicholson et al., 1999; Patti et al., 2012). Modern analytical platforms such as nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry (GC–MS), and liquid chromatography–mass

spectrometry (LC–MS) have significantly improved the accuracy and coverage of metabolite profiling (Dettmer et al., 2007).

Metabolomics has been widely applied across diverse animal species, including ruminants, poultry, and companion animals, where it supports both diagnostic and therapeutic advancements in veterinary medicine (Goldansaz et al., 2017). As the field has evolved, metabolomics has become an important diagnostic and research tool, enabling the identification of biomarkers associated with infectious, metabolic, and nutritional disorders in livestock and companion animals (Goldansaz et al., 2017). While metabolomics is widely used in human medicine, its application in livestock and veterinary science continues to expand (Goldansaz et al., 2017; Wishart, 2019; Tran et al., 2020).

Metabolomics has become a powerful method for detecting metabolic shifts in drug-resistant pathogens, enabling the identification of biomarkers and therapeutic targets (Zhao et al., 2024). Studies show that resistance is often linked to disrupted antioxidant, amino acid, carbohydrate, and energy metabolism in organisms such as *Eimeria tenella*, *Giardia*, and *Leishmania* (Müller et al., 2020; Popruk et al., 2023; Pountain and Barrett, 2019). Host-focused metabolomics also reveals metabolic changes during infections like canine babesiosis, reflecting host–parasite adaptive interactions (Kuleš et al., 2021). Broader analyses of trypanosomatid diseases confirm that metabolic remodeling is a central resistance mechanism and emphasize metabolomics as a key tool for discovering new biomarkers and therapeutic targets (Yuan et al., 2023; Ungogo et al., 2024).

Metabolomic analyses can identify unique metabolic signatures in both the host and the parasite due to the disruption of multiple metabolic processes caused by protozoan infections. These metabolic signals enhance comprehension of infection progression, help in the identification of alternative diagnostic biomarkers, and improve disease monitoring. Metabolomics is also crucial for identifying biochemical indicators of drug resistance, explaining how parasites adjust to therapy, and directing the development of effective antiparasitic therapies. Therefore, the aims of this review are to highlight current applications of

metabolomics in the diagnosis and pathophysiological understanding of protozoan diseases in animals. It also aims to summarize recent advances in metabolomics-based approaches for detecting and characterizing antiprotozoal drug resistance

To achieve the aims of this review, a comprehensive literature search was conducted to identify studies on the application of metabolomics in protozoan diseases of veterinary importance and antiparasitic drug resistance from 02 November 2025 to 28 December 2025. Electronic databases, including PubMed, Scopus, Web of Science, and Google Scholar, were searched using combinations of keywords related to metabolomics, metabolomic profiling, protozoan diseases, biomarkers, metabolic pathways, and antiprotozoal drug

resistance, together with specific diseases such as trypanosomosis, babesiosis, theileriosis, cryptosporidiosis, coccidiosis, and toxoplasmosis. Reference lists of relevant articles and recent reviews were also screened to identify additional studies. Peer-reviewed original research articles reporting metabolomic profiles, altered metabolites or metabolic pathways, biomarkers, host–parasite metabolic interactions, or metabolomics-based investigations of drug resistance were prioritized. Studies were screened based on their relevance to veterinary protozoan infections, and findings were synthesized descriptively, focusing on analytical platforms, biological samples, identified biomarkers, altered metabolic pathways, diagnostic or prognostic potential, and associations with antiprotozoal drug resistance.

FUNDAMENTALS OF METABOLOMICS

Metabolomics, a rapidly advancing “omics” science, investigates small molecules (metabolites) to understand biological states and disease processes. The metabolome the complete set of metabolites reflects both host and parasite physiology; during infection, a shared co-metabolome emerges, revealing resource utilization, metabolic disruption, and potential therapeutic targets (Holmes et al., 2008; Kafsack and Llinás, 2010). Selecting appropriate specimens is critical: urine, exhaled breath, serum/plasma, and erythrocytes provide complementary perspectives on infection-related metabolism, while other matrices (feces, milk, ruminal fluid, saliva, tissue) add distinct metabolic fingerprints (Getahun et al., 2022; Rossi et al., 2021; Liu et al., 2020; Ogunrinde et al., 2022; Lamour et al., 2019; Le Roy et al., 2016; Matysik et al., 2016).

Modern metabolomics uses NMR, LC-MS, and GC-MS, each with trade-offs in reproducibility, sensitivity, and coverage. In livestock research, metabolomics studies have used a range of analytical platforms, with nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography–mass spectrometry (LC–MS), and gas chromatography–mass spectrometry (GC–MS) among the most commonly employed approaches (Goldansaz et al., 2017). Strategically, untargeted approaches broadly profile the metabolome to discover patterns and generate hypotheses, whereas targeted approaches quantify predefined metabolites often with MS/MS for hypothesis testing and validation (Wang et al., 2010; Hyötyläinen and Wiedmer, 2013).

METABOLOMICS IN THE DIAGNOSIS OF PROTOZOAN DISEASE/INFECTIONS IN ANIMALS

The discovery of diagnostic biomarkers is a key potential benefit of using metabolomics in parasite research. The identification and quantification of small-molecule biomarkers like amino acids, lipids, and organic acids in biofluids are made possible by metabolomics, which has developed into a potent, high-throughput technique for identifying protozoan infections in animals. This technique can detect unique metabolic fingerprints that differentiate infected from uninfected animals,

frequently in the early stages of infection, by offering a top-down perspective of metabolic disturbances (Kafsack, and Llinás, 2010; Holmes *et al.*, 2008; Hunter and Wilson, 2020).

Metabolomics of Blood Protozoan Parasitic Diseases

Vector-borne blood protozoan infections cause profound and characteristic metabolic reprogramming in their vertebrate hosts, driven by

intense nutrient scavenging, immune-inflammatory responses, erythrocyte destruction, and tissue injury. These infections generate reproducible metabolic fingerprints across biofluids (blood, urine, saliva), exhaled breath, and within infected cells, enabling metabolomics to illuminate parasite biology, reveal drug mechanisms, and provide diagnostic, prognostic, and monitoring biomarkers (Creek et al., 2012; Igbokwe, 2018). By capturing coordinated shifts in amino acids, lipids, carbohydrates, nucleotides, and energy metabolism, metabolomics provides a systems-level perspective on host–parasite interactions and may help identify candidate biomarkers and metabolic pathways for further validation.

Untargeted GC–MS metabolomics identified infection-associated volatile metabolites in cattle experimentally infected with *Trypanosoma congolense* and *T. vivax* (Getahun et al., 2022). In the experimental component, four cows were infected with each trypanosome species, and urine and breath profiles were compared with samples collected from the same animals before infection. Dihydro- β -ionone and junenol were induced, while dihydro- α -ionone, p-cresol, and 3-propylphenol showed increased abundance in infected cattle. Several of these metabolites were also detected in urine from naturally infected cattle, supporting their reproducibility across experimental and field settings. Metabolite levels declined toward pre-infection levels following trypanocide treatment, suggesting an association with active infection. For preliminary field validation, an independent set of 34 cattle was assessed using microscopy, PCR, and a phenolic biomarker-based assay. The authors reported agreement between the biomarker assay and reference methods, including a κ value of 0.64 for agreement with PCR; however, the study did not establish diagnostic thresholds or provide conventional sensitivity, specificity, or AUC estimates for the GC–MS biomarker panel. In addition, although comparisons involving naturally infected cattle exposed to different populations, feeding conditions, and environments provided some evidence that the metabolite changes were not solely attributable to these factors, broader evaluation against other infectious and noninfectious conditions is required. Thus, these metabolites should be regarded as promising candidate biomarkers that require further validation in larger, independent, field-relevant cattle

populations. Similar studies have reported alterations in lipid, amino acid, and carbohydrate metabolism during blood protozoan infections, consistent with changes in parasite nutrient acquisition and host inflammatory responses (Igbokwe, 2018; Creek et al., 2012).

In canine leishmaniasis, metabolomic and volatile organic compound (VOC) analyses have identified infection-associated alterations in host metabolic profiles. VOC profiling of dogs infected with *Leishmania infantum* has identified compounds including octanal, nonanal, decanal, and heptadecane as potential infection-associated biomarkers. In a subsequent behavioral study, Magalhães-Junior et al. (2019) evaluated these compounds individually and in combinations using wind-tunnel assays with *Lutzomyia longipalpis*. Octanal, nonanal, decanal, and heptadecane elicited activation and/or attraction responses in male sand flies, whereas decanal and nonanal produced activation responses in females; a combination of octanal, decanal, and heptadecane increased activation and attraction in males. These findings provide experimental evidence that selected VOCs associated with infected dogs can influence sand-fly behavior, but they do not by themselves establish these VOCs as validated diagnostic biomarkers. At the intracellular level, *Leishmania* infection alters central carbon and amino acid metabolism, including the tryptophan–kynurenine pathway, and affects membrane lipid metabolism involving phosphatidylcholine and phosphatidylethanolamine (Ferreira et al., 2021; Qin et al., 2022; Yuan et al., 2023; Muxel et al., 2019). These metabolic changes provide mechanistic insight into host immune responses and parasite nutrient acquisition and may identify candidate pathways and biomarkers for further investigation. Current evidence therefore indicates that canine leishmaniasis is associated with both extracellular VOC profiles and intracellular metabolic remodeling, with the former showing potential relevance to vector behavior and the latter providing mechanistic insight into host–parasite interactions.

Babesiosis, characterised by intraerythrocytic infection and haemolysis, produces stage-dependent and organ-specific metabolic alterations detectable in serum, saliva, and urine. In experimental *Babesia microti* infection, LC–MS

serum metabolomics revealed acute-phase disturbances in taurine/hypotaurine, histidine, and arachidonic acid pathways, with taurocholic acid, anserine, and arachidonic acid identified as metabolites associated with acute infection (Shen et al., 2023). In naturally infected dogs, saliva profiling identified differential metabolites associated with inflammation and erythrocyte damage (Kuleš et al., 2025), while urine metabolomics identified seventeen significantly altered metabolites related to amino acid, fatty acid, and energy metabolism, as well as urea cycle and ammonia detoxification pathways, potentially reflecting metabolic alterations associated with babesiosis and renal involvement (Rubić et al., 2022). In another study, Kuleš et al. (2021) combined untargeted and targeted metabolomics to evaluate urinary changes in dogs infected with *Babesia canis*. They observed pronounced disturbances in amino acid and energy metabolism, particularly in the tricarboxylic acid (TCA) cycle and oxidative-stress-related metabolites. Although the study did not directly measure parasite metabolism, the altered host metabolite patterns suggest that *Babesia* infection triggers adaptive metabolic responses that could influence treatment efficacy and drug susceptibility (Kuleš et al., 2021).

Collectively, these findings demonstrate that babesiosis is associated with measurable metabolic perturbations across multiple biofluids and provide candidate biomarkers and pathways for further investigation. However, the diagnostic and treatment-monitoring utility of these signatures requires validation in larger independent cohorts, including appropriate disease controls, defined sampling time points, and assessment using diagnostic-performance measures such as sensitivity, specificity, and AUC.

To clarify how *T. annulata* alters host cell metabolism during infection, Zhao et al. (2022) identified key metabolic indicators associated with parasite-induced cellular transformation. The study also demonstrates that BW720c acts by disrupting specific metabolic pathways, particularly those involving leucine, arginine, and L-carnitine, ultimately leading to apoptosis of the transformed cells. In doing so, the research offers valuable insights into the molecular interactions between the parasite and its host, highlighting potential

metabolic targets for improved strategies to combat tropical theileriosis.

Another study by Ünal et al. (2025) found that cattle infected with *Theileria annulata* exhibit significant alterations in 21 amino acids, with many including methylhistidines, alanine, leucine, and tyrosine increased compared to healthy animals, while amino acids such as arginine, citrulline, glutamine, threonine, and tryptophan were decreased. The study also identified key prognostic markers: high isoleucine and tyrosine levels and low arginine and asparagine levels were strongly associated with increased mortality risk, indicating their value as predictors of disease outcome (Ünal et al., 2025). Beyond lymphatic pathology, broader metabolomic disturbances correspond with the multi-system impact of blood parasites (El-Deeb and Younis, 2009).

Across these blood protozoan diseases, several common biomarker themes emerge. Firstly, amino acid dysregulation is widespread. Arginine, tryptophan, tyrosine, leucine, and valine frequently change in trypanosomiasis, leishmaniasis, babesiosis, and theileriosis, reflecting immune activation, nitric oxide/arginine pathway shifts, oxidative stress, and parasite nutrient capture (Ferreira et al., 2021; Qin et al., 2022; Ünal et al., 2025; Shen et al., 2023). For example, arginine depletion occurs in babesiosis and theileriosis and may relate to NO-dependent immune dynamics; tryptophan and kynurenine pathway alterations are prominent in leishmaniasis and are also noted in trypanosome infections, indicating immunometabolic regulation; and tyrosine perturbations are seen in leishmaniasis and theileriosis (Qin et al., 2022; Ünal et al., 2025; Muxel et al., 2019). Secondly, lipid pathway remodelling is common. Phosphatidylcholine and phosphatidylethanolamine dysregulation, altered arachidonic acid metabolism, and bile acid changes (e.g., taurocholic acid) are observed across infections, aligning with membrane remodelling, eicosanoid-mediated inflammation, and hepatobiliary involvement (Qin et al., 2022; Shen et al., 2023). Third, VOC changes represent a cross-parasite hallmark, even if compound identities differ: ionones and phenolics characterise trypanosome infections, while aldehydes and hydrocarbons characterise leishmaniasis – together supporting the development of non-invasive breath

or skin diagnostics and suggesting possible effects on vector behaviour (Getahun et al., 2022). Finally, markers of oxidative stress and inflammation, including taurine/hypotaurine, phenolic metabolites, and arachidonic acid derivatives, appear repeatedly, consistent with the haemolysis, immune activation, and tissue injury that accompany blood parasite infections (Shen et al., 2023; Rubić et al., 2022).

Importantly, metabolomics provides value across the entire translational spectrum. Untargeted discovery defines broad fingerprints and identifies candidate biomarkers; targeted validation refines quantitative assays suitable for clinical use; longitudinal profiling confirms normalisation with effective therapy; and integration with cellular metabolomics clarifies mechanisms of drug action and parasite survival strategies (Creek et al., 2012; Zhao et al., 2022). In practice, this enables earlier and more reliable diagnosis (e.g., VOCs in breath, saliva, or urine), differentiation of disease stages (e.g., acute-phase markers in babesiosis), monitoring of treatment response (e.g., normalisation of ionones in trypanosomiasis), risk stratification (e.g., amino acid prognostics in theileriosis), and insights into host–vector ecology (e.g., VOCs modulating sand fly attraction in leishmaniasis) (Getahun et al., 2022; Ünal et al., 2025).

Metabolomics of Intestinal and Tissue Protozoan Diseases

Intestinal and tissue-dwelling protozoan parasites impose strong metabolic pressures on their hosts due to their nutrient dependence, intracellular localization, and the inflammatory and microbial disruptions they cause (Ewald et al., 2024).

Cryptosporidiosis, caused by *Cryptosporidium* spp., exemplifies how intestinal protozoa can reshape host and gut microbial metabolism. Owing to its limited biosynthetic capacity, the parasite depends substantially on nutrients derived from the host and intestinal environment (Ng Hublin et al., 2013; Khan et al., 2021; Karpe et al., 2021), potentially contributing to alterations in host and microbial metabolic profiles. In a *Cryptosporidium muris* infection model, Wang et al. (2023) used BALB/c mice and longitudinal fecal sampling at 0, 7, 14, 21, and 28 days post-infection to demonstrate infection-associated alterations in gut microbiota

and metabolites, including changes in short-chain fatty acid and bile-acid-related pathways. Thus, these findings provide experimental evidence for metabolic and microbiome perturbations during cryptosporidiosis. Fecal metabolomics comparing healthy, infected non-diarrheic, and diarrheic calves showed that diarrheic animals had reduced short-chain fatty acids such as valerate and increased ethanol, formate, lactate, and succinate, suggesting altered microbial fermentation and gut metabolic homeostasis (Pinto, 2024). Microbiome analyses have similarly demonstrated infection-associated changes in microbial communities and transient alterations in calf body condition, suggesting metabolic disturbance followed by partial recovery as infection resolves (Hares et al., 2025).

In poultry, serum metabolomics of *Cryptosporidium baileyi*-infected chickens identified 138 differential metabolites at 5 days post-infection, with enrichment of glycerophospholipid, sphingolipid, energy-metabolism, and immune-related pathways (Wu et al., 2021). Nine metabolites, including choline, sphinganine, and sirolimus, were selected for ROC analysis and showed AUC values >0.7 in the study cohort. However, these findings should be interpreted as preliminary biomarker evidence because the study was based on a comparison of infected and mock-infected chickens and did not provide independent external validation. In particular, sirolimus (rapamycin) is an exogenous pharmaceutical rather than a conventional endogenous metabolite; therefore, its identification should be independently confirmed and its biological origin established before it is considered a biologically meaningful biomarker of cryptosporidiosis. Collectively, current evidence indicates that *Cryptosporidium* infection is associated with alterations in host and microbial metabolism, while the diagnostic value of individual metabolites requires further analytical confirmation and validation in independent animal populations. Toxoplasmosis caused by *Toxoplasma gondii* is associated with alterations in host metabolism arising from the parasite's intracellular lifestyle, nutrient requirements, and host immune responses. Metabolomics studies have identified changes in multiple metabolic pathways during infection, although the available evidence differs substantially in experimental design and analytical

scope. In acute infection, metabolomic profiling has revealed alterations in taurine, hypotaurine, taurocholic acid, and glutathione-related metabolites, suggesting perturbation of bile acid metabolism and oxidative stress-related pathways (Chen et al., 2018). Biochemical and metabolic studies have also shown that *T. gondii* tachyzoites convert substantial amounts of glucose to lactate and acetate under both aerobic and anaerobic conditions, providing evidence for parasite-associated alterations in carbon metabolism (Denton et al., 1996). However, this experimental metabolic study should be distinguished from untargeted metabolomics because it assessed specific metabolic products rather than performing broad metabolite profiling.

Evidence from small ruminant studies also indicates metabolic and biochemical alterations during *T. gondii* infection. Khaier et al. (2024) reported changes in metabolites related to protein turnover, mineral metabolism, glucose regulation, and serotonin, whereas Castello et al. (2018) reported alterations in serum serotonin, glucose, and protein concentrations. These findings provide evidence of systemic biochemical and metabolic disturbances, but they should not be classified as metabolomics unless the analytical methods involved comprehensive metabolite profiling. Similarly, reported changes in albumin, alanine aminotransferase (ALT), progesterone, electrolytes, and hematological variables during infection (Roland et al., 2022; Khaier et al., 2024) represent conventional clinical chemistry or hematological evidence rather than metabolomics. Collectively, these findings indicate that *T. gondii* infection induces measurable metabolic perturbations, with metabolomics providing broader pathway-level insights that complement, rather than substitute for conventional biochemical and hematological assessments.

In murine models, metabolomics highlights the systemic breadth of *T. gondii* effects (Ma et al., 2019). Urine metabolomics detected major shifts in amino acid, fatty acid, and nicotinate - nicotinamide pathways (Zhou et al., 2022). Serum profiling during acute and chronic infection revealed extensive disturbances in glycerophospholipids, amino acid biosynthesis, and tyrosine metabolism (Zhou et al., 2018). Neural metabolomics identified time-dependent changes in cerebellar metabolites,

with arachidonic acid, docosahexaenoic acid, oleic acid, and tabersonine proposed as biomarkers, highlighting parasite-induced remodeling of neural lipid and amino acid pathways (Ma et al., 2020).

Coccidiosis caused by *Eimeria* spp. is associated with substantial alterations in host metabolism. In chickens repeatedly exposed to low-dose *Eimeria mitis*, metabolomic profiling identified 286 differential metabolites and changes in metabolic pathways, including carbohydrate metabolism, ABC transporters, and MAPK- and ErbB-related signaling (Wang et al., 2024). In *Eimeria acervulina* infection, metabolomic analyses revealed alterations in fatty acid metabolism and β -oxidation, including changes in stearyl carnitine, palmitoyl carnitine, and linoleoyl carnitine, along with alterations in nucleotide metabolism and inflammatory mediators such as thromboxane B₂ and itaconate (Aggrey et al., 2019). Multi-omics analyses of *Eimeria tenella* infection further distinguished healthy, infected, vaccinated, and recovering birds, demonstrating infection-associated enrichment of amino acid and lipid metabolic pathways, vaccination-associated changes in sphingolipid metabolites, and recovery-associated alterations in microbial vitamin B pathways and short-chain fatty acid production (Liu et al., 2024). Collectively, these findings show that *Eimeria* infection is associated with coordinated alterations in host and microbial metabolism and highlight the potential of metabolomics and multi-omics approaches to characterize disease-associated metabolic responses and recovery-related changes.

Common metabolic themes are found in metabolomic studies of *Cryptosporidium*, *Toxoplasma*, and *Eimeria* infections: changes in amino acid composition, extensive lipid remodeling (particularly of glycerophospholipids and long-chain fatty acids), altered energy and carbohydrate metabolism, oxidative stress signatures, and fermentation product shifts driven by the microbiota. These shared and parasite-specific metabolic fingerprints provide valuable insights into disease mechanisms and offer promising biomarkers for diagnosis, staging, and monitoring of intestinal and tissue protozoan diseases.

METABOLOMICS IN ANTIPROTOZOAL DRUG RESISTANCE DETECTION

Recent research has increasingly applied metabolomics to drug resistance, drug-induced metabolomic responses, and host metabolic changes associated with protozoan infections of veterinary relevance. These applications can be distinguished into three categories: direct comparisons of drug-resistant and drug-susceptible parasite populations, characterization of metabolic responses to drug exposure, and assessment of host metabolic responses during infected and treated states.

Direct comparison of resistant and susceptible parasite lines provides the most direct metabolomics-based evidence for resistance-associated metabolic changes. In this regard, Zhao et al. (2024) employed an integrated transcriptomic and metabolomic approach to compare maduramycin-resistant and maduramycin-sensitive *Eimeria tenella* strains. Untargeted and targeted metabolomics identified significant differences in amino acid, carbohydrate, and antioxidant-related pathways such as glycolysis, glutathione metabolism, and fructose/mannose metabolism. These findings identify metabolic pathways associated with the resistant phenotype and provide candidate mechanisms for further investigation. However, such differences should be considered resistance-associated metabolic signatures rather than validated markers of resistance unless their ability to discriminate resistant from susceptible populations is independently confirmed.

Characterization of metabolomic responses following drug exposure is another application. Such studies can reveal biochemical pathways affected by drug action, metabolomic stress, or cellular adaptation, but changes following exposure do not by themselves establish resistance. For example, Zhao et al. (2022) used LC-QTOF metabolomics to examine *T. annulata*-transformed bovine leukocytes treated with buparvaquone (BW720c). Metabolic profiles differed between treated and DMSO control cells at 24, 48, and 72 h, with treatment-associated alterations involving amino acid and other metabolic pathways. The study therefore illustrates how metabolomics can characterize the cellular metabolic response to an antiprotozoal drug, but it does not directly

demonstrate drug resistance because the experiment compared drug-treated and control cells rather than resistant and susceptible parasite populations. A third application involves host metabolic responses during treatment of infected hosts. In this context, metabolomics can capture changes arising from the interaction among parasite infection, host responses, and drug treatment. Li et al. (2022), for example, investigated the cecal microbiome and metabolome of chickens infected with *E. tenella* in the presence or absence of ethanamizuril, sulfachlorpyridazine, or their combination. Using 16S rRNA sequencing and LC-MS/MS, the study showed treatment-associated changes in cecal microbial and metabolic profiles and reported that changes in metabolites such as N-carbamoylglutamic acid were consistent with the anticoccidial effects of ethanamizuril. These findings illustrate the potential of host and gut metabolomics to characterize treatment-associated metabolic responses and to generate candidate markers of drug efficacy; however, they do not directly establish parasite drug resistance.

Metabolomics has also proven valuable in elucidating protozoal responses to nitro-drug exposure, particularly in *Giardia* species, a parasite of zoonotic and veterinary relevance. Popruk et al. (2023) used mass spectrometry-based metabolomics to profile the metabolic perturbations induced by metronidazole in *Giardia duodenalis*. Their study revealed that metronidazole exposure caused broad disruptions in energy metabolism, amino acid utilization, and oxidative stress regulation, indicating pathways potentially associated with the development of metronidazole resistance. Complementary findings were reported earlier by Müller et al. (2020), who applied high-resolution magic-angle-spinning (HR-MAS) NMR spectroscopy to compare wild-type and transgenic *Giardia lamblia* strains, including those with altered nitroreductase expression linked to nitro-drug resistance. Distinct metabolite profiles, particularly in arginine catabolism and central carbon metabolism, were associated with resistance phenotypes, confirming that metabolomic fingerprints can differentiate resistant and sensitive

parasite strains (Müller *et al.*, 2020; Popruk *et al.*, 2023).

Direct evidence of antiparasitic drug resistance has also been demonstrated in *Babesia* species using experimental and molecular approaches. In *Babesia gibsoni*, in vitro selection under atovaquone pressure generated a resistant parasite population carrying an M121I substitution in the mitochondrial cytochrome b gene, while a separate study developed a diminazene aceturate-resistant strain with increased drug tolerance (Iguchi *et al.*, 2012; Hwang *et al.*, 2010). More recently, Keroack *et al.* (2024) used comparative chemical genomics and parallel in vitro evolution in *Babesia bovis* and *B. divergens* to investigate resistance to the experimental antibabesial compound MMV019266. Independent resistant lines acquired mutations in the conserved phoD gene, which encodes a predicted membrane-bound metallodependent phosphatase. Reverse genetic experiments in *B. divergens* confirmed that mutations in bdphoD increased resistance to MMV019266, while conditional knockdown of BdPhoD increased resistance and overexpression increased parasite susceptibility. These findings identified PhoD as a determinant of resistance to MMV019266 in *Babesia* species (Keroack *et al.*, 2024). However, these studies primarily used phenotypic, genomic, and functional genetic approaches rather than metabolomics. Therefore, although they experimentally define resistance phenotypes and mechanisms in *Babesia*, they do not identify metabolomic signatures that distinguish resistant from susceptible parasites. Comparative metabolomic profiling of independently characterized resistant and susceptible *Babesia* populations thus remains an important area for further investigation. Similarly, Metabolomics has provided direct evidence of metabolic alterations associated with drug resistance in *Leishmania*. Shaw *et al.* (2016) investigated experimentally selected miltefosine-resistant *Leishmania donovani* strains and identified genetic changes involving the *LdMT* transporter alongside alterations in phosphatidylcholine and lysophosphatidylcholine metabolism, implicating the Kennedy pathway in resistance. These changes also varied according to the parasite's background susceptibility to antimonial drugs.

Further evidence comes from amphotericin B-resistant *Leishmania mexicana*. Pountain *et al.* (2019) identified genomic instability at the sterol C24-methyltransferase locus and associated changes in parasite sterol composition. Using these characterized resistant lines, Pountain and Barrett (2019) applied untargeted LC–MS metabolomics and found substantial differences in metabolite abundance, including increased reduced trypanothione and glutathione in several resistant lines and changes in oligohexoses. However, these metabolic alterations varied among resistant lines and were considered more likely to represent resistance-associated phenotypic changes than the primary resistance mechanism. Overall, these studies show that metabolomics can identify metabolic signatures associated with drug-resistant *Leishmania*, but such signatures vary with the drug, parasite species, genetic background, and resistance mechanism. Therefore, they should not be considered universal markers of resistance without validation in independently characterized resistant and susceptible populations. Metabolomic studies of drug exposure, such as the analysis of miltefosine action in *L. infantum* (Vincent *et al.*, 2014), provide complementary information on drug response but should be distinguished from direct resistant-versus-susceptible comparisons.

Broader reviews of metabolomics applications in veterinary protozoan diseases further support these trends. For instance, Ungogo *et al.* (2024) reviewed mechanisms of drug resistance in animal trypanosomiasis, emphasizing the role of metabolic adaptation and energy-pathway remodeling in trypanosome survival under drug pressure. They highlighted that metabolomics, although underutilized in livestock trypanosomes, represents a promising frontier for identifying resistance biomarkers and novel chemotherapeutic targets (Ungogo *et al.*, 2024).

Collectively, these studies indicate that metabolomics can identify metabolic alterations associated with drug resistance and provide insights into pathways potentially involved in parasite adaptation. However, altered metabolite profiles alone are insufficient to establish diagnostic biomarkers of resistance. Candidate signatures should be validated using independently characterized resistant and susceptible isolates, blinded classification, and appropriate phenotypic

and/or genotypic reference standards, followed by assessment of diagnostic performance using sensitivity, specificity, and other relevant metrics.

Validation in diverse field populations is also required to determine their robustness and practical applicability.

CONCLUSION AND RECOMMENDATIONS

This review summarizes the application of metabolomics in the diagnosis of protozoan diseases and antiprotozoan drug resistance in naturally and experimentally infected animals. The reviewed studies indicate that metabolomics can identify disease-associated metabolic alterations, reveal systemic metabolic responses, and provide insights into pathways potentially involved in protozoan adaptation and drug resistance. However, translation into routine veterinary diagnostics remains limited by substantial methodological and biological challenges. These include pre-analytical variation, effects of diet, age, breed, sex, and physiological status, differences in sample matrices, batch effects, incomplete metabolite annotation and identification, multiple-testing and model-overfitting risks, small study populations, inadequate comparator disease groups, limited external validation, and poor portability of metabolic signatures across analytical platforms. High instrumentation costs and limited standardization further constrain application in

resource-limited settings. Therefore, candidate metabolomic biomarkers require validation in adequately powered and independently sampled populations before translation into affordable, field-ready diagnostic assays for veterinary use.

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Contributions

MM, MAG, MCK and NYA conceived and designed this review. MM, MYA and AT read and wrote the manuscript. All authors read and approved the manuscript. MM wrote the manuscript. All authors read, reviewed and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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