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Lipopolysaccharide-induced alterations in the liver metabolome of St. Croix and Suffolk sheep

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The development of resistance in parasites due to overuse of anthelmintics has resulted in a marked decrease in the efficacy of these drug classes. Recent research efforts have focused on exploring alternatives such as selection for parasite-resistant breeds with the implication that immunocompetence may align with parasite resistance. Two breeds that are often investigated are the St. Croix (STC), a resistant hair breed, and Suffolk (SUF), a susceptible wool breed sheep. The liver plays a vital role in metabolism in the body and metabolizes lipopolysaccharide (LPS), which triggers whole body response through the production of appropriate metabolites, cytokines and immune cells. The objective of this study was to investigate the breed differences in liver metabolome of sheep, with divergent resistance to parasites, in response to LPS. Both STC and SUF sheep (n = 9/breed) were challenged with LPS intravenously. Rectal temperatures and sheep grimace score (SGS) were recorded hourly, for each animal, and averaged across the study for both breeds. The average rectal temperature throughout the study was similar for STC and SUF sheep (40.4°C and 40.2°C respectively), but the pattern of response was different. STC had an average SGS of 0.8 while SUF had an average of 3.3. Liver biopsies were collected from 3 sheep that were not challenged with LPS (HR0; n = 3/breed), two hours post-challenge (HR2; n = 3/breed), and six hours post-challenge (HR6; n = 3/breed). Liver tissue samples were subjected to quantitative untargeted metabolome analysis using chemical isotope labeling/liquid chromatography-mass spectrometry. Pathway analysis of the HR0 metabolome data revealed that 8 pathways (and their associated metabolites) including beta-alanine metabolism, arginine and proline metabolism and glutathione metabolism were altered (false discovery rate-adjusted *P*-value (FDR) ≤ 0.05) between STC and SUF sheep. At HR2, 10 altered pathways such as folate biosynthesis, taurine and hypotaurine metabolism, and glutathione metabolism. At HR6, only 2 pathways (glycerophospholipid metabolism and

purine metabolism) were altered (FDR ≤ 0.05) between STC and SUF sheep. Results highlight the differences in hepatic metabolome and physiological response to LPS challenge that exist between SUF and STC. These findings suggest breed-specific differences in metabolic response to immune challenge, potentially influencing the divergent resistance of the two breeds to parasitic infections.

KEYWORDS

metabolism, parasite, LPS, liver, oxidative stress

Introduction

Parasite infections have consistently been one of the greatest challenges facing small ruminant production and were listed as one of the primary causes of non-predatory death loss in sheep in 2019 (Benford, 2022). Traditionally, the treatment of parasites depends on the administration of anthelmintics. This approach has, however, led to resistance in worms (Traoré et al., 2017). The development of resistance in these worms has resulted in a marked decrease in the efficacy of several anthelmintic drug classes (Fleming et al., 2006). Recent research efforts have focused on exploring alternatives including nutritional supplementation and selection for parasite-resistance within breeds (Sayers and Sweeney, 2005).

In sheep, St. Croix and Suffolk are two popular breeds with different resistance to parasites. St. Croix sheep respond quickly to *Haemonchus contortus* (*Hc*) larvae which prevents establishment of adult worms (Bowdridge et al., 2015). Conversely, Suffolk sheep's delayed response favors greater fecal egg counts and adult worm establishment, making them more susceptible to infection (Weaver et al., 2021). In response to *Hc* larval antigen, St. Croix peripheral blood mononuclear cells (PBMC) upregulated significantly more genes associated with signal transduction, response to stress and immune system processes compared to Suffolk PBMC (Jacobs et al., 2020). Efficient and rapid response, in hair breeds, to parasites favors a Th2 response and may suggest STC are more immunocompetent than SUF (MacKinnon et al., 2015).

Lipopolysaccharide, a component of the outer membrane of gram-negative bacteria, stimulates immune cells to produce proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 1-beta (IL-1β) (Lynn and Golenbock, 1992). Stimulation with LPS can reflect a bacterial infection and presents a model for research into immune response (Lynn and Golenbock, 1992). In a recent study, Bentley et al., 2023 evaluated the differences in immune response to LPS in St. Croix and Suffolk sheep. They observed an early decline in blood concentration of TNFα, a pro-inflammatory cytokine in the parasite-resistant group, compared to the susceptible group, which was associated with a reduced duration of sick behaviors in the former. The liver plays a vital role in the clearance of antigens such as LPS from the body, as Toll-like receptors (TLRs) recognize and help trigger the appropriate release of cytokines (Jirillo et al., 2002). However, the effect of LPS stimulation on liver metabolic profile of St. Croix and Suffolk sheep has not been fully described. The application of metabolomics has facilitated the characterization of metabolic phenotyping, providing insights into disease studies and metabolic mechanisms (Kenéz et al., 2016). Metabolomics enables the study of both exogenous and endogenous compounds as intermediate and final products of biological processes. Therefore, this study aimed to determine the effect of LPS on the liver metabolome profile in sheep breed with divergent parasite resistance (St. Croix and Suffolk breeds). We hypothesized that there would be discernible alterations in liver metabolome profiles in response to LPS between the two breeds.

Materials and methods

Animals

Animal use for this experiment was approved by the Institutional Animal Care and Use Committee of West Virginia University (protocol number # 2303064503). Nine (9) St. Croix wethers (BW= 66.8 ± 8.2 kg; 2.5 years of age) and 9 Suffolk (7 wethers and 2 ewes; BW = 83.0 ± 8.7 kg; 2.5 years of age) were housed and raised at West Virginia University Agronomy Farm, under parasite free conditions. A licensed veterinarian evaluated the health of all sheep 24 hours prior to start of experiment and only healthy animals were allowed to participate in the study. Live weights were recorded and used to calculate LPS dosage (*E. coli* O111:B4, Sigma-Aldrich, St Louis, MO). The LPS was administered via jugular venipuncture at 2.5 µg/kg then animals were euthanized via captive bolt gun at the following time points; HR0 (n = 3/breed; prior to LPS challenge), HR2 (n = 3/breed; 2 hours post- LPS challenge), and HR6 (n = 3/breed; 6 hours post-LPS challenge). This was immediately followed by exsanguination then liver and spleen were retrieved. Animal carcasses were disposed of via composting.

Sample collection and metabolome analysis

Rectal temperatures and sheep grimace score (SGS) were taken for each animal every hour from the start of the experiment using rectal thermometer. The orbital tightening, ear and head position, flehming of each animal were scored between 0 and 3 and the total of the three components were used to assess pain on a scale of 0 - 7 (Häger et al., 2017).

The liver was removed, weighed and photographed for each animal. Duplicate biopsy punches were taken from the same area of the right lobe and placed in cryogenic tubes for storage. Samples were stored in liquid nitrogen until time of analysis. A total of eighteen liver samples were subjected to untargeted metabolome analysis. To extract metabolites from the liver tissue, the samples were placed in plastic tubes with 6 ceramic beads, weighed, and homogenized at 6 m/s for 15 seconds. Exactly 500 µL LC-MS grade MeOH/water (4:1 v/v) was added before homogenizing again at 4 m/s for 10 seconds. Samples were then incubated at -20°C for 10 minutes and then centrifuged at 15,000 g for 10 minutes. All solvent was then transferred to a new vial and centrifuged for an additional 1 minute before the supernatants were removed and dried. Sample extracts were then re-suspended in 50 µL of LC-MS grade water. Metabolome analysis of the liver sample extracts were performed using chemical isotope labeling/liquid chromatography- mass spectrometry (Zhao et al., 2019). The technique uses ^{12}C and ^{13}C-isotope dansylation labeling to identify metabolites according to their chemical groups (amines/phenols, carboxylic acids, carbonyls, and hydroxyls (Zhao et al., 2019). Comprehensive details of the technique, encompassing sample preparation and analysis, have been previously documented (Zhao et al., 2019). A total of 18 raw LC-MS data files (n = 6/time point) were processed using IsoMS Pro 1.2.14 to remove redundant pairs (adduct ions and dimers) and singlet peaks. The peak pairs were identified as metabolites using CIL metabolite library (containing 1060 unique endogenous metabolites) at tier 1 and linked identity (LI) library (containing over 2000 metabolic-pathway-related metabolites extracted from the KEGG database at tier 2 (Li et al., 2013). All other metabolite features that were not identified at tiers 1 or 2 were removed from subsequent data analyses.

Statistical analysis

The intensity values for the metabolites were analyzed using MetaboAnalyst 6.0 software (metaboanalyst.ca). Metabolome data for each time point were analyzed separately. The data at each time were normalized and pareto-scaled. Principal component analysis (PCA) score plots were used to visualize differences between the breeds of sheep at each time point. Volcano plot analysis was used to determine the metabolites that were different (false discovery rate-adjusted *P*-values (FDR) ≤ 0.05; Benjamini and Hochberg, 1995) between the two breeds at each time point. Pathway enrichment analyses of all the metabolites were conducted using the KEGG database, to determine pathways (and their associated metabolites) that were significantly altered (FDR ≤ 0.05) between STC and SUF at each time point.

Results

Physiological response to LPS

The average rectal temperature at HR0 was 39.5°C for STC and 39.0°C for SUF. STC sheep exhibited a more pronounced initial temperature response to LPS, with a 1.1°C change compared to a 0.6°C change in SUF, between hour 0 and hour 1. By HR2, STC showed minimal change in rectal temperature, which persisted for the remainder of the study. In contrast, SUF displayed a gradual increase in temperature, with the most significant change occurring between hours 5 and 6 (Supplementary Figure 1). Despite STC initially having a higher temperature than SUF, they maintained a consistent average rectal temperature for the duration of the study. Similarly, the average sheep grimace score (SGS) remained constant throughout the study for STC, with the highest score recorded as 2 on a 0 -7 scale. The average SGS for SUF peaked at HR2, averaging 3.3 compared to 0.8 for STC throughout the study (Supplementary Figure 2).

Liver metabolome prior to lipopolysaccharide challenge

A total of 874 metabolites were detected and identified. The PCA score plot showed a clear separation between SUF and STC, indicating that there were clear breed differences (Figure 1) in the liver metabolome of the two breeds HR0. The volcano plot analysis revealed no metabolites with significant differential abundance

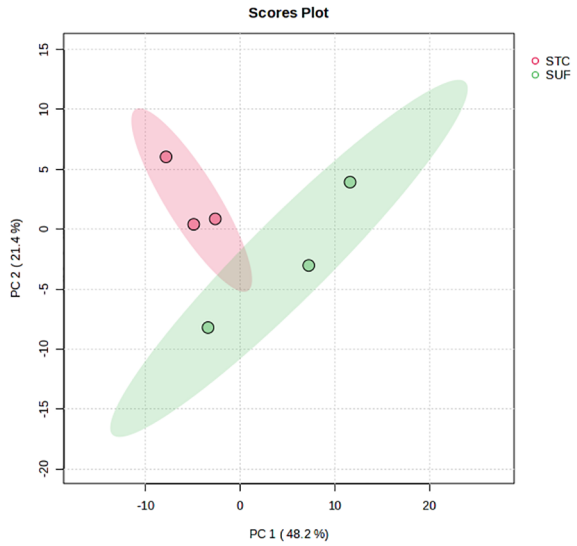

FIGURE 1
PCA scores plot of the liver metabolome of STC and SUF sheep at HR 0.

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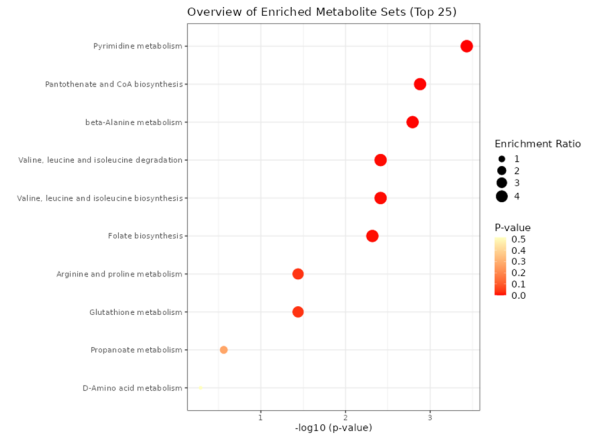

FIGURE 2
Pathways altered between STC and SUF at HR 0.

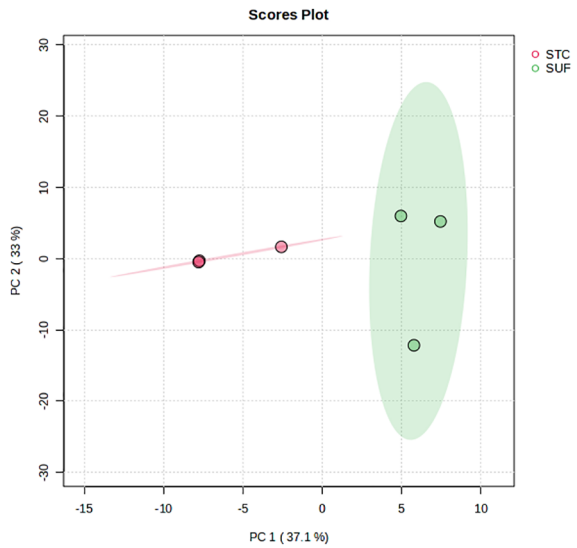

FIGURE 3
PCA scores plot of the liver metabolome of STC and SUF sheep at HR 2.

(FDR > 0.05). However, the results of the pathway enrichment analysis showed that eight pathways (and their associated metabolites such as uridine, uracil, and valine), including pyrimidine metabolism, pantothenate and CoA biosynthesis, folate biosynthesis, arginine and proline metabolism, and glutathione metabolism, were altered (FDR ≤ 0.05) between STC and SUF sheep (Figure 2; Table 1).

Liver metabolome two hours post lipopolysaccharide challenge

The PCA score plot showed a clear separation between SUF and STC at HR2 (Figure 3). The volcano plot analysis revealed no differentially abundant metabolites (FDR > 0.05). Results of the pathway enrichment showed that 10 pathways (and their associated metabolites such as tetrahydrobiopterin, L-cysteine and tyramine) including folate biosynthesis, glycine, serine and threonine metabolism were altered (FDR ≤ 0.05) between STC and SUF sheep (Figure 4; Table 2).

Liver metabolome six hours post lipopolysaccharide challenge

The PCA score plot showed a clear separation between SUF and STC, indicating that there were clear breed differences at HR6 (Figure 5). The volcano plot analysis revealed no differentially abundant metabolites (FDR > 0.05). However, pathway enrichment analysis revealed alterations (FDR ≤ 0.05) in two pathways, glycerophospholipid metabolism, and purine metabolism, along with their associated metabolites like

TABLE 1 Pathways altered between STC and SUF prior to LPS challenge (HR 0).

Pathway Name	FDR	Associated metabolites
Pyrimidine metabolism	0.01	Uridine ↑, uracil ↓, beta-alanine ↓
Pantothenate and CoA biosynthesis	0.01	Uracil ↓, L-valine ↑, beta-alanine ↓
beta-alanine metabolism	0.01	Uracil ↓, beta-alanine ↓
Valine, leucine and isoleucine degradation	0.01	L-Valine ↑
Valine, leucine and isoleucine biosynthesis	0.01	L-Valine ↑
Folate biosynthesis	0.08	Tetrahydrobiopterin ↑
Arginine and proline metabolism	0.05	Putrescine ↓
Glutathione metabolism	0.05	Putrescine ↓

Only pathways with false discovery rate-adjusted (FDR)-P-values ≤ 0.05 are shown. Arrows indicate associated metabolites that were greater (↑) or lower (↓) in STC compared to SUF.

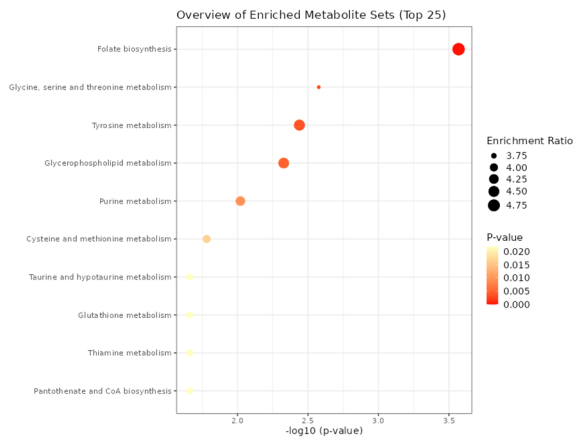

FIGURE 4
Pathways altered between STC and SUF at HR 2.

TABLE 2 Pathways altered between STC and SUF at 2 hours post-LPS challenge.

Pathway Name	FDR	Associated metabolites
Folate biosynthesis	0.01	Tetrahydrobiopterin ↑
Glycine, serine and threonine metabolism	0.01	L-Cysteine ↓, sarcosine ↓
Tyrosine metabolism	0.01	Tyramine ↑
Glycerophospholipid metabolism	0.01	Ethanolamine ↓
Purine metabolism	0.02	Xanthine ↓
Cysteine and methionine metabolism	0.02	L-Cystine ↓, L-cysteine ↓
Taurine and hypotaurine metabolism	0.02	L-Cysteine ↓
Glutathione metabolism	0.02	L-Cysteine ↓
Thiamine metabolism	0.02	L-Cysteine ↓
Pantothenate and CoA biosynthesis	0.02	L-Cysteine ↓

Only pathways with false discovery rate-adjusted (FDR)-adjusted P-values ≤ 0.05 are shown. Arrows indicate associated metabolites that were greater (↑) or lower (↓) in STC compared to SUF.

deoxyguanosine, dGDP, and ethanolamine, between STC and SUF sheep (Figure 6; Table 3).

Discussion

Hair breeds like STC demonstrate greater adaptability to harsh climate conditions and increased resistance to internal parasites when compared to their wool counterparts (Wildeus, 1997). Though SUF sheep breeds are susceptible to parasites which

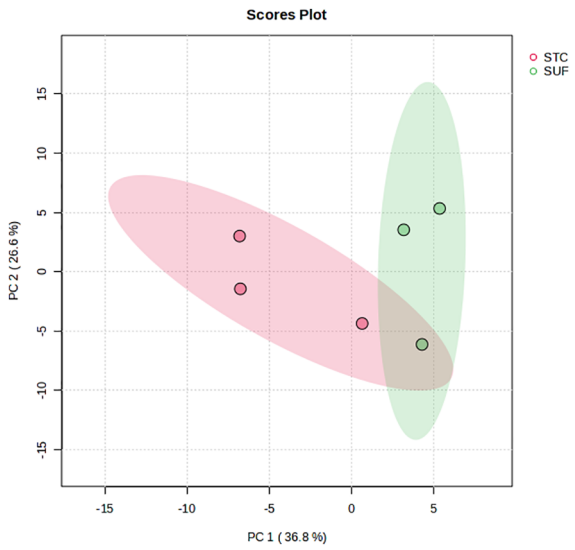

FIGURE 5
PCA scores plot of the liver metabolome of STC and SUF sheep at HR 6.

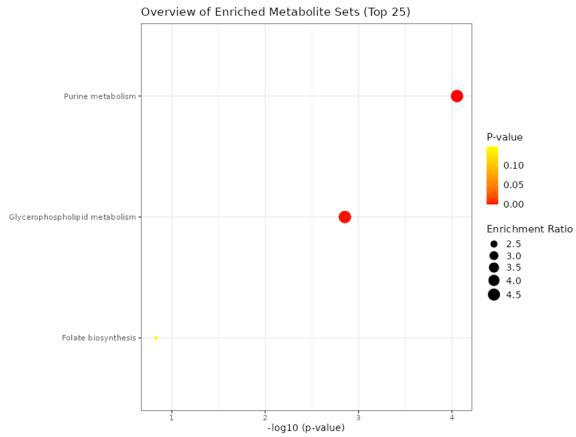

FIGURE 6
Pathways altered between STC and SUF at HR 6.

poses a significant challenge (Leymaster, 1991; Bahirathan et al., 1996), they exhibit profitable growth rates and favorable carcass traits. They have emerged as a prominent meat breed in North America, leading to extensive efforts in generating crosses due to their impressive performance and maternal abilities (Shrestha et al., 2008). Fever response to LPS between breeds indicated a more rapid but less prolonged change in rectal temperature for STC sheep compared to SUF sheep, that presented longer lasting and slower response in temperature for this study. Assessment of pain, through SGS, revealed a more pronounced pain response to LPS in SUF sheep. Similar physiological responses were observed by Hadfield et al. (2018) in Dorset and Suffolk ewes' response to LPS. They reported a greater and more frequent display of sick behavior in SUF ewes compared to Dorset ewes, and LPS treatment increased rectal temperature, peaking at hour 4 post-administration. A response akin to that of STC, as observed in this experiment, would be more desirable, where the immune system can promptly respond and resolve insults.

In this study, eight metabolic pathways, including those associated with some amino acid metabolism were found to be different between SUF and STC prior to LPS stimulation, suggesting increased altered metabolic activity. Amino acid metabolism in ruminants holds paramount importance, influencing various physiological functions crucial for their health and productivity (Titgemeyer and Löest, 2001). Serving as the fundamental building blocks of proteins, amino acids are indispensable for protein synthesis, and overall growth performance (Wu, 2009).

Wool production, a notable aspect of sheep husbandry, relies heavily on amino acids, particularly those containing sulfur, such as

TABLE 3 Pathways altered between STC and SUF at 6 hours post-LPS challenge.

Pathway Name	FDR	Associated metabolites
Purine metabolism	0.01	Deoxyguanosine ↑, dGDP ↓
Glycerophospholipid metabolism	0.01	Ethanolamine ↓

Only pathways with false discovery rate-adjusted (FDR)-P-values ≤ 0.05 are shown. Arrows indicate associated metabolites that were greater (↑) or lower (↓) in STC compared to SUF.

cysteine and methionine, which are pivotal for synthesizing keratin —the principal component of wool (Hynd and Masters, 2002). Furthermore, amino acids play a key role in supporting reproductive processes, from fertility to fetal development, highlighting their significance in sustaining the sheep population (Kwon et al., 2003; McCoard et al., 2016). The vast differences in metabolic profiles and pathways observed in this study may be attributed to the larger liver size and likely support the better growth performance, wool production, and maternal ability of SUF compared to STC.

In response to LPS challenge, the traditional reaction involves the activation of proinflammatory cytokines and mediators like TLR4, IL-1β, and TNF-α, accompanied by the generation of reactive oxygen species (ROS) and the initiation of NF-kB activation (Page et al., 2022). NADPH oxidase is implicated in driving proinflammatory responses to LPS, such as NF-kB activation (Koay et al., 2001; Qin et al., 2004). NADH oxidase, particularly in phagocytic cells like neutrophils and macrophages, is crucial for ROS production, contributing to inflammatory responses against invaders like LPS (Forman and Torres, 2002; Qin et al., 2004). Franchini et al. (2013) identified NADPH oxidase, specifically the NADPH oxidase complex (NOX2), as a crucial element in the complex essential for ROS production in macrophages, leading to subsequent IL-6 production in response to insults such as bacterial invasion. In this study, L-Cysteine drives most of the pathways (such as cysteine and methionine metabolism, taurine and hypotaurine metabolism, and glutathione metabolism) altered at HR2, and its relative concentration was lower in STC compared to SUF. Cysteine is directly derived from homocysteine and is required for glutathione and taurine synthesis, which both play significant roles in combating oxidative stress (Malmezat et al., 2000). Cysteine is also one of the products of methionine degradation in the liver (Yin et al., 2016; Coleman et al., 2020). It has been demonstrated that dietary methionine supplementation improved oxidative status in ruminants, through increases in glutathione transferase activity and glutathione concentrations in plasma and liver (Osorio et al., 2014; Tsiplakou et al., 2017). Batistel et al. (2018) further underscored the importance of methionine in combating oxidative stress by facilitating ROS scavenging, antioxidant activity, increased neutrophil phagocytic activity, and oxidative burst activity, through taurine and glutathione metabolism. Glutathione is an important component of living cells and more specifically plays a protective role in red blood cells against oxidative stress (Tucker et al., 1981). Given the central role cysteine plays in combating oxidative stress, it can be concluded that by HR2, STC sheep are actively combating LPS-induced oxidative stress. Reduced cysteine concentration in STC suggests a shift in metabolic functions to defend against LPS through oxidative stress interventions as a result of inflammation.

The relative concentration of tetrahydrobiopterin (BH4) was greater in STC, relative to SUF at 2 hours post LPS stimulation, and this metabolite is associated with folate biosynthesis. Tetrahydrobiopterin is a cofactor in nitric oxide (NO) production and helps mediate excessive oxidative stress, through reduction in superoxide radical anions (Gamal et al., 2018). Coupling of endothelium nitric oxide synthases is facilitated by BH4, which ameliorates oxidative stress (He et al., 2012). Folate is essential in many metabolic processes such as nucleic acid synthesis, methionine regeneration and purine and pyrimidine synthesis (Bailey and Gregory, 1999). Xu et al., 2014 showed that folate metabolism is affected by biopterins, such as BH4, through increases in oxidative stress abatement pathways. Also, at HR2, purine metabolism was driven by xanthine, which was lower in STC. Xanthine is oxidized into uric acid, catalyzed by xanthine oxidoreductase (XOR), as the end product of purine metabolism (Al-Shehri et al., 2020). This enzyme, XOR, is most abundant in the liver (hepatocytes) and is rate-limiting in the degradation of nucleic acid (Harrison, 2002; Battelli et al., 2016). Xanthine oxidase, a form of XOR, generates ROS. The elevated level of BH4 and reduced relative concentration of xanthine in STC probably suggest a mitigation of LPS-induced oxidative stress.

Tyramine was the only other metabolite that was enriched in STC at HR2. Tyramine is a biogenic amine derived from the decarboxylation of tyrosine (Scherer et al., 2015). (Glymenaki et al., 2023) reported the negative impacts of tyramine on colon cells include cytotoxicity, DNA damage, necrosis and upregulation of oxidative stress-related genes. Despite its detrimental effects, tyramine exerts an indirect influence on the nervous system, promoting pupil dilation, respiration elevation, and blood sugar increase in humans (Shalaby, 1996). Tyramine has been reported to have a dose-dependent effect on immune regulation, energy uptake, and feed uptake modulation in response to starvation in marine shrimp (Kuo et al., 2024). The result of this study suggests potential immunological implications of tyramine concerning its response to LPS in sheep.

Purine metabolism and glycerophospholipid metabolism were the only two pathways altered at HR 6 post-LPS challenge. These two pathways were driven by deoxyguanosine and ethanolamine, respectively. The relative concentration of ethanolamine was lower in STC, compared to SUF sheep, and its associated glycerophospholipid metabolism was found to be a strong indicator of copper toxicity in pig kidney (Qiao et al., 2021; Bi et al., 2022; Mukhopadhyay and Trauner, 2023). Reduced relative concentration of ethanolamine in STC may suggest non-toxic liver tissue. The relative concentration of deoxyguanosine was greater in STC. Deoxyguanosine is derived from guanine, a very sensitive base nucleotide, which is known to be very susceptible to oxidative damage (Nikolova et al., 2022). Increased relative concentration of deoxyguanosine suggests that STC was able to resolve the LPS insult with no oxidative damage to the liver tissue which aligns with the results of sick behavior and rectal temperature pattern of STC compared to SUF sheep breed.

Conclusion

This study highlights hepatic metabolic distinctions between STC and SUF sheep breeds before and after an LPS challenge. The enriched amino acid metabolism before the LPS challenge supports the better growth performance, wool production, and reproduction of the SUF, compared to STC breed. The observed variations in the relative concentrations of several metabolites associated with important metabolic pathways further contribute to understanding the responses of SUF and STC to the LPS challenge. Notably, the lower concentration of ethanolamine in STC indicates potentially non-toxic liver tissue, while the increased relative concentration of deoxyguanosine suggests the effective resolution of the LPS insult

without oxidative damage. These findings enhance our understanding of the hepatic metabolome changes of SUF and STC sheep breeds to inflammatory challenges.

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 studies were approved by Institutional Animal Care and Use Committee of West Virginia University (protocol number # 2303064503). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

SJ: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. KB: Data curation, Investigation, Methodology, Project administration, Writing – review & editing. SB: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. IO: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fanim.2024.1407533/full#supplementary-material

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