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Metabolomic Profiling, Volatile Fatty Acids, and Greenhouse Gas Emissions of Beef Cattle Infused with Different Essential Oil Blends

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Simple Summary: Essential oil blends show promise as natural ruminant feed additives, potentially modulating rumen fermentation and mitigating greenhouse gas emissions. The present study investigated the metabolite profiles, methane production, and fermentation dynamics of five novel essential oil blends in Black Angus beef cattle. The blends, composed of four distinct essential oils in varying proportions, were directly infused into the rumen to simulate practical application. Advanced metabolomics techniques were employed to analyze and elucidate the EOB-induced changes in the rumen fluid. Results demonstrated that the infusion of the blends modified the rumen metabolism, fermentation patterns, and metabolite production. The study provides insights into how the essential oil blends influenced the specific metabolic processes and fermentation characteristics in the rumen. This research contributes to the development of bio-based strategies for optimizing rumen function, offering valuable information for animal nutritionists and producers aiming to improve feed efficiency and minimize the environmental footprint of ruminant livestock production.

Abstract: Essential oils are natural feed additives that improve animal health and enhance their performance. This study investigated the effects of the rumen infusion of five essential oil blends (EOBs) on greenhouse gas (GHG) emissions, rumen fermentation parameters, and rumen metabolome and metabolic pathways in Black Angus cows. Using a 6 × 6 Latin Square experimental design, a 90-day study was conducted with six cattle. A daily dosage of 4 mL of EOBs was administered during each infusion. Volcano plot analyses between the control (CON) and each of the EOBs (EOB1, EOB2, EOB3, EOB4, and EOB5) revealed several differentially abundant ($p \leq 0.05$; absolute fold change ≥ 1.5) metabolites. The EOB5 treatment exhibited the most significant impact, with 26 differentially abundant metabolites, including elevated valine and reduced gallic acid. Volatile fatty acids (VFAs), including valerate, isobutyrate, and isovalerate, were significantly increased ($p < 0.05$). GHG emissions were not significantly affected, but a numerical decrease was observed in the animals infused with the EOB5 treatment. Ammonia nitrogen concentrations remained within the suitable range for rumen microbes' growth, indicating a normal internal environment for microbial crude protein synthesis. In conclusion, the study has demonstrated that the direct infusion of EOBs significantly improved the generation of VFAs and impacted the energy production, protein synthesis, and microbial activity of the animals.

Keywords: essential oil blends; volatile fatty acids; metabolic pathways

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1. Introduction

Essential oils (EOs) are natural feed additives that improve animal health and enhance their performance. They have been identified as alternatives to antibiotics in some livestock production [1–3]. Despite several studies conducted on EOs, there exist discrepancies and irregularities, including inconsistent effects, transient responses, and potential adverse consequences, which might have been influenced by factors ranging from compositions, dosages, and stages of the animal during administration [4]. Recent advancements in metagenomics, transcriptomics, proteomics, and metabolomics techniques have opened new avenues for studying the rumen microbiomes in unprecedented detail. By integrating phylogenetic information with metabolic insights, researchers are gaining a deeper understanding of the complex interactions within the rumen ecosystem [5,6].

Metabolomics has enabled researchers to fully elucidate the chemical phenotype of an organism, thereby making it a useful tool in systems biology [7]. The metabolic content of the rumen fluid reflects the interaction between rumen microbes and diets, which could be identified and quantified using advanced analytical tools such as mass spectrometry (MS)- and nuclear magnetic resonance (NMR) spectroscopy-based techniques. However, the type of metabolite detected and the level of sensitivity and accuracy achievable are unique to each technique [8]. Zhang et al. [9] reported that the metabolome analysis of rumen fluid in cattle showed that oregano essential oil (OEO) supplementation modified metabolic pathways such as CoA biosynthesis and pantothenate. In another study by Li et al. [10], metabolomics analysis by LC-MS and multivariate/univariate analysis was employed, where they utilized metabolomics coupled with microbiome analysis to investigate the effects of essential oils on the intestinal metabolome and microbiota in piglets.

Rumen fermentation is essential for ruminant productivity; however, it also presents challenges in terms of greenhouse gas (GHG) emissions, excessive nitrogen excretion, and potential impacts on the nutritional quality of ruminant-derived products [11]. Dietary modification including dietary supplementation with EOs, synthetic methanogen inhibitors derived from plant lipids, and genetic selection for low-emission animals are just a few of the mitigation strategies used to reduce methane emissions [6,12–14].

The optimal dose of EOs for improving rumen fermentation and animal performance has long been a debate and an ongoing area of research. Kholif et al. (2018) [15] reported that a blend of capsicum/thymus EOs at 2 mL improved the feed digestion and increased milk production of lactating Farafra ewes. Cardozo et al. [16] reported that anise oil at 2 g/day significantly reduced the acetate-to-propionate ratio, branched-chain volatile fatty acids (VFAs) and ammonia nitrogen concentrations, and protozoal count in beef heifers. While previous in vitro studies have shed light on the effects of EOs on rumen fermentation parameters, gas production, and nutrient digestibility, few in vivo studies have been conducted to assess the replicability of these findings. The present study sought to bridge this gap by conducting in vivo studies that closely mimic real-life conditions, providing valuable information on EO effects in a practical setting. This study aimed to provide comprehensive insights into the complex interactions between EO infusion and metabolite production. Four different EOs having various bioactive components were mixed in varying proportions to form five distinct essential oil blends (EOBs). Hence, the study was carried out to investigate the effects of the EOBs on methane emissions, the modulation of rumen fermentation characteristics, and metabolic pathways in Black Angus beef cattle.

2. Materials and Methods

2.1. Study Ethical Approval

Ethical clearance for this study was obtained from the Institutional Animal Care and Use Committee (Approval No: LA22-0019) at North Carolina A&T State University, Greensboro. The cattle were managed according to the established standards of the University Farm.

2.2. Animals, Experimental Design, and Diet Composition

Six Black Angus beef cows with rumen cannulas from the North Carolina A&T State University Beef Research Farm were used for the study following a 6 × 6 Latin Square experimental design. These cows were screened to ensure that they were free from diseases or infections. The average weight of the cows was 627.5 ± 58.8 kg. Throughout the study, the cows had access to a mixed-grass pasture. Adequate shelter, ad libitum hay (consisting of oats and triticale), and water were provided to the animals. The study spanned six cycles, with each cycle encompassing 15 days. In each cycle, the animals were infused for 9 days straight and were sampled on the 8th, 9th, and 10th days (no infusion on the 10th day). A rest/wash-out period of five days was observed before they were switched to a different treatment. The rest period minimized potential carry-over effects between treatments. Five distinct EOBs were formulated, which were composed of anise, clove, oregano, and peppermint in specific ratios, as follows: EOB1 [1:1:1:1], EOB2 [1:2:3:4], EOB3 [2:3:4:1], EOB4 [3:4:1:2], and EOB5 [4:1:2:3]. The outline of the experiment is shown in Table 1.

Table 1. Dietary treatment distribution in the 6 × 6 Latin Square experimental design.

Cycles	A	B	C	D	E	F
1	CON	EOB1	EOB2	EOB3	EOB4	EOB5
2	EOB5	CON	EOB1	EOB2	EOB3	EOB4
3	EOB4	EOB5	CON	EOB1	EOB2	EOB3
4	EOB3	EOB4	EOB5	CON	EOB1	EOB2
5	EOB2	EOB3	EOB4	EOB5	CON	EOB1
6	EOB1	EOB2	EOB3	EOB4	EOB5	CON

Cows (A–F) received a different treatment in each period of the experiment. A daily dosage of 4 mL of the EOBs was directly infused into the rumen at 08:00 h, except on the last day of sampling. The control animal (CON) did not receive any EOB infusion. Samples of hay fed to the animals were collected during each cycle for subsequent chemical and proximate analyses.

The chemical composition of the diet during the experiment was determined following standard protocols [17]. Details of the dry matter (DM), nitrogen, crude protein (CP), ash content, organic matter (OM), neutral detergent fiber (NDF), and acid detergent fiber (ADF) determination have been reported in our previous study [18]. The chemical composition of the diet during the study is presented in Table 2.

Table 2. Chemical composition of grass hay (oats and triticale) fed during the study.

	(As Submitted, %)	Dry Matter Basis (%, Unless Stated)
DM	86.49	---
Moisture	13.51	
CP	9.18	10.61
Fat	1.19	1.38
Ash	6.66	7.71
NDF	57.75	66.77
ADF	34.86	40.30
ADL	22.61	19.55
Unavailable protein	1.44	1.66
Adjusted crude protein	8.66	10.01
Total digestible nutrients	55.64	64.32
Nitrate ion	0.09	0.10
Non-fiber carbohydrates	15.36	17.76
Calcium	0.26	0.30
Phosphorus	0.23	0.27
Sulfur	0.14	0.16
Sodium	0.05	0.06
Magnesium	0.13	0.15
Potassium	1.83	2.12
Copper (ppm)	13.00	15.01
Manganese (ppm)	42.00	48.15
Iron (ppm)	338.00	390.86
Zinc (ppm)	29.00	33.87

DM, dry matter; NDF, neutral detergent fiber; CP, crude protein; ADF, acid detergent fiber; ADL, acid detergent lignin.

2.3. Rumen Fluid Sample Collection

On the 8th, 9th, and 10th days of each cycle period, gas samples were aspirated from the rumen using a 300cc syringe and promptly transferred into a portable gas analyzer

(Biogas 5000, Landtec, Dexter, MI, USA) to quantify the greenhouse gases (CH_4, CO_2, NH_3, and H_2S). Additionally, rumen samples were obtained from different regions of the rumen of the cannulated Black Angus beef cows at 08:00 h. This process involved passing the rumen content through four layers of cheesecloth for filtration. The pH of the resulting fluid was measured immediately with a portable pH meter (model 507, Crison Instruments SA, Barcelona, Spain), and the values were recorded. Thereafter, 15 mL of the fluid subsamples was collected for the VFA analysis, and 25 mL for the ammonia nitrogen (NH_3-N) determination. Samples (45 mL of rumen fluid) for the metabolome analysis were collected over the 3-day sampling period and stored in a −80 °C freezer.

2.4. Short-Chain Fatty Acid Analysis

Thawed rumen fluid samples, preserved with 25% metaphosphoric–crotonic acid, were centrifuged at 10,000 rpm for 15 min at 4 °C using a Thermo Fisher Scientific centrifuge (model Sorvall X4R Pro-MD; Thermo Electron LED GmbH, Osterode, Germany). Analysis of acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate concentrations followed the procedure outlined in [19]. Volatile fatty acid (VFA) profiles in rumen fluid were quantitatively analyzed using gas chromatography–mass spectrometry (GC-MS). The analytical system consisted of an Agilent 7890B gas chromatograph coupled to a 5977B mass selective detector and integrated with a 7693 autosampler (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved on a Zebron ZB-FFP capillary column (Phenomenex Inc., Torrance, CA, USA). The quantification procedure utilized an internal standard method, incorporating a mixture of metaphosphoric acid and crotonic acid (trans-2-butenoic acid). An external calibration standard, which was comprised of acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids, was used for the quantification of individual VFAs [20]. The injector port adopted a split ratio of 1:12 at a temperature of 250 °C, with a helium flow rate of 1 mL/min. Column and detector temperatures were maintained at 120 °C and 140 °C, respectively.

2.5. Ammonia Nitrogen Analysis

The determination of the NH_3-N content in the rumen liquid involved the application of the Kjeldahl method [21]. Rumen samples (25 mL) were carefully mixed with 5 mL of diluted H_2SO_4 (72%). The resulting solution was promptly stored at −20 °C for subsequent analysis. Upon thawing, the samples were blended with 50 mL of NaOH (32%) and subjected to distillation using a BÜCHI Distillation Unit (K-355, BÜCHI Lyovapor, New Castle, DE, USA). The distilled output was then titrated against diluted HCl (0.1 N) until the solution reverted to its original color, marking the completion of the analytical process.

2.6. Metabolome Analysis of Rumen Fluid

The rumen fluid samples underwent untargeted metabolome analysis using chemical isotope labeling (CIL) coupled with liquid chromatography–mass spectrometry (LC-MS). This method involves labeling with ^{12}C and ^{13}C-isotope dansylation to identify metabolites based on their chemical groups, including amines/phenols, carboxylic acids, carbonyls (primarily aldehydes and ketones), and hydroxyls [22]. Detailed information on the technique, including sample preparation and analysis, has been reported by [23]. A total of 21 raw (18 rumen fluid samples which consisted of 3 replicates from each treatment and 3 quality control samples) LC-MS data files were generated. The raw LC-MS data files were processed using IsoMS Pro 1.2.16 to eliminate redundant peaks attributed to the same metabolite, including adduct ions, dimers, and multimers. Peak pairs lacking data in at least 80% of samples within any group were excluded from further analysis. Metabolite identification for the peak pairs was carried out by mass and retention time matching with the CIL and linked identity libraries [23,24].

2.7. Statistical Analysis

The metabolome data for all 18 samples were analyzed using MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca/ accessed on 13 November 2023). Differentially abundant metabolites (p-values ≤ 0.05 and absolute fold change (FC) ≥ 1.5) [25] were determined using a volcano plot analysis. Score plots using Partial Least Squares Discriminant Analysis (PLS-DA and OPLS-DA) were created to illustrate the variation in the metabolome between treatments (see Supplementary Materials). To elucidate the distinct metabolic pathways in the rumen, a pathway enrichment analysis of the metabolome data was conducted. This analysis aimed to identify metabolic pathways that exhibited significant differences ($p \leq 0.05$) in beef cattle infused with each of the EOBs compared with the CON.

The other data were analyzed using the PROC MIXED of SAS as a 6 × 6 Latin Square design with 6 treatments and 6 periods. The model included the fixed effect of the treatment and the random effect of the period, as follows:

Yijk = μ + Ti + Pj + Eijk, where Yijk is individual observation for a given variable, μ is the overall mean, Ti is the treatment effect, Pj is the period effect, and Eijk is the residual error. Differences between specific treatments were identified using the probability of difference (PDIFF) option of the least squares means statement, with statistical significance set at $p \leq 0.05$.

3. Results

3.1. Volatile Fatty Acids

Table 3 shows the effect of the essential oil blends on volatile fatty acid production in the rumens of the experimental animals. The inclusion of EOBs at 4 mL/cow produced significant effects ($p < 0.05$) on isovalerate, valerate, and isobutyrate. Compared to the control, the EOB2 and EOB5 treatments increased ($p < 0.05$) the molar proportion of isobutyrate by 37% and 41%, respectively, and isovalerate by 36% and 43%, respectively. A higher ($p < 0.05$) valerate proportion was observed in the EOB2 group, while the EOB5 group had the lowest value. The EOBs had no effect ($p > 0.05$) on total volatile fatty acids (TVFAs), acetate, propionate, butyrate, as well as the acetate/propionate ratio.

Table 3. Effect of essential oil blends on volatile fatty acid production (mM).

Treatments	TVFA	Acetate	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate	A:PR
CONTROL	95.4	0.711	0.178	0.099	0.0046 ^{b}	0.0062 ^{ab}	0.0014 ^{b}	3.99
EOB1	93.4	0.713	0.178	0.098	0.0039 ^{b}	0.0058 ^{bc}	0.0011 ^{b}	4.02
EOB2	88.1	0.709	0.180	0.096	0.0063 ^{a}	0.0064 ^{a}	0.0019 ^{a}	3.94
EOB3	93.3	0.710	0.179	0.099	0.0046 ^{b}	0.0061 ^{ab}	0.0013 ^{b}	3.99
EOB4	94.6	0.719	0.176	0.094	0.0040 ^{b}	0.0055 ^{c}	0.0011 ^{b}	4.12
EOB5	86.1	0.714	0.177	0.094	0.0065 ^{a}	0.0062 ^{ab}	0.0020 ^{a}	4.05
SEM	3.460	0.0317	0.0020	0.0175	0.0001	0.0002	0.0001	0.0635
p-value	0.312	0.196	0.665	0.171	0.000	0.011	0.0001	0.473

A:PR, acetate/propionate ratio; SEM, standard error of mean; $^{a–c}$ Within a row, treatment means with different superscripts differ, $p \leq 0.05$.

3.2. Greenhouse Gas Emissions, Rumen pH, and NH_3-N Concentrations

The effects of infusing the EOBs on greenhouse gas emissions, rumen pH, and NH_3-N concentration are presented in Table 4. Across all of the treatments, there was no significant effect ($p > 0.05$) on CH_4, CO_2, NH_3, and H_2S. The EOB5 and EOB3 treatments numerically decreased the CH_4 by 28.4% and 20%, respectively, while the CO_2 was decreased by 24% and 18%, respectively. The effects of the direct infusion of the EOBs had no significant effect ($p > 0.05$) on the rumen pH and NH_3-N concentration. The rumen pH ranged from 6.48 to 6.62 in the present study.

Table 4. Effects of the EOBs on greenhouse gas emissions, rumen pH, and NH_3-N concentration.

Treatments	CH_4	CO_2	NH_3	H_2S	Rumen pH	NH_3-N (mg/dL)
CONTROL	11.6	22.3	54.8	234	6.48	12.3
EOB1	11.6	19.9	45.2	101	6.60	14.1
EOB2	11.4	12.0	70.6	299	6.62	14.7
EOB3	9.29	18.3	57.3	180	6.55	16.1
EOB4	10.8	21.3	62.1	222	6.57	14.6
EOB5	8.31	16.9	50.3	179	6.59	12.4
SEM	1.613	3.318	10.169	65.278	0.0771	0.5773
p-value	0.610	0.771	0.570	0.404	0.0700	1.364

CH_4, methane; CO_2, carbon dioxide; NH_3, ammonia; H_2S, hydrogen sulfide; SEM = standard error of mean.

3.3. Rumen Metabolites

A total of 1350 metabolites were detected and identified in the rumen (see Supplementary Materials).

3.3.1. CON vs. EOB1

The results of the volcano plot analysis revealed that a total of eight differentially abundant (FC $\geq$ 1.5, $p \leq 0.05$) metabolites were detected between the CON and EOB1 groups (Figure 1A). Compared to the CON, rumen concentrations of four metabolites, including glyceraldehyde, N-acetyl-beta-alanine, L-threonine/L-allo threonine, and hydroxyproline, were greater ($p \leq 0.05$) in the EOB1 group, whereas four metabolites, including two isomers of acetic acid, phloroglucinol and 2,5-dihydroxypyridine, were lower ($p \leq 0.05$) in the EOB1 group. The results of the pathway enrichment analysis of all of the metabolites showed the enrichment ($p \leq 0.05$) of the propanoate metabolism, selenocompound metabolism, steroid hormone biosynthesis, glycolysis/gluconeogenesis, pyruvate metabolism, and pyrimidine metabolism pathways in the EOB1 group (Figure 2A).

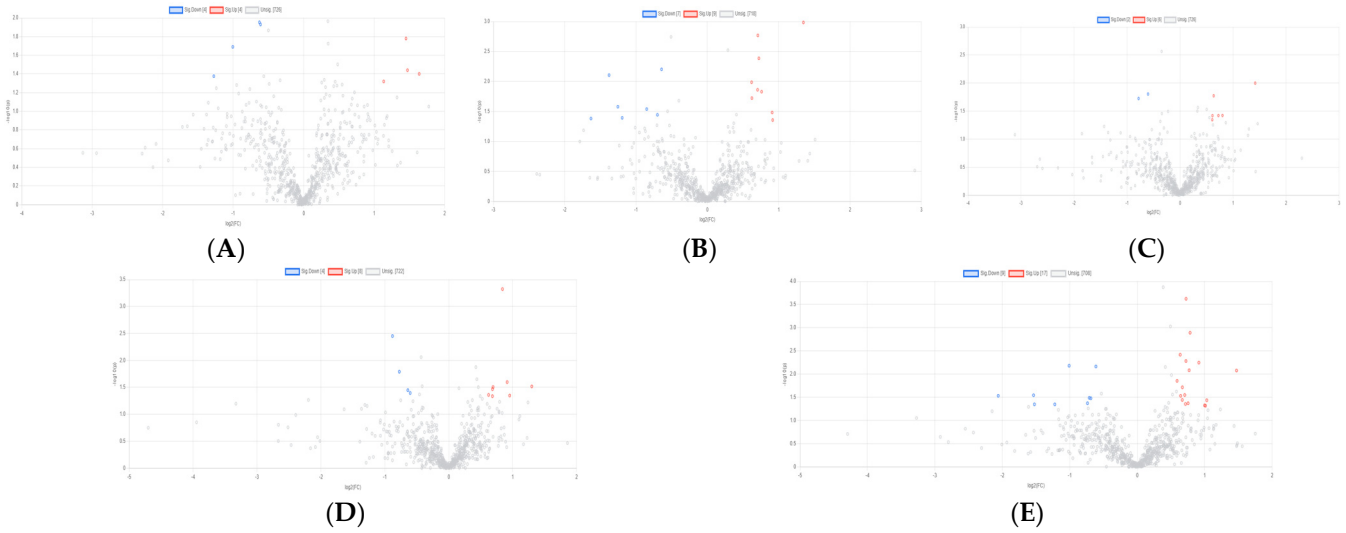

Figure 1. Volcano plot ((**A**) CON and EOB1; (**B**) CON and EOB2; (**C**) CON and EOB3; (**D**) CON and EOB4; (**E**) CON and EOB5) showing the number of the differentially abundant metabolites between the EOBs and CON ($p \leq 0.05$, FC > 1.5). Metabolites with a false discovery ratio of $\leq$0.05 (red or blue) are differentially increased (red dot) or reduced (blue dots) in the EOB groups relative to the CON group.

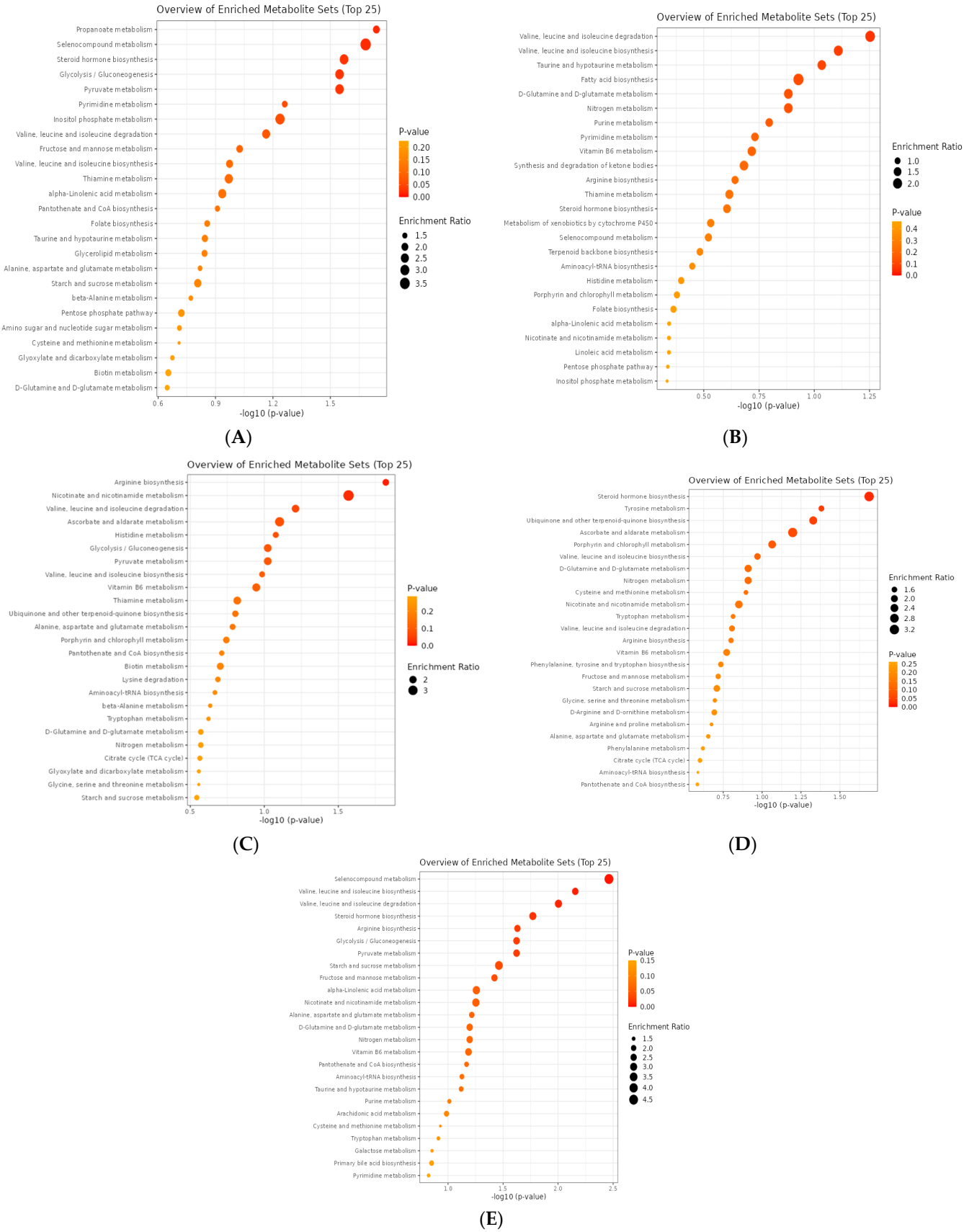

Figure 2. Pathway enrichment analysis ((**A**) CON and EOB1; (**B**) CON and EOB2; (**C**) CON and EOB3; (**D**) CON and EOB4; (**E**) CON and EOB5) of the metabolome between the EOB groups and control group. Pathways with $-\log10(P) \geq 1.3$ (equivalent to $p \geq 0.05$) were altered.

3.3.2. CON vs. EOB2

The results of the volcano plot analysis revealed that a total of 16 differentially abundant (FC $\geq$ 1.5, $p \leq 0.05$) metabolites were detected between the CON and EOB2 groups (Figure 1B). Compared to the CON, the rumen concentrations of nine metabolites, such as aminoacrylic acid, salidroside, prunasin, isomer 1 of salidroside, nopaline, 3-methyl-2-oxovaleric acid, N(6)-methyllysine, 3-sulfocatechol, and isomer 2 of 2-hydroxy-6-oxo-(2′-aminophenyl)-hexa-2,4-dienoic acid, were greater ($p \leq 0.05$) in the EOB2 group, whereas seven metabolites, including gamma-glutamyl-gamma-aminobutyraldehyde, gallic acid, 3,4-dihydroxystyrene, 4-hydroxycinnamyl aldehyde, L-4-hydroxyglutamate semialdehyde, 5-hydroxyferulic acid methyl ester, and isomer 2 of aspartic acid, were lower ($p \leq 0.05$) in the EOB2 group (Figure 3). The results of the pathway analysis of all of the metabolites showed that the EOB2 group enriched ($p \leq 0.05$) the valine, leucine, and isoleucine degradation pathways (Figure 2B).

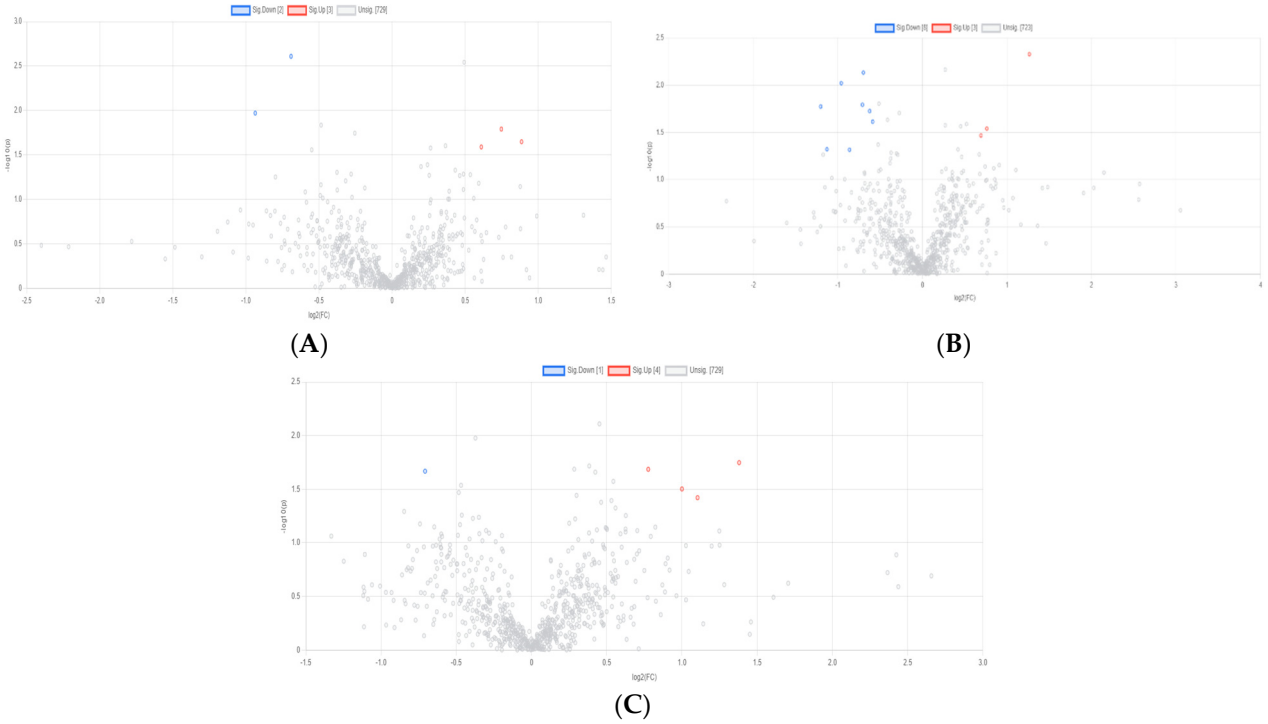

Figure 3. Volcano plot between (**A**) EOB4 vs. EOB3, (**B**) EOB5 vs. EOB3, and (**C**) EOB5 vs. EOB4 showing the number of the differentially abundant metabolites between the EOB4 group and EOB3 group ($p \leq 0.05$, FC > 1.5). Metabolites with a false discovery ratio of $\leq$0.05 (red or blue) are differentially increased (red dot) or reduced (blue dots) in the EOB groups.

3.3.3. CON vs. EOB3

The results of the volcano plot analysis revealed that a total of eight differentially abundant (FC $\geq$ 1.5, $p \leq 0.05$) metabolites were detected between the CON and EOB3 groups (Figure 1C). Compared to the CON, the rumen concentrations of six metabolites, such as guanosine, isomer 1 of methionine sulfoxide, N-hydroxy-L-valine, N(gamma)-acetyldiaminobutyric acid, salidroside, and aminoacrylic acid, were greater ($p \leq 0.05$) in the EOB3 group, whereas two metabolites, including prostaglandin C2 and homogentisic acid, were lower ($p \leq 0.05$) in the EOB3 group. The results of the pathway analysis of all of the metabolites showed that the EOB3 group enriched ($p \leq 0.05$) the following pathways: arginine biosynthesis, and the nicotinate and nicotinamide metabolism pathways (Figure 2C).

3.3.4. CON vs. EOB4

The results of the volcano plot analysis revealed that a total of 12 differentially abundant (FC $\geq$ 1.5, $p \leq 0.05$) metabolites were detected between the CON and EOB4 groups (Figure 1D). Compared to the CON, the rumen concentrations of eight metabolites, such as estradiol-17, N-hydroxy-L-valine, 3-chloro-cis-1,2-dihydroxycyclohexa-3,5-diene, isomer 4 of 5-aminopentanal, nopaline, N(6)-methyllysine, 3-(4-hydroxyphenyl)pyruvic acid, and hydroxyproline, were greater ($p \leq 0.05$) in the EOB4 group, whereas four metabolites, including gamma-glutamyl-beta-cyanoalanine, guanidoacetic acid, gallic acid, and oxamic acid, were lower ($p \leq 0.05$) in the EOB4 group. The results of the pathway analysis of all of the metabolites showed that the EOB4 group enriched ($p \leq 0.05$) the following pathways: steroid hormone biosynthesis, the tyrosine metabolism, and ubiquinone and other terpenoid–quinone biosynthesis pathways (Figure 2D).

3.3.5. CON vs. EOB5

The results of the volcano plot analysis revealed that a total of 26 differentially abundant (FC $\geq$ 1.5, $p \leq 0.05$) metabolites were detected between the CON and EOB5 groups (Figure 1E). Compared to the CON, the rumen concentrations of 17 metabolites, such as valine, 3-sulfino-L-alanine, isomer 1 of beta-alanine, leucine, nopaline, N-(6-aminohexanoyl)-6-aminohexanoic acid, 3-methyl-2-oxovaleric acid, citrulline, isomer 4 of 5-aminopentanal, hydroxyproline/cis-4-hydroxy-D-proline, homovanillic acid, acrylic acid, coniferyl acetic acid, pyridoxal, c6-amino-2-oxohexanoic acid, isomer 1 of feruloylputrescine, and feruloylputrescine, were greater ($p \leq 0.05$) in the EOB5 group, whereas nine metabolites, including gallic acid, (S)-2-(hydroxymethyl)glutaric acid, 2′-deamino-2′-hydroxyneamine, glycochenodeoxycholic acid, glycerone, 2-dehydro-3-deoxy-L-fuconic acid/2-dehydro-3-deoxy-L-rhamnonic acid, 7-cyano-7-carbaguanine, and 2,5-dihydroxypyridine, were lower ($p \leq 0.05$) in the EOB5 group. The results of the pathway analysis of all of the metabolites showed that the EOB5 group enriched ($p \leq 0.05$) the following pathways: the selenocompound metabolism; valine, leucine, and isoleucine biosynthesis; valine, leucine, and isoleucine degradation; steroid hormone biosynthesis; arginine biosynthesis; glycolysis/gluconeogenesis; the pyruvate metabolism; the starch and sucrose metabolism; and the fructose and mannose metabolism pathways (Figure 2E).

3.3.6. EOB4 vs. EOB3

The results of the volcano plot analysis revealed that a total of five differentially abundant (FC $\geq$ 1.5, $p \leq 0.05$) metabolites were detected between the EOB4 and EOB3 groups (Figure 3A). Compared to the EOB3 group, the rumen concentrations of three metabolites, such as 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoic acid, 4-hydroxy-3-methylbenzoic acid, and tabtoxinine-delta-lactam, were greater ($p \leq 0.05$) in the EOB4 group, whereas two metabolites, isomer 1 of deoxyuridine and thymidine, were lower ($p \leq 0.05$) in the EOB4 group and higher in the EOB3 group. The results of the pathway analysis of all of the metabolites showed that the EOB4 group enriched ($p \leq 0.05$) the terpenoid backbone biosynthesis (Figure 4A).

3.3.7. EOB5 vs. EOB3

The results of the volcano plot analysis revealed that a total of 11 differentially abundant (FC $\geq$ 1.5, $p \leq 0.05$) metabolites were detected between the EOB5 group and EOB3 group (Figure 3B). Compared to the EOB3 group, the rumen concentrations of three metabolites, such as O-phosphoethanolamine, 5-hydroxykynurenine, and 6-amino-2-oxohexanoic acid, were greater ($p \leq 0.05$) in the EOB5 group, whereas eight metabolites, including deoxyuridine, thymidine, cis-2-methyl-5-isopropylhexa-2,5-dienoic acid/trans-2-methyl-5-isopropylhexa-2,5-dienoic acid, isomer 1 of alpha-aminobutyric acid, L-rhamnono-1,4-lactone, 2-hydroxy-6-oxo-(2′-aminophenyl)-hexa-2,4-dienoic acid, p-coumaroyl quinic acid, and ethylene glycol, were lower ($p \leq 0.05$) in the EOB5 group and higher in the EOB3 group. The results of the pathway analysis of all of the metabolites showed that the EOB5 group enriched

($p \leq 0.05$) the following pathways: the pyrimidine metabolism; valine, leucine, and isoleucine degradation; the sphingolipid metabolism; valine, leucine, and isoleucine biosynthesis; and lysine degradation (Figure 4B).

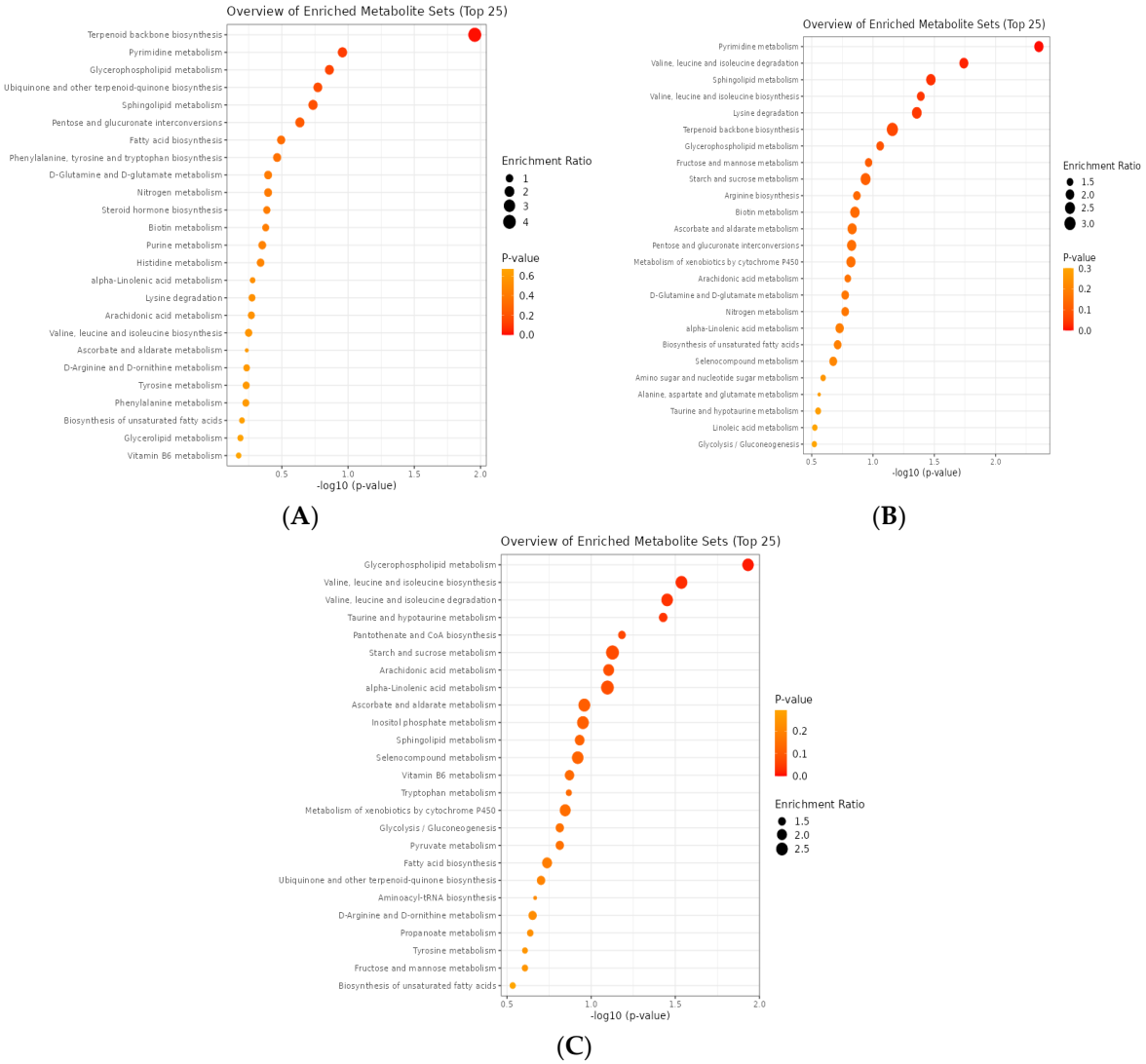

Figure 4. Pathway enrichment analysis of the metabolome between EOB groups ((**A**) EOB4 vs. EOB3; (**B**) EOB5 vs. EOB3; (**C**) EOB5 vs. EOB4). Pathways with $-\log10(P) \geq 1.3$ (equivalent to $p \geq 0.05$) were altered.

3.3.8. EOB5 vs. EOB4

The results of the volcano plot analysis revealed that a total of five differentially abundant (FC ≥ 1.5, $p \leq 0.05$) metabolites were detected between the EOB5 and EOB4 groups (Figure 3C). Compared to the EOB4 group, the rumen concentrations of four metabolites, such as 3-methyl-2-oxovaleric acid, O-phosphoethanolamine, N-(6-Aminohexanoyl)-6-aminohexanoic acid, and leukotriene A4, were greater ($p \leq 0.05$) in the EOB5 group, whereas one metabolite, 2-hydroxymuconate semialdehyde, was lower ($p \leq 0.05$) in the EOB5 group and was greater in the EOB4 group. The results of the pathway analysis of all of the metabolites showed that the EOB5 group enriched ($p \leq 0.05$) the following pathways: the glycerophospholipid metabolism; valine, leucine, and isoleucine biosynthesis; valine, leucine, and isoleucine degradation; and the taurine and hypotaurine metabolism (Figure 3C).

4. Discussion

4.1. Effects of EOBs on Volatile Fatty Acids

The generation of VFAs is a pivotal component of rumen fermentation, exerting a significant impact on the overall energy provision to the animal [26]. It is argued that maintaining the appropriate balance of VFAs in the rumen is crucial for the preservation of rumen health and the achievement of optimal animal performance [27]. VFAs are produced from dietary carbohydrates by rumen fermentation. Thus, they serve as the main source of metabolizable energy for ruminants, and enhancing their production from the diet is advantageous for the animals. Phytochemicals, including natural EOs, have been documented to modify the composition of rumen microbiota, consequently influencing the end-products of rumen fermentation, such as VFAs [28]. Castillejos et al. [29] observed a significant effect on the propionic acid concentration with the inclusion of 500 mg/L thymol. Alabi et al. [18] reported that the inclusion of anise, clove, oregano, and peppermint oils improved the production of VFAs. The inclusion level of 4 mL in this present study might not be sufficient to induce similar effects on the in vivo total VFAs, acetate/propionate ratio, as well as butyrate production. However, there was an improvement in the production of valerate, isovalerate, and isobutyrate by the EOB2 and EOB5 treatments. Isoacids, denoting the VFAs with branched-chain structures (BCVFAs), specifically isovaleric acid (isovalerate), isobutyric acid (isobutyrate), and 2-methylbutyric acid (2-methylbutyrate), along with the linear-chain valeric acid (valerate), are microbial by-products arising from the metabolism of amino acids. These amino acids include valine, leucine, isoleucine, and proline [30]. While found in limited amounts in the rumen, the minor BCVFAs isobutyrate and isovalerate play a crucial role as substrates for specific microbial species within the rumen [31]. Increased concentrations of valerate, isobutyrate, and isovalerate suggest that these treatments have the potential to enhance cellulolytic bacteria populations and improve fiber digestibility in the rumen. Contrary to this, Roy et al. [32] reported that a 600 ppm dose of clove oil lowered total VFAs and the acetate/propionate ratio in in vitro studies conducted with inoculum from buffaloes fed a wheat straw-based diet

4.2. Effects of EOBs on Rumen pH and NH_3-N Concentration

The rumen pH serves as a crucial indicator for assessing microbial populations and maintaining the internal environment homeostasis in the rumen. The optimal range is typically between 5.5 and 7.0, ensuring normal microbial activity in the rumen [33]. The results obtained in this study (Table 4) indicated that the rumen pH values did not differ between treatment groups. In the present study, the rumen fluid pH in each group fell within the normal range, varying from 6.48 to 6.62. This is consistent with a previous study by Tager and Krause [34], who reported that cinnamaldehyde, eugenol, and capsicum essential oils did not influence the rumen pH. Hristov et al. [14] discovered that oregano leaves had no notable impact on the rumen pH in Holstein cows, even when fed varying amounts of oregano leaves. The Black Angus cows used in the present study were fed a high-roughage diet, which tends to produce more saliva, which can help buffer the rumen and maintain a stable pH. Significantly lower or elevated pH values over a long period could affect rumen microbial populations and fermentation because cellulolytic bacteria in the rumen are pH sensitive [18]. In addition to this, lower rumen pH values caused by an increase in volatile fatty acids in the rumen could lead to acidosis [35]. Hence, keeping the rumen pH within the optimum range is critical for efficient rumen function and microbial activity.

NH_3-N is not only the final product for nitrogenous substance degradation but also a raw material for microbial crude protein synthesis [36]. Studies have shown that EO inclusion significantly affects rumen fermentation [37]. The NH_3-N was not significantly different among the treatments, which may be due to the low dosage of the essential oils that was infused. The suitable range for the NH_3-N concentration for rumen microbe growth is 6–30 mg/100 mL [38]. In the present study, the NH_3-N concentration was 12.31–14.74 mg/100 mL, which is sufficient for normal microbial protein (MCP) synthesis by rumen microorganisms. The additive treatment did not lead to significant differences among the experimental groups. These results are similar to

those reported by Li et al. [38], who investigated the effects of oregano essential oil, cobalt, and the synergistic effects of both of them on the rumen degradation rate and fermentation characteristics of corn silage in sheep. Cardozo et al. [39] indicated that a concentration of 0.22 mg/L of capsicum oil had no effect on ammonia fraction concentrations. Similarly, Benchaar et al. [40] reported that capsicum oil, administered in doses ranging from 3 to 300 mg/L, had no impact on the ammonia concentration in a dairy-type rumen environment. Conversely, Cardozo et al. [16], in an in vitro batch culture fermentation using rumen fluid from heifers fed a 10:90 forage/concentrate diet, found that doses from 0.3 to 30 mg/L of CAP decreased the ammonia concentration.

4.3. Effect of EOBs on Greenhouse Gases

The influence of the EOBs on greenhouse gas emissions in the present study was assessed to achieve a deeper understanding of their potential impact on rumen microbial activity and methane production, a crucial environmental concern associated with livestock agriculture [41]. Methane is directly involved in global warming, and it correlates with a loss of energy and a reduction in production efficiency in ruminants [42]. It is synthesized through the reduction in CO_2 using hydrogen, a product generated by diverse rumen microbial populations during the process of feed digestion [18]. The synergistic modulation of rumen fermentation and methane reduction can be achieved by combining multiple EOs harboring diverse bioactive compounds [18,42]. Within such combinations, individual EOs may exhibit targeted effects on specific microbial populations or metabolic pathways within the rumen, thereby contributing to a more comprehensive strategy for mitigating methane emissions. It is crucial to recognize that the efficacy of specific EO combinations is contingent upon various factors, including the specific types and proportions of EOs used, the composition of the dietary regimen, and the particularities of the animal species under consideration [43]. The effects of EOBs on CH_4, CO_2, HN_3, and H_2S in this study were not significant.

These results are consistent with the results of Castro-Montoya et al. [44], who reported that in vivo supplementation of some essential oils at a dose of 0.2 g/d in beef cattle did not affect daily methane emissions. This result differs from that of Staerfl [45], who reported that gallic bulb administration in vivo reduced methane emissions in lactating Brown Swiss cows. This might be because of the higher inclusion levels (150 mg/kg of the diet). The results in the present study showed that there were numerical decreases in CH_4 and CO_2 in the EOB5 and EOB3 treatments, while the EOB1 treatment reduced NH_3 and H_2S, which implied their potentials to mitigate GHG emissions. This might be due to the higher concentration of anise in these treatments, which has been proven to reduce methane when administered individually [46]. This could also imply that, despite the low volume (4 mL) used, the EOB5 treatment, which had a higher concentration of anise, can be very effective in reducing methane production.

Similarly, H_2S emissions were reduced in all EOB groups when compared to the control, with the EOB2 group as the only exception. This is supported by Shah et al. [47], who stated that, at a rumen pH of 6.5, the production of H_2S gas is deemed to be favorable. As indicated earlier, all of the EOBs had a pH greater than 6.5, hence the reason for a lower H_2S emission. The substantial decrease in H_2S emissions in the EOB1 group by 56.8% as compared to the control is especially noteworthy, as a high concentration of H_2S in the rumen can lead to polioencephalomalacia in ruminants [47].

4.4. Effect of EOBs on Rumen Metabolites

Comparing the rumen metabolites of the CON and EOB1 obtained from the volcano plot analysis, the relative concentrations of glyceraldehyde, N-acetyl-beta-alanine, L-threonine/L-allothreonine, and hydroxyproline were increased in the EOB1 group. Glyceraldehyde, one of the first three intermediates in the glycolytic pathway, is a key player in converting glucose to pyruvate and eventually generating energy (ATP and NADH). Through glycolysis, glucose becomes two molecules of glyceraldehyde-3-phosphate (G3P), leading to energy production. G3P is a precursor in biosynthetic pathways (nucleotides,

amino acids, lipids, and glycogen) essential for cell growth and function [48,49]. N-acetyl-beta-alanine is a product of the beta-alanine metabolism, which is involved in the synthesis of carnosine, predominantly found in the muscles [50], and has been reported to exhibit antioxidant activities in several animal models [51]. Threonine is an essential amino acid [52] which is involved in the formation of proteins, including collagen, elastin, etc., and is a precursor for the synthesis of isoleucine and methionine. An optimal threonine concentration in animals promotes their growth and aids immune functions [53]. Hydroxyproline is an amino acid derivative that is abundant in collagen. This is the main structural protein in connective tissues, including the skin, tendons, and bones [54]. The addition of trans-4-hydroxy-L-proline in diets for humans and animals has been found to improve intestinal, joint, bone, and skin health [55]. The decrease in the concentration of 2,5-dihydroxypyridine, which is involved in thymidine synthesis, in the EOB1 group could have been independently influenced by factors like specific regulatory mechanisms, the activation of alternate pathways, or metabolic flux redistribution based on cellular demands in the EOB1 combination.

The results of the pathway enrichment analysis revealed that the propanoate metabolism, selenocompound metabolism, steroid hormone biosynthesis, glycolysis/gluconeogenesis, pyruvate metabolism, and pyrimidine metabolism pathways were all affected in the EOB1 group. The process by which propionic acid is broken down to produce propionyl-CoA is known as the propanoate metabolism. Moreover, hepatic gluconeogenesis utilizes propionic acid as a substrate via its conversion to succinyl-CoA [56]. The enrichment of the glycolysis/gluconeogenesis pathways may be a result of the action of glyceraldehyde and threonine, which were increased. The carbon released from the breakdown of threonine provides the building blocks for the synthesis of glucose [52]. Pyruvate is a key intermediate in the glycolytic pathway and its metabolism is crucial to the generation of energy [57]. The enrichment of these pathways in the EOB1 group suggests improved energy metabolism and utilization, which can support better growth performance and health compared to the CON group. Together, the enriched pathways and the metabolites in the EOB1 group suggest an improved energy metabolic efficiency, the successful elimination of oxidative stress in the digestive system, which promotes strong bone health, and a general boost to the immune system of the animals.

The volcano plot analysis showed that the EOB2 group led to nine differentially abundant metabolites. N(6)-methyllysine, a derivative of lysine methylation, was differentially increased in the EOB2 group when compared to the CON. DNA repair, transcription, and replication are all affected through the regulation of effector molecules by lysine methylation [58]. Sidney et al. [59] reported a lower level of N(6)-methyllysine in the stomach fluid of less-efficiently fed cattle compared to more efficiently fed ones, and this affected the growth performance. Lysine, as an essential amino acid, plays a significant role in tissue protein synthesis and the energy metabolism [60]. The study of Idowu et al. [23] identified high concentrations of 4-chlorolysine (a derivative of lysine) as a potential biomarker for residual feed intake in beef cattle. The high concentration of N(6)-methyllysine could result in tissue protein synthesis, including collagen synthesis, the production of antibodies for various immune responses, enzyme and hormone synthesis, and nitrogen balance, in the EOB2 group. Prunasin is a cyanogenic glycoside chemical compound found in the Prunus genus plant, including apricot kernels, almonds, and cherries. Anise, clove, oregano, and peppermint are not known to contain prunasin. However, some bioactive compounds, including phenolic compounds, terpenoids, essential oils, and alkaloids, found in the herbs may have interacted with certain enzymes and influenced the metabolic pathways involved in the synthesis of secondary metabolites such as prunasin [61–63]. Gallic acid is a bioactive phenolic compound found in plants. It has been found to possess antioxidant, anti-inflammatory, antimicrobial, and anticancer effects [64,65]. Gallic acid was, however, found to be differentially reduced in the EOB2 group. The effectiveness of the EOB2 treatment, concerning gallic acid, could have been influenced by the dosage used in the present study. The interactions between EOs and specific bioactive compounds, such as gallic

acid, can vary, and their effectiveness is dependent on factors such as the composition and dosage administered [66,67].

The degradation pathway of branched chain amino acids (BCAAs), including valine, leucine, and isoleucine, was enriched in the EOB2 group. These are essential amino acids and play significant roles in protein synthesis, glucose homeostasis, anti-obesity, and nutrient-sensitive signaling pathways [68,69]. The EOB2 combination may have initiated a series of enzymatic reactions involved in the degradation of these amino acids, which may also result in the synthesis of proteins, energy generation, and several other physiological processes linked with BCAAs in the EOB2 group. Together, the enriched pathways and the metabolites in the EOB2 group suggest increased tissue protein synthesis, improved energy production, and increased microbial activity in the animals. This was further confirmed by the increased VFA production in the EOB2 group.

Guanosine, isomer 1 of methionine sulfoxide, N-hydroxy-L-valine, N(gamma)-acetyldiaminobutyric acid, salidroside, and aminoacrylic acid were greater in the EOB3 group. Guanosine is a nucleoside belonging to the purinergic system and has been established to proffer several kinds of biological importance. Guanosine is involved in the synthesis of nucleic acids, including RNA and DNA [70]. Guanine nucleotides have been implicated in intracellular signaling via G-protein-coupled receptors, as they are involved in cellular hormonal and neurotransmitter signals, and also therapeutic targets [70]. Reports from studies have also revealed that guanosine-based nucleotides play significant roles in extracellular signaling activating molecular pathways, resulting in neuroprotection and development of the central nervous system [71,72]. The increase in the concentration of guanosine in the EOB3 group suggests improved intracellular signaling, neuroprotection, and the synthesis of purinergic nucleic acids in the animals. N-hydroxy-L-valine, a product of N-hydroxylation of L-valine (an essential amino acid), is found in certain plants and microorganisms [73]. The enzymes responsible for N-hydroxylation possess varying capabilities ranging from antibacterial activity to anti-inflammatory, anti-protease, and neuroprotective activities [73]. The result suggests that N-hydroxy-L-valine may have contributed to tissue protein synthesis, increased antimicrobial activity, reduced inflammation, and protection of the CNS in the EOB3 animals. The low concentration of homogentisic acid of the EOB3 group probably implies that the animals possess the homogentisate 1,2-dioxygenase enzyme, resulting in the normal metabolism of tyrosine amino acid [74].

The arginine biosynthesis and nicotinate and nicotinamide metabolism pathways were found to be enriched in the EOB3 group. The immunological system and ammonia detoxification are two metabolic processes in which arginine is involved. It acts to maintain the host's survival by playing an effective role in the prevention and treatment of diseases [75]. The arginine biosynthesis pathway exhibits antimicrobial and biocidal activities [76]. Nicotinate and nicotinamide are components of vitamin B12, which have been linked to cellular respiration, the energy metabolism, and redox reactions, including the synthesis of nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate [77,78]. NAD+ is essential to cell and organismal function and its level reduces with age. A study by Elhassan et al. [79] reported that improving NAD+ availability via nicotinamide results in the improved skeletal muscles of aged humans. These findings suggest that animals in the EOB3 group may exhibit robust skeletal muscle development as they age, potentially enhancing their disease resilience. This resilience can be attributed to the role of arginine in supporting overall survival.

The EOB4 group had 12 differentially abundant metabolites, with higher levels of estradiol-17beta/17alpha and nopaline. The more active form of estradiol (estradiol-17beta) can be found in the brain and is a strong neuroprotective agent. Its presence could proffer neuroprotection to the animals administered the EOB4 treatment. The study by Wang et al. [80] revealed that astrocyte-derived 17β-estradiol is involved in the regulation of reactive astrogliosis, microglial activation, and neuroprotection after cerebral ischemia in mice. This is made possible by the upregulation of aromatase in astrocytes after a brain injury. Nopaline is an opine found in the tissues of certain plants infected with *Agrobacterium*

tumefaciens and causes crown gall disease in the plants [81]. EOs are known to impact rumen microbial activity and overall animal health. The bioactive compounds present in these oils may influence the rumen microbial population, resulting in an increased concentration of nopaline, which is usually associated with the plant–bacteria interaction [82,83]. The enrichment pathway analysis revealed that the EOB4 group enriched the tyrosine metabolism and ubiquinone and other terpenoid–quinone biosynthesis pathways. The metabolism of tyrosine (an aromatic amino acid) results in the generation of neurotransmitters, including dopamine, epinephrine, and norepinephrine, and compounds for hormonal regulation (thyroid) and pigmentation (melanin) [84]. The biosynthesis of ubiquinone and other terpenoid–quinone pathways play significant roles in cellular respiration, antioxidant defense, and cellular signaling. The enrichment of pathways involved in these processes suggests a potential enhancement, which could lead to improved overall cellular function and health in the EOB4 group [85].

The EOB5 group displayed 26 differentially abundant metabolites. However, most of the metabolites expressed in the EOB5 group were also expressed in the groups above, including elevated levels of valine, nopaline, and leucine, and reduced levels of gallic acid and 2,5-dihydroxypyridine metabolites. The enrichment pathway analysis revealed that the EOB5 group enriched the starch and sucrose metabolism and fructose and mannose metabolism pathways. These pathways are involved in energy generation [86], further suggesting that the EOB5 treatment could result in improved energy production in the animals.

Derivatives of benzoic acid (2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoic acid and 4-hydroxy-3-methylbenzoic acid) and tabtoxinine-beta-lactam all had increased concentrations in the EOB4 group when compared to the EOB3 group. Benzoic acid (BA) belongs to the aromatic carboxylic acid family that is naturally found in plants and animal tissues, and which is often produced by microorganisms [87]. BA and its derivatives are generally used as food preservatives and feed supplements, resulting from their antimicrobial (antifungal and antibacterial) activities [87,88]. BAs are known to improve the growth and health of animals, especially by improving their gut functions. This is achieved by the inhibition of *E. coli* challenge, as well as promoting the production and activation of digestive enzymes, and facilitating nutrient absorption in the intestine [89,90]. The increase in the concentration of these metabolites in animals in the EOB4 group implies that the EOB4 treatment is rich in BA and the animals in this group experienced better gut function compared to those in the EOB3 group. Tabtoxinine-beta-lactam (TBL), a phytotoxin produced by certain strains of *Pseudomonas syringae*, is thought to play a role in inhibiting the biosynthesis of amino acids, including glutamine [91]. Tabtoxinine-delta-lactam (TDL) differs from TBL by just the lactam ring, and information on TDL is sparse. However, if they both were to present with similar functions, TDL might have played a role in preventing the activation of the glutamine biosynthetic pathway in the EOB4 group via the inhibition of the enzyme (glutamine synthetase) involved in the processes. Deoxyuridine and thymidine are deoxyribose sugars utilized in the synthesis of DNA. The increased concentration of deoxyuridine and thymidine in the EOB3 group may be attributed to the need for cellular requirements for proliferation or DNA damage repair. This increased concentration may contribute to the growth of animals, tissue regeneration, and an enhanced ability to respond to environmental stresses [92].

The terpenoid backbone biosynthesis pathway that was enriched in the EOB4 group is responsible for the synthesis of various backbones for terpenoid molecules [93]. Terpenoids, as the predominant compounds in many natural products, play significant roles in both the growth and development of plants, as well as in shaping their responses to environmental and physiological conditions [94]. They have also been found to exhibit neuroprotective abilities [95]. The enrichment of this pathway suggests that animals in the EOB4 group may possess better immunity against various environmental and physiological stressors, and experience better growth. O-phosphoethanolamine (PEA), a derivative of ethanolamine, plays a significant role in the cellular membrane structure and function, as it

is a major player in the synthesis of phospholipids [96]. The increase in the concentration of PEA in the EOB5 group suggests an improved structural integrity and fluidity of the cell membranes in the animals. Moreover, 5-hydroxykynurenine is a metabolite in the tryptophan degradation pathway. It is a key intermediate in the kynurenine pathway [97], and the kynurenine pathway has been implicated in various central nervous system diseases, including Alzheimer's disease, Parkinson's disease, and Huntington's disease [98]. The increase in the rumen concentration of the 5-hydroxykynurenine of animals in the EOB5 group reflects variations in the activity of enzymes involved in the kynurenine pathway, which may result in varying impacts on the neurobiology or oxidative balance of the animals. The increase in the rumen concentration of ethylene glycol (EG) in the animals in the EOB3 group raises concerns about toxicity in these animals, as EG is toxic to animals and humans even in low quantities. The metabolism of EG can generate metabolites that are toxic to certain organs in the animals, including the kidneys and liver [99]. Additionally, p-coumaroyl quinic acid, a phenolic acid compound found in plants, contributes to a plant's defense mechanism by increasing its antioxidant properties [100,101]. The result suggests that p-coumaroyl quinic acid may have contributed to the antioxidant properties of the animals and reduced inflammation in the EOB3 animals.

The pyrimidine metabolism, the biosynthesis and degradation of BCAAs, including valine, leucine, and isoleucine, the sphingolipid metabolism, and lysine degradation pathways were enriched in the EOB5 group. The EOB5 treatment may have impacted the regulation of several metabolic processes or significantly affected the enzyme activity of enzymes involved in these pathways more than the EOB3 group, as indicated by the enrichment in these pathways. The pyrimidine metabolism results in the synthesis, breakdown, and utilization of nitrogenous bases (cytosine, thymine, and uracil), which are essential components of nucleic acids (DNA and RNA) [102]. This process is critical in the maintenance of pyrimidine nucleotides' cellular pool, required for DNA and RNA synthesis and other fundamental cellular functions, including DNA replication and repair, and protein synthesis [103].

The enrichment of the biosynthesis and degradation pathways of the BCAAs in the rumen concentration of the animals administered the EOB5 treatment suggests an increased protein synthesis, energy production, or the generation of various precursors for other essential molecules [68,104]. Studies have also revealed the potential links between BCAAs and milk yield in dairy cows. The study of Xue et al. [105] on the relationship between the rumen microbiota, metabolites, and milk protein yield in dairy cows revealed the enrichment of the BCAA biosynthesis in high-milk-yielding cows, whereas BCAA degradation was enriched in low-milk-yielding cows. The enriched pathways of the sphingolipid metabolism and lysine degradation suggest an enhancement in cellular signaling, cell growth, membrane dynamics, and energy production within the EOB5 group. This enrichment suggests a potential positive impact of the EOB5 treatment on key cellular processes, contributing to improved cellular function and metabolism [106,107].

Comparing the volcano plot analyses of the EOB5 and EOB4 groups, 3-methyl-2-oxovaleric is a keto acid generated from the breakdown of BCAAs [108]. Increased concentrations of this acid can result in metabolic acidosis in the animals in the EOB5 group, thereby disrupting the normal pH balance [108]. As a result of this, the administration of the EOB5 treatment needs to be monitored. Additionally, N-(6-aminohexanoyl)-6-aminohexanoic acid is made up of two repeating units of 6-aminohexanoic acid (aminocaproic acid) linked together by an amide bond, and aminocaproic acid is a derivative of lysine. While lysine is involved in tissue protein synthesis and the energy metabolism, 6-aminohexanoic acid is a synthetic amino acid involved in the inhibition of fibrin breakdown, thereby improving blood clots and reducing excessive loss of blood [60,109]. The increase in this metabolite in the EOB5 group, when compared to those in the EOB4 group, may be beneficial to the animals, as it could increase tissue protein synthesis, contribute to energy generation, and potentially aid in preventing excessive bleeding due to injuries. Leukotriene A4 (LKA4) is an intermediate metabolite in the synthesis of leukotrienes [110]. Leukotrienes (LKA4, LKB4, LKC4, LKD4, and LKE4) play significant roles

in inflammation and immune responses. They are involved in recruiting leukocytes to sites of inflammation and have also been implicated in cancer and several neurological diseases [111]. The increase in the concentration of LKA4 in the EOB5 group suggest an increased immune and inflammatory activities in the animals.

2-Hydroxymuconate semialdehyde was higher in the EOB4 group, which is an intermediate metabolite in the degradation pathway of aromatic compounds. It is generated from the action of catechol-2,3-dioxigenase and can be utilized as a carbon source for microbial growth [112,113]. The prevalence of 2-Hydroxymuconate semialdehyde in the rumen concentration suggests an increased microbial activity. Interestingly, the EOB4 group displayed a more substantial presence of this intermediate, suggesting an enhanced microbial activity in their rumen compared to the EOB5 group. The glycerophospholipid metabolism, BCAA metabolism, and taurine and hypotaurine metabolism pathways were found to be enriched in the EOB5 group. The glycerophospholipid metabolism results in the production of several glycerophospholipids, including phosphatidylcholine, phosphorethanoamine, phosphatidylserine, and phosphatidylinositol. These metabolites are involved in signal transduction, and cell structure and function [114,115]. Taurine and hypotaurine are two sulfur-containing amino acids, and the metabolism of these amino acids involves L-cysteine, which is also a sulfur-containing amino acid. L-cysteine is implicated in redox homeostasis by its function in the regulation of glutathione synthesis [116,117]. The BCAA metabolic pathways were also enriched when the EOB5 group was compared with the EOB3 group, as shown above. The enrichment of these aforementioned pathways and their associated metabolites in the EOB5 group implies that the EOB5 treatment enhanced the cellular structure and function, contributed to the antioxidant defense of the animals, and also promoted the tissue protein synthesis and energy production of the animals.

5. Conclusions

The results from the present study showed that the EOBs can confer several benefits to animal health and influence various aspects of the rumen metabolome. Metabolomic analysis revealed that the direct infusion of the EOBs significantly modified the rumen metabolite profiles and altered the molar proportions of isovalerate, valerate, and isobutyrate, indicating shifts in microbial activity and fermentation patterns. The EOB1, EOB4, and EOB5 groups showed metabolome changes suggestive of enhanced energy metabolism, as evidenced by the increases in the glyceraldehyde and threonine levels, and the enrichment of the energy production pathways. The EOB2 and EOB3 groups exhibited metabolite profiles are potentially supportive of tissue protein synthesis, with the EOB2 treatment increasing the levels of N(6)-methyllysine. Furthermore, the EOB2, EOB4, and EOB5 treatments appeared to impact the microbial activity based on changes in the metabolites associated with the microbial metabolism. The EOB3 and EOB4 treatments showed potential neuroprotective properties, as indicated by the elevated levels of guanosine and estradiol-17beta/17alpha, respectively. These metabolomic findings suggest that the EOB1, EOB3, EOB4, and EOB5 treatments may influence energy production, protein synthesis, microbial activity, and neuroprotection, though direct effects on animal health were not measured.

These findings provide insights into the diverse metabolic effects of different EOBs on rumen function and the potential systemic impacts. However, further research is necessary to directly correlate these metabolic alterations with animal performance metrics and to investigate optimal EOB compositions for mitigating greenhouse gas emissions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ruminants4030024/s1, Figure S1: Figure showing data of metabolome of CON and EOB1 being normalized using Normalization by median, cube root transformation and auto scaling. Figure S2: PLS-DA scores plot of the metabolome of CON group and EOB1. Figure S3: OPLS-DA scores plot of the metabolome of CON group and EOB1. Figure S4: Figure showing the permutation test statistics of the metabolome of the CON group and EOB1 group at $p = 0.61$. Figure S5: PLS-DA scores plot of the metabolome of CON group and EOB2 group. Figure S6: OPLS-DA scores

plot of the metabolome of CON group and EOB2 group. Figure S7: Figure showing the permutation test statistics of the metabolome of the CON group and EOB2 group at $p = 0.78$. Figure S8: PLS-DA scores plot of the metabolome of CON group and EOB3 group. Figure S9: OPLS-DA scores plot of the metabolome of CON group and EOB3 group. Figure S10: Figure showing the permutation test statistics of the metabolome of the CON group and EOB3 group at $p = 0.82$. Figure S11: PLS-DA scores plot of the metabolome of CON group and EOB4 group. Figure S12: OPLS-DA scores plot of the metabolome of CON group and EOB4 group. Figure S13: Figure showing the permutation test statistics of the metabolome of the CON group and EOB4 group at $p = 0.63$. Figure S14: PLS-DA scores plot of the metabolome of CON group and EOB5 group. Figure S15: OPLS-DA scores plot of the metabolome of CON group and EOB5 group. Figure S16: Figure showing the permutation test statistics of the metabolome of the CON group and EOB5 group at $p = 0.44$. Figure S17: PLS-DA scores plot of the metabolome of EOB4 group and EOB3. Figure S18: OPLS-DA scores plot of the metabolome of EOB4 group and EOB3 group. Figure S19: Figure showing the permutation test statistics of the metabolome of the EOB4 group and EOB3 group at $p = 0.92$. Figure S20: PLS-DA scores plot of the metabolome of EOB5 group and EOB3 group. Figure S21: OPLS-DA scores plot of the metabolome of EOB5 group and EOB3 group. Figure S22: Figure showing the permutation test statistics of the metabolome of the EOB5 group and EOB3 group at $p = 0.55$. Figure S23: PLS-DA scores plot of the metabolome of EOB5 group and EOB4 group. Figure S24: OPLS-DA scores plot of the metabolome of EOB5 group and EOB4 group. Figure S25: Figure showing the permutation test statistics of the metabolome of the EOB5 group and EOB4 group at $p = 0.59$.

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