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RESEARCH ARTICLE

Dietary β-hydroxybutyrate sodium alters rumen microbiome and nutrient metabolism in the rumen epithelium of young goats

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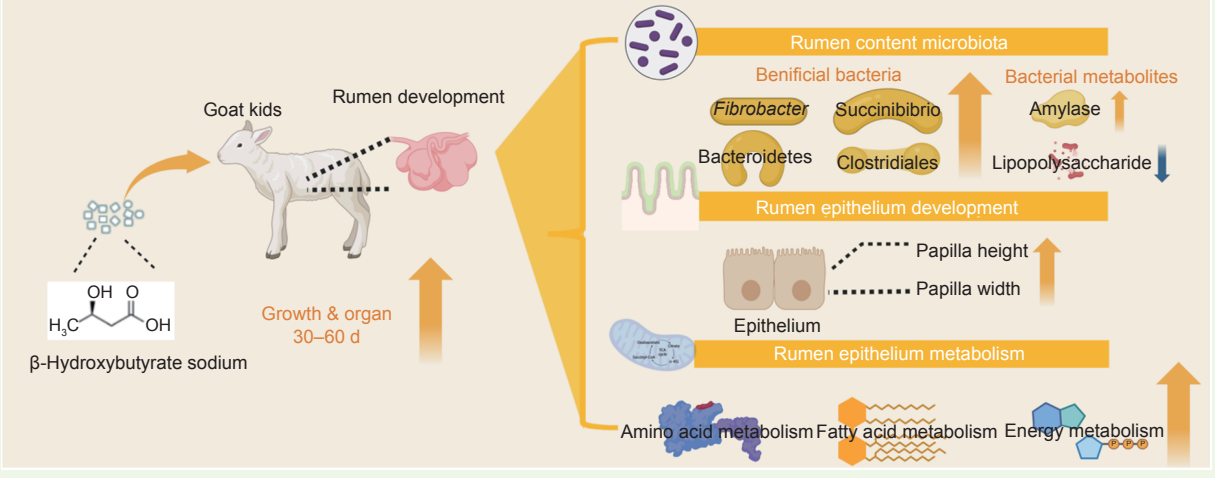
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Highlights

- β-Hydroxybutyric acid (BHBA) -Na promoted the growth performance and rumen development of young goats.
- BHBA-Na increased the abundance of beneficial bacteria in the rumen.
- BHBA-Na regulated the metabolisms of VFA and amino acid in the rumen epithelium positively.

Graphical abstract



Abstract

The role of β-hydroxybutyric acid (BHBA) includes providing energy, regulating signaling pathways, and ameliorating the gut microbiota in the host, while its nutrient mechanism to improve rumen epithelium development in young ruminants is still unclear. In this study, a total of 12 female Haimen goats with 30 d of age were chosen and divided into two groups. One group was fed with basic diet (CON), and the other group was fed a basal diet supplemented with 6 g d^{-1} dietary

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β-hydroxybutyrate sodium (BHBA-Na). The experimental period was 30 d, and all goats were slaughtered at 60 d of age. The joint analysis of multi-omics, including rumen microbiota, rumen epithelial transcriptome and rumen epithelial metabolomics in young goat model, was performed to systematically investigate the effect of dietary BHBA-Na on rumen development in young goats. As the results, we found that dietary BHBA-Na improved the growth performance of young goat including body weight, average daily gain (ADG) and dry matter intake (DMI) (P<0.05). Dietary BHBA-Na also increased the weight of rumen, and promoted the growth of rumen epithelium development (P<0.05). The abundance of several beneficial bacteria was increased (*Fibrobacter*, *Succinivibrio*, *Clostridiales*, etc.). The rumen epithelium transcriptome and metabolomics indicated that BHBA-Na supplementation showed a remarkable effect on the nutrient metabolism of the rumen epithelium. Specifically, the pathways of "fatty acid metabolism", "cholesterol homeostasis", "reactive oxygen species (ROS) pathway" and "peroxisome" were activated in response to BHBA-Na addition (P<0.05). Moreover, the genes (*HMGCS2*, *ECSH1*, *ACAA2*, *ECH1*, *ACADS* etc.) and metabolites (succinic acid, alpha-ketoisovaleric acid, etc.) involved in these pathways were also regulated positively (P<0.05). The rumen epithelium obtained the energy for its development from the process of volatile fatty acids (VFAs) decomposition. Finally, we observed the close correlations among the phenotypes, ruminal microbiota, host genes and epithelial metabolites. Overall, our results revealed that the BHBA-Na promoted the growth and rumen development of young goats possibly by enhancing DMI and regulating the rumen microbiota and the metabolisms of VFA and amino acid in the rumen epithelium.

Keywords: goats, β-hydroxybutyrate acid, rumen microbiota, rumen transcriptome, rumen epithelial metabolomic, rumen epithelium development

1. Introduction

Ruminants, one of the most common and critical livestock in the world, provide high quality proteins from meat and milk for human beings. With the increases of the needs of high-quality food, greater production efficiency of ruminants is necessary (Broderick 2018). Rumen, the unique and important digestive organs, is colonizing various microbiotas facilitating the host's digestion and utilization of nutrients (Zhang *et al*. 2021; Chen *et al*. 2022). This process culminates in the production of significant volumes of VFAs, which serves as a crucial energy source for the host's growth and development (Huang W Q *et al.* 2022). Thus, rumen is a unique and essential organ of ruminants and closely related to the development and productivity of ruminants.

As one of the major energy sources in rumen, β-hydroxybutyrate acid (BHBA) exerts a crucial role in regulating energy metabolism of host. Numerous studies showed that increased BHBA can improve reproductive performance by ameliorate metabolic stress conditions caused by negative energy balance (Missio *et al*. 2022). As the metabolic substrate of TCA cycle, BHBA can reduce NADP/NADPH ratio and promote the expression of reactive oxygen species (ROS) detoxification genes such as metallothionine, thus delaying cell aging (Sharma and Ramanathan 2020). In addition, Oxidative metabolism of BHBA in microglia cells can increase NADH/NAD ratio, enhance mitochondrial respiratory function, and promote glycolysis and lactic acid output (Achanta and Rae 2017; Benito *et al.* 2020). BHBA also acts as a precursor of milk fat in dairy cows to improve the milk quality (Hostens *et al.* 2011) and it can even alleviate excessive negative energy balance in postpartum in dairy cows (Sun *et al.* 2020; Islam *et al.* 2022). In addition to being an energy carrier, for juvenile ruminants, BHBA is an indispensable stimulus for the growth of gut epithelium (Deelen *et al.* 2016). In the host, BHBA also acts as a regulator of cell signals, which can inhibit or activate various signaling pathways and regulate gene expression (Han *et al.* 2020). It has been found that BHBA alleviates colon inflammation by promoting polarization of M2 macrophages through a STAT6-dependent signaling pathway (Huang C *et al.* 2022). BHBA inhibits the proliferation of colonic crypt cells and effectively inhibits the growth of intestinal tumors by acting on the surface receptor Hcar2 and inducing the transcription regulatory factor Hopx (Dmitrieva-Posocco *et al.* 2022). BHBA can inhibit the damage of midgut intestinal stem cells and enterocytes induced by age and oxidative stress to maintain stem cell homeostasis (Park and Kim 2020). Recently, Kim's team reported that supplementation of BHBA alleviates tumor necrosis factor-alpha (TNF-α) induced intestinal epithelial cell apoptosis and helps to prevent intestinal inflammation (Broderick 2018). Previous study have also confirmed that supplementation of BHBA and overexpression of *HMGCS2* can promote intestinal cell differentiation, regulate and maintain intestinal epithelial homeostasis (Wang *et al.* 2017).

Nowadays, some studies have confirmed dietary BHBA could have the ability to change the microbiota in host. As reported, exogenous BHBA can reduce the level of intestinal proinflammatory Th17 cells and selectively inhibit the growth of *Bifidobacterium* (Ang *et al.* 2020). Similarly, in children with severe epilepsy, the addition of BHBA was observed to be associated with a decrease in the abundance of *Bifidobacterium* and an increase in *E. coli* (Lindefeldt *et al.* 2019). Also, metagenomics showed that intravenous injection of BHBA could change the microbial diversity into *Clostridium botulinum* of the respiratory tract and increase the risk of respiratory diseases in yaks (Qi *et al.* 2020). In Alzheimer's patients, as the concentration of BHBA in plasma rises, there is an observed increase in the abundance of Christensenellaceae, applied alongside a decrease in the abundance of *Bifidobacterium* and *Lachnobacterium* (Nagpal *et al.* 2019). These above studies suggested that BHBA could change the gut microbial community and stimulate the

proliferation and metabolism of cells. Besides, BHBA exerted important roles in the host being an energy metabolite, signaling molecule and epigenetic regulatory factor (Wang *et al.* 2021). Our previous study has demonstrated that BHBA significantly improved the growth performance of post-weaning goats (Abdelsattar *et al.* 2022a). However, whether and how BHBA affects rumen microbiome in pre-weaned ruminants is unclear. In addition, understanding of the BHBA impacts on rumen gene expression and metabolism as well as even after rumen is urgent.

In this study, we recorded integral data of growth performance in young goats with β-hydroxybutyrate sodium (BHBA-Na) treatment and further applied multi-omics technologies (rumen 16S rRNA gene sequencing, rumen epithelial transcriptome and metabolomics) to do the comprehensive analysis of the effects of BHBA-Na on the rumen of young goats at the level of metabolic mechanism. In this study, we hypothesized that BHBA-Na could promote rumen development of young goats by regulating rumen microbiota and nutrient metabolism in the rumen epithelium. This comprehensive study provided insights into the potential use of BHBA-Na to improve ruminant growth performance and health.

2. Materials and methods

2.1. Animals trial and sample collection

A total of 12 female Haimen goats, aged 30 d and with an average body weight of (5.48±0.30) kg, were chosen from a commercial goat farm located in Haimen, Jiangsu, China. The additive of BHBA was selected as the chelate form of BHBA-Na with the purity of 99% purity (Wuhan Huajiu Pharmaceutical Technology Co., Ltd., China). The goats were randomly assigned into two groups (n=6) which were either supplied dietary BHBA-Na or not (CON *vs.* BHBA-Na group). BHBA-Na supplement amount was set as 6 g d^{-1} per goat by referring to our pre-experiment and previous study (Zhuang *et al.* 2023). Prior to the study (from 0 to 30 d), the young goats were raised alongside their dams and solely nourished with milk. On 30 d of age, all goat kids were gradually weaned and transitioned to a milk replacer (Patent number: ZL201210365927.6) and solid starter feed (nutrient composition shown in Tables 1 and 2). The diet was prepared according to the basic diet nutrition level as per the feeding standard of NY/T816–2004 feeding standard of meat-producing sheep and goats (Ministry of Agriculture of the People's Republic of China, 2004). The goats lived in an environment with a controlled temperature of (28±2)°C and a relative humidity of 60±5% and were housed in separate pens (one goat per pen). Each goat kid was fed milk replacer triple times daily and offered 300 mL at each time (the ratio of milk replacer to water is 1:5) from 30 to 60 d of age. During the experiment, young goats had free access to water and starters. In the morning, 50 g of starter feed was thoroughly mixed with BHBA-Na to ensure complete consumption by the young goats. At the end of this experiment (60 d of age), the 6 young goats in each group were fasted 12 h before slaughter. Animals were weighed before slaughter and rumen weight were also recorded after slaughter. Rumen content samples were collected for microbial analysis, while rumen tissue from the ventral sac were obtained and preserved for morphological, transcriptomic, and metabolomic analyses.

Table 1 Nutritional composition of milk replacer (%, DM basis)[1)]

Item	Milk replacer
DM	95.50
CP	25.52
EE	15.96
NDF	–
ADF	–
NFC	51.98
Ash	6.54
Ca	1.02
P	0.66

[1)] DM, dry matter; CP,crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; NFC, non-fiber carbohydrates; Ash, crude ash. NFC=100–(NDF+CP+EE+Ash).

Table 2 Ingredients and nutritional composition of starter (%, DM basis)[1)]

Item	Starter
Ingredients	
Corn	50.0
Soybean meal	25.0
Corn husk	10.0
Wheat bran	10.0
Premix	2.5
Salt	0.5
Lime stone	1.0
Calcium phosphate	1.0
Chemical composition	
DM	95.25
CP	19.63
EE	3.40
NDF	29.62
ADF	8.64
NFC	39.36
Ash	7.99
Ca	0.95
P	0.70

[1)] DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; NFC, non-fiber carbohydrates; Ash, crude ash. NFC=100–(NDF+CP+EE+Ash). Provided per kilogram of diet: vitamin A, 12,000 IU; vitamin D3, 2,000 IU; vitamin E, 30 IU; Cu ($CuSO_4$), 12 mg; Fe ($FeSO_4$), 64 mg; Mn ($MnSO_4$), 56 mg; Zn ($ZnSO_4$), 60 mg; I (KI), 1.2 mg; Co ($CoCl_2$), 0.4 mg; Se (Na_2SeO_3), 0.04 mg.

2.2. Analysis of nutrient composition of starter

The starter was milled to achieve a particle size suitable for passing through a 1 mm sieve, and then transferred to an oven at 135°C for 2 h (method 930.15; AOAC, 1990) to measure the dry matter (DM) content. Moreover, crude protein (CP), ether extract (EE) and crude ash (ASH) were measured based on previous methods (Abdelsattar *et al.* 2022a). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured with the ANKOM fiber analyzer (A2000i; American ANKOM, Macedon, NY, USA) followed by Van Soest *et al.* (Van Soest *et al.* 1991). The calcium, and total phosphorus were determined using spectrometry (ZA3000, Polarized Zeeman Atomic Absorption Spectrophotometer, Hitachi High-Tech Atomic Absorption

Scientific Solutions Co., Ltd., Tokyo, Japan).

2.3. Analysis of rumen metabolites

The analysis of rumen BHBA, LPS and α-AMY concentrations were assessed according to a previously documented procedure by Abdelsattar *et al.* (2022b). Initially, samples were extracted using 1 mL of ice-cold methanol (80% v/v, 0.1% formic acid] and subsequently homogenized. Following centrifugation, the supernatants underwent vacuum drying and were reconstituted in 100 mL of methanol (3% v/v)). Quantitative analysis was executed using an LC-MS system controlled by Xcalibur 2.2 software provided by Thermo Fisher Scientific, Waltham, MA. The targeted analytes were quantified and normalized utilizing a 13C stable isotope-labeled internal standard sourced from Cambridge Isotope Laboratories, Inc., Tewksbury, MA. Finally, concentrations were determined *via* a standard curve spanning from 0.1 to 50 mmol L^{-1}.

For the determination of VFA concentrations in the rumen, samples containing metaphosphoric acid were thawed at room temperature and subsequently centrifuged at 12,000×g for 15 min at 4°C. The supernatant was injected into a gas chromatography (GC) system. The GC apparatus utilized methyl valerate as the internal standard and operated on an Agilent 6890 series platform equipped with a capillary column (HPFFAP19095F-123, 30 m length, 0.53 mm diameter, and 1mm thickness, manufactured by Agilent Technologies, Santa Clara, CA, USA).

2.4. Morphology measurement

A 2 cm×2 cm section of rumen epithelial tissue from ventral sac of rumen within each goat kid was collected, and after that, the samples were promptly rinsed with physiological saline and then immersed in a 250 mL jar filled with 10% neutral formalin solution for fixation. The rumen epithelial tissue samples underwent dehydration using varying concentrations of ethanol, followed by embedding in paraffin and cutting into 6 μm sections. Subsequently, the rumen papilla structure was examined under a light microscope at a magnification of 4×10 (Olympus BX-51; Olympus Corporation, Tokyo, Japan) after staining with Yihong-hematoxylin (H.E.). The Image-Pro Express Image Analysis Processing System (Image-Pro Plus 6.0, Media Cybernetics, Silver Spring, MD, USA) was utilized to assess rumen papilla height, papilla width, lamina propria thickness, and epithelial thickness.

2.5. Rumen microbial sequencing and bioinformatics

A DNeasy PowerSoil Kit (cat. no. 12888, Qiagen, Valencia, CA, USA) was used to extract total genomic DNA from collected rumen samples. The concentration and quality of DNA were measured using a Thermo NanoDrop 2,000 UV microphotometer (ThermoFisher Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis. The genomic DNA was diluted to the optimal concentration and used as a template for PCR process. The V3–V4 region was amplified using adaptor-linked universal primers (341F: CCTACGGGRSGCAGCAG; 806R GGACTACVVGGGTATCTAATC). Following manufacturer's instructions for a high-fidelity enzyme in the KAPA HiFi Hotstart ReadyMix PCR Kit (Kapa Biosystems, Wilmington, MA, USA), PCR was performed. Next, the AxyPrep DNA Gel Recovery Kit (AXYGEN Inc., Union City, CA, USA) was employed to cut the gel and obtain the PCR products. To prevent contamination from reagents, negative controls for DNA extraction and PCR amplification were included, and no PCR products of the negative controls were detected from agarose gel. Then, after making the sequencing library, its quality was checked using bioanalyzer (Bioanalyzer 2100, Agilent, Santa Clara, USA). The library was quantified using a Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, MA, USA). Subsequently, the prepared amplicon library was sequenced on an Illumina Miseq PE250 platform (Realbio Technology Genomics Institute, Shanghai, China).

QIIME2 platform (2021.4 release) was applied to analysis the raw data of sequencing (Bolyen *et al.* 2019). The Deblur method was chosen to handle the demultiplexed sequences with default parameters setting including the steps of paired reads merging, barcode and length trimming, quality filtering, denoising (Deblur), classification (Greengenes reference database; 13–8 version; 99% similarity), and sequence clustering. After removing the chimeric sequences and singletons with the official pipeline, the amplicon sequence variants (ASV) were clustered based on 100% identity.

2.6. Transcriptomics of rumen epithelium and data processing

RNA extraction from the rumen epithelial tissue was conducted using TRIzol reagent (Invitrogen, CA, USA) following the manufacturer's instructions. The concentration and quality of the RNA were assessed by measuring the absorbance optical density ratio (OD260/280) using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Madison, WI), ensuring values fell within the range of 1.80 to 2.10. Additionally, RNA integrity was confirmed by electrophoresis on a 1.4% agarose-formaldehyde gel.

DNase I was utilized to eliminate both double-stranded and single-stranded DNA contaminants from all RNA samples, followed by purification of mRNA molecules from total RNA using oligo(dT)-attached magnetic beads. Following to fragmentation of the mRNA into small pieces using a fragmentation reagent, the first-strand cDNA was synthesized through random hexamer-primed reverse transcription, and succeeded by second-strand cDNA synthesis. The synthesized cDNA underwent end-repair and 3´ adenylation, after which adaptors were ligated to the ends of these 3´ adenylated cDNA fragments. The ligated products were then amplified by PCR using specific primers. Subsequently, the PCR product was thermally denatured into single strands, and the single-stranded DNA was cyclized with a single segment bridge primer to yield a single-strand cyclic DNA library, initially on a BGISEQ-500 platform (Beijing Genomics Institute, Shenzhen, China). A quality inspection of the constructed library was conducted, and sequencing was carried out following library qualification. The RNA library underwent sequencing at the Beijing Genomics Institute Co., Ltd. The Pacbio RS II sequencer (Pacific Biosciences,

CA, USA) was employed to obtain 150 bp paired end reads according to the manufacturer's instructions. Following quality control of the raw reads, clean reads were obtained, and HISAT2 (https://ccb.jhu.edu/software/hisat2/index.shtml) was utilized for alignment of the clean reads to the host genome. StringTie (version 1.3.4d) was used for mapping reads to elucidate the expression profiles of gene transcripts (Pertea *et al.* 2016). Subsequently, the fragments per kilobase of transcript per million fragments mapped (FPKM) method were employed to calculate the expression levels of genes (Trapnell *et al.* 2010). Differentially expressed genes (DEGs) were identified using the DESeq method, with threshold criteria set at a Q-value of <0.05. Only genes with detectable expression (FPKM>0.1 in each sample) were included for further analysis. KEGG pathway enrichment analysis was conducted using KOBAS (version 3.0, http://kobas.cbi.pku.edu.cn/kobas3). Using the KEGG Mapper, the dominant metabolic pathways enriched by high-abundance enzymes in the rumen epithelium were visualized.

2.7. Metabolites identification and quantification in rumen tissues

The metabolites in rumen epithelium tissue were analyzed using metabolomics technology. Each sample, weighing 100 mg, was ground using a SCIENTZ-48 tissue grinder (SCIENTZ, Ningbo, China). Subsequently, the grounded tissue was transferred into a 5 mL tube along with 2 mL of tissue extract fluids consisting of 75% methanol and 25% ddH_2O, and 3 steel beads. After sonication at room temperature for 30 min followed by chilling on ice for 1 h, the mixture was centrifuged at 6,000×g for 20 min at 4°C to separate the supernatant. The supernatant was then transferred into 2 mL centrifuge tubes and concentrated to dryness under vacuum. The residue was dissolved in 200 µL of a 50% acetonitrile solution containing 2-chlorobenzalanine, and the supernatant was filtered through a 0.22 µm membrane. Quality control (QC) samples were prepared by mixing equal volumes (20 µL) from each sample to calibrate the analysis for errors. The remaining sample was subjected to LC-MS detection using a Thermo Q Exactive Plus and Thermo Vanquish (both from Thermo Fisher Scientific, Waltham, MA) to detect metabolites.

2.8. Statistical analyses

In this study, one goat was the experimental unit. The phenotypes including growth performance, rumen epithelium morphological parameters and VFAs were compared between the two groups using *t*-test. Statistical significance was determined for results with a *P*-value less than 0.05. The Wilcoxon rank sum test was used to compare the alpha diversity (Chao1 and Shannon index), gene expression and metabolite abundance between the two groups. Beta diversity was assessed using Bray-Curtis distances, and an analysis of similarity (ANOSIM) was conducted. The diversity metrics were then visualized using the "ggplot2" package in R.

The linear discriminant analysis (LDA) effect size (LEfSe), an analytical tool for discovering and interpreting biomarkers of high-dimensional data, was used to identify the signature microbiota and metabolites differentiating three ages. LDA score>2 and *P* value<0.05 was used as a criterion for judging the significant effect size.

Gene set enrichment analysis (GSEA) (Subramanian *et al.* 2005) was performed to identify the critical gene modules related rumen epithelium development.

The network of genes was constructed by according to the STRING database (https://string-db.org/) (Szklarczyk *et al.* 2021) and visualized by Cytoscape (version 3.7.1, Bethesda, MD, USA). Hub genes in the network were further selected using the MCODE plugin in Cytoscape (version 2.0, http://apps.cytoscape.org/apps/mcode) with programmed parameters (degree cutoff: 2; K-Core: 2; and max depth: 100). The functional enrichment analysis of metabolites was conducted using MetaboAnalyst 5.0 platform (www.metaboanalyst.ca). The Spearman's analysis was conducted to assess correlations among features using the 'psych' package in R. The correlations showed in figures were depicted via a heatmap. The edges of network were set as significance with *P*<0.05 and |R|>0.5.

3. Result

3.1. Dietary BHBA-Na improved the growth performance and rumen development in young goats

The effect of dietary supplementation of BHBA-Na on the growth performance of young goat was shown in Table 3. The BHBA-Na group exhibited a significantly higher body weight and ADG compared to the CON group (*P*<0.05). Similarly, the DMI was significantly higher in the BHBA-Na group than that in the CON group (*P*<0.001). while no significant difference was observed in feed conversion ratio between the two groups. Simultaneously, the rumen weight was also improved in response to dietary BHBA-Na addition (*P*=0.017) (Fig. 1).

3.2. Dietary BHBA-Na altered the composition of rumen microbiota and fermentation

Rumen microbiota and their fermented products provided nutrition and energy for rumen epithelial metabolism, so as to promote rumen development and improve goat growth performance. Therefore, deep investigation of rumen microbiota and fermentation parameters affected by dietary BHBA-Na could implement the mechanism how BHBA-Na improved the developments of rumen. Using 16S rRNA

Table 3 Effects of BHBA-Na supplementation on the growth performance of young goats[1)]

Parameters[2)]	Groups		SEM	*P*-value
	CON (*n*=6)	BHBA-Na (*n*=6)		
Initial body weight (kg)	5.45	5.50	0.10	0.745
60 d body weight (kg)	7.42	8.36	0.18	0.004
ADG (g)	65.67	95.44	3.61	<0.001
DMI (g)	160.87	238.78	4.20	<0.001
Feed conversion ratio	2.49	2.51	0.21	0.851

[1)] BHBA, β-hydroxybutyric acid.
[2)] ADG, average daily gain; DMI, dry matter intake.

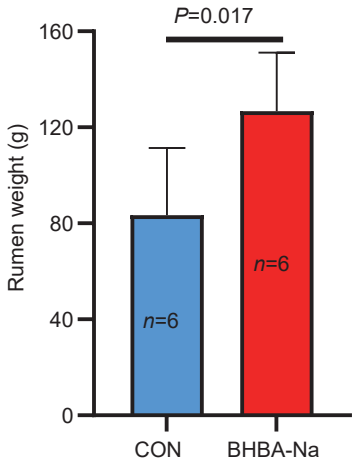


Fig. 1 comparison of rumen weight of young goats between the CON and β-hydroxybutyric acid (BHBA)-Na group. Data are mean±SE.

sequencing, a total of 35,548 high-quality reads at the single-nucleotide resolution were generated. After the rarefaction of sample reads to a minimum, a total of 1,085 ASVs were included for downstream analysis of the rumen microbial community. As shown in Fig. 2-A and B, there were no significant difference of microbial diversity (Chao1, Shannon and Simpson) and structure (Based on Bray-Curtis; ANOSIM R=0.013, P=0.344).

It is worth noting that BHBA-Na altered the rumen microbial compositions at different taxonomic levels. At the phylum level, the dominant bacteria were Proteobacteria, Firmicutes and Bacteroidetes in all groups. Compared with the CON group, BHBA-Na decreased the relative abundances of Bacteroidetes while increased the abundances of Firmicutes and Proteobacteria (Fig. 2-C). At the genus level, the top 20 bacterial genera in abundance were compared between the CON group and BHBA-Na group, with *Prevotella* being the predominant bacterium (Fig. 2-D). The results showed that BHBA-Na increased the relative abundance of *Clostridium* and *Succinivibrio*. In contrast, the abundance of *Ruminococcus* and *Selenomonas* decreased by dietary BHBA-Na.

To further identify the signature microbiota differentiating these two groups, LEfSe was performed at the ASV level (LDA>2.0, P<0.05) (Fig. 2-E). The result revealed that, in the BHBA-Na group, *Lachnospiraceae* (ASV28, ASV147), *Clostridiales* (ASV457), *Intestinimonas* (ASV1253), *Prevotellaceae* (ASV470), *Clostridium_IV* (ASV174), *Schwwartzia* (ASV254), *Bacteroidetes* (ASV1536), and *Fibrobacter* (ASV262) were identified as signature bacteria. *Olsenella* (ASV195), *Lachnospiraceae* (ASV32, ASV1615, ASV401), *Clostridiales* (ASV502), *Prevotellaceae* (ASV460), *Porphyromonadaceae* (ASV1453), *Prevotella* (ASV117), *Streptophyta* (ASV212), *Clostridium XIVa* (ASV148) and *Paraprevotella* (ASV144) were abundant in the CON group.

Moreover, the co-occurrence network of bacteria was constructed to reveal the relationship between the microbial community at genus level (Fig. 2-F and G). In the networks of both two groups, most of the connections were negatively correlated, indicating a general competitive relationship between bacteria. Furthermore, compared with the CON group, the BHBA-Na group had a simpler network with a lower number of degrees (41 *vs.* 51) and edges (70 *vs.* 165). The beneficial bacteria including genera *Butyrivibrio*, *Clostridium sensu stricto* and *Bifibacterium* were the central nodes connecting the other bacteria in the BHBA-Na group.

We then further analyzed the changes of microbial fermentation parameters. There were no significant differences in the main VFAs, including acetate, propionate, and butyrate between the CON and BHBA-Na group (Fig. 2-H). However, the BHBA concentration of ruminal content in the experimental group was significantly higher than that in the CON group (P=0.049). In addition, BHBA-Na also significantly increased the rumen α-amylase (α-AMY) activity (P=0.041) and decreased the concentration of lipopolysaccharide (LPS) (P=0.003) (Fig. 2-I).

3.3. Dietary BHBA-Na benefited rumen epithelial development and altered transcriptomics

As expected, dietary BHBA-Na was beneficial to the rumen epithelium development. In terms of epithelial morphology, a greater anatomical structure in the BHBA-Na group was observed (Fig. 3-A). Dietary BHBA-Na significantly increased the height and width of epithelial papilla (P<0.05) while no differences of the rumen muscle and epithelial thickness were found (Fig. 3-B).

We next sought to investigate the effects of dietary BHBA-Na on the transcriptomics in the rumen epithelium. The rumen epithelial transcriptome produced 58.44 Gb of clean data. The expression profiles of 12,143 genes were determined in all rumen epithelial tissue samples. We identified 51 DEGs (25 upregulated and 26 downregulated) between the two groups (Appendix A-a). Then, using KEGG enrichment analysis, our results revealed that these DEGs were mainly enriched in the pathways related to metabolism of amino acid, glucose and lipid, which included the pathways of “valine, leucine and isoleucine degradation”, “lysine degradation”, “pyruvate metabolism” and “propanoate metabolism” (Appendix A-b). Next, we performed gene set enrichment analysis (GSEA), a computational method used to determine whether a priori defined gene set displays a statistically significant and consistent difference between two biological states (e.g., phenotypes), to assign genes to gene sets based on their known function. We screened out four activated transcriptomic modules (P<0.05) in response to dietary BHBA-Na addition including “fatty acid metabolism”, “cholesterol homeostasis”, “reactive oxygen species (ROS) pathway” and “peroxisome” (Fig. 4-A), and the expression levels of related genes in these 4 modules were significantly increased (P<0.05) (Fig. 4-B). Then according to string database which contains direct physical binding and indirect function-related interactions between known and predicted genes (Szklarczyk *et al.* 2021), an interaction network of signature genes in these four metabolic modules was constructed to reveal the affinity between genes at the level of biochemical metabolism (Appendix B). Moreover, using the MCODE plugin in Cytoscape, a subnetwork was

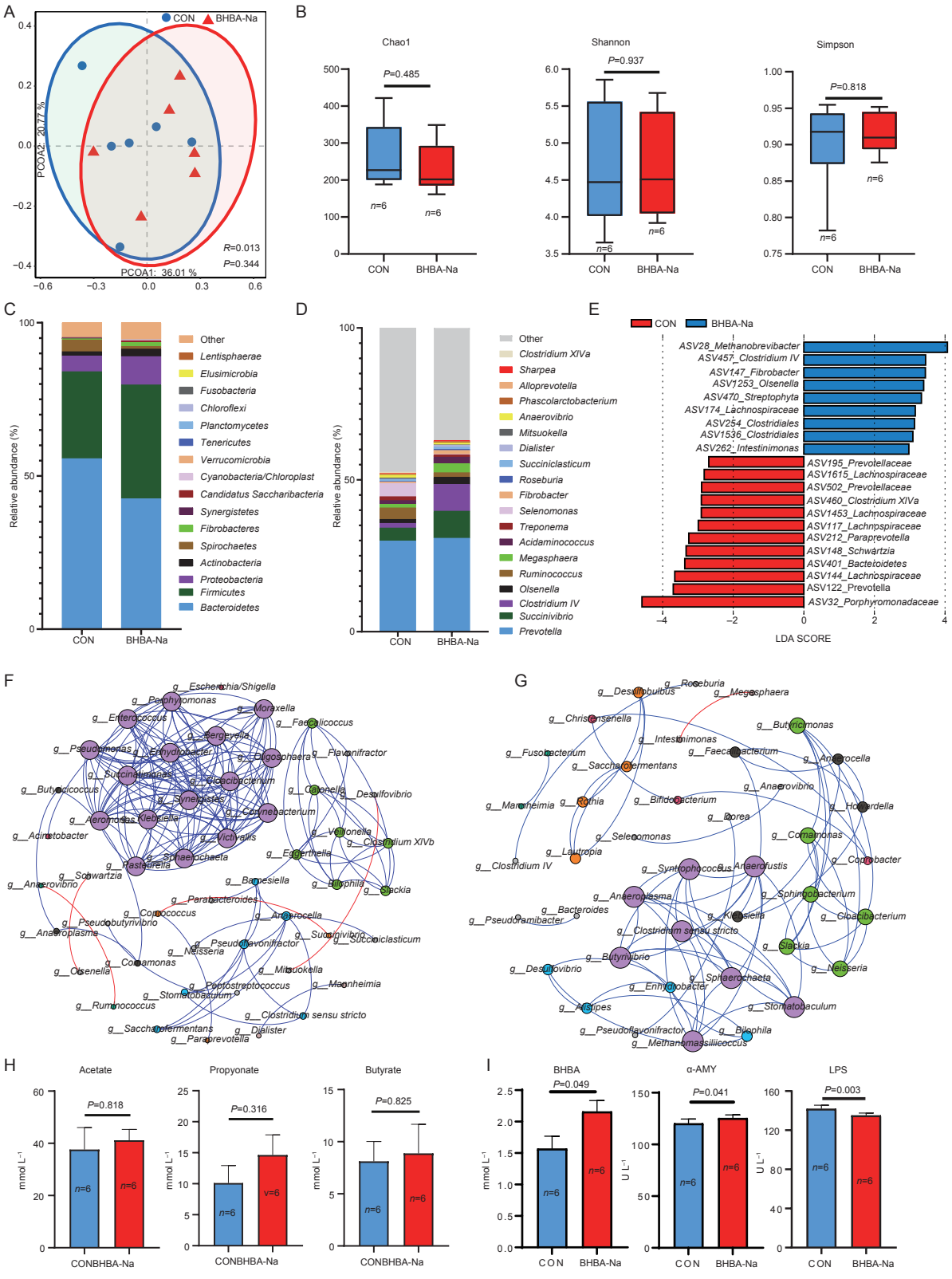


Fig. 2 Effects of dietary β-hydroxybutyric acid (BHBA)-Na on the microbial community and metabolites in the rumen of young goats. A, PCoA based on Bray-Curtis distance between rumen microbiota of CON and BHBA-Na group. B, microbial diversity and richness in rumen fluid samples of young goats between the CON and BHBA-Na groups. The five horizontal lines in the box plots represented the upper limit, the upper quartile, the median, the lower quartile and the lower limit. C, relative abundance of the main phyla of rumen bacteria between the CON and BHBA-Na groups. D, relative abundance of the top 20 genera of rumen bacteria between the CON and BHBA-Na groups. E, identification of signature taxa at the ASV level according to LEfSe analysis. F, co-occurrence network of bacteria in the CON groups. G, co-occurrence network of bacteria in the BHBA-Na groups. The size of nodes represented the degree of connection, and the color of the line represented the positive (red) or negative correlation (blue). H, concentrations of acetate, propionate and butyrate. I, the average concentrations of BHBA, α-AMY and LPS in the rumen. The error pounds in the charts were represented by mean±SE.

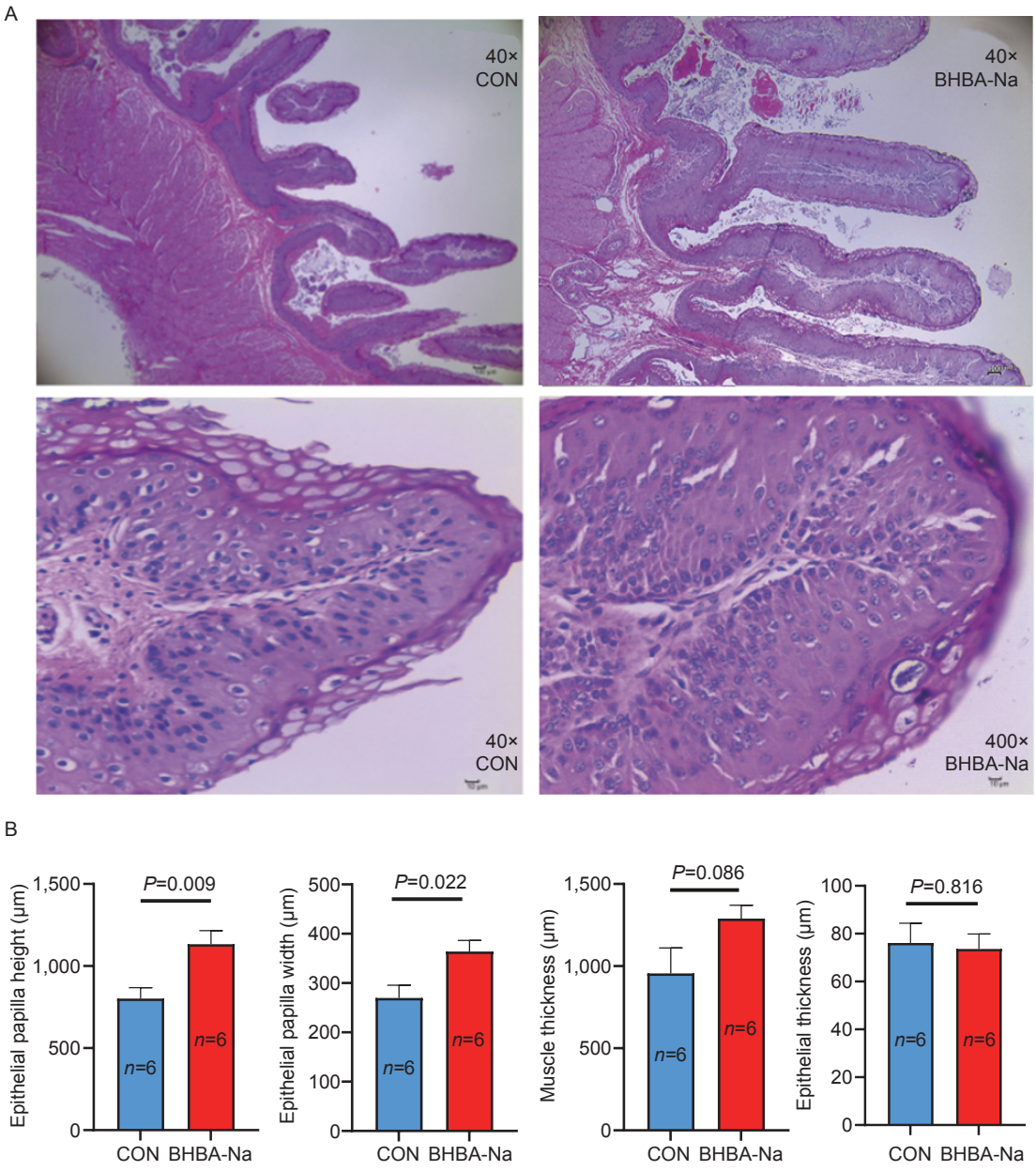


Fig. 3 Effects of dietary β-hydroxybutyric acid (BHBA)-Na on rumen epithelium development. A, morphological differences of rumen epithelial papilla between the CON and BHBA-Na groups. B, comparison of rumen epithelial parameters between the CON and BHBA-Na groups. The error pounds in the charts were represented by mean±SE.

identified with 10 hub genes (acetyl-CoA acyltransferase 1 (*ACAA1*), acetyl-CoA acyltransferase 1 (*ACAA2*), acetyl-CoA acyltransferase 2 (*ACAT2*), enoyl CoA hydratase 1 (*ECHS1*), acyl coenzyme A dehydrogenase (*ACADS*), 3-hydroxymethyl-3-methylglutaryl coenzyme A lyase (*HMGCL*), enoyl-CoA delta isomerase 1 (*ECI1*), *HMGCS2*, enoyl CoA hydratase 1 (*ECH1*) and NAD(P)-dependent steroid dehydrogenase-like (*NSDHL*)) (Fig. 4-C). Specifically, among these 10 hub genes, the expression of *HMGCS2*, *ECSH1*, *ACAA2*, *ECH1*, *ACADS* and *ACAA1* involved in the process of lipid metabolism and ketogenesis in the BHBA-Na group was greater (P<0.05) (Fig. 4-D). Then, the KEGG enrichment analyses of these critical genes were visualized using the KEGG Mapper. As shown in Fig. 4-E, VFA and energy metabolism were activated which were based on the reaction substrate of butanoate and propanoate in the BHBA-Na group. The expression of related enzymes was increased including *EC 1.3.8.1*, *EC 4.2.1.17*, *EC 2.3.1.9*, *EC 2.3.3.10*, *EC 4.1.3.4*, *EC 6.2.11* and *EC 5.1.99.1*.

3.4. Dietary BHBA-Na drove the changes of the rumen epithelial metabolism

After identifying the changed gene expression in rumen epithelium, we further analyzed the effects of dietary BHBA-Na on the rumen epithelial metabolic profile by untargeted metabolomics. Unsupervised principal component analysis (PCA) in the positive and negative ion modes was all performed to establish the significant difference

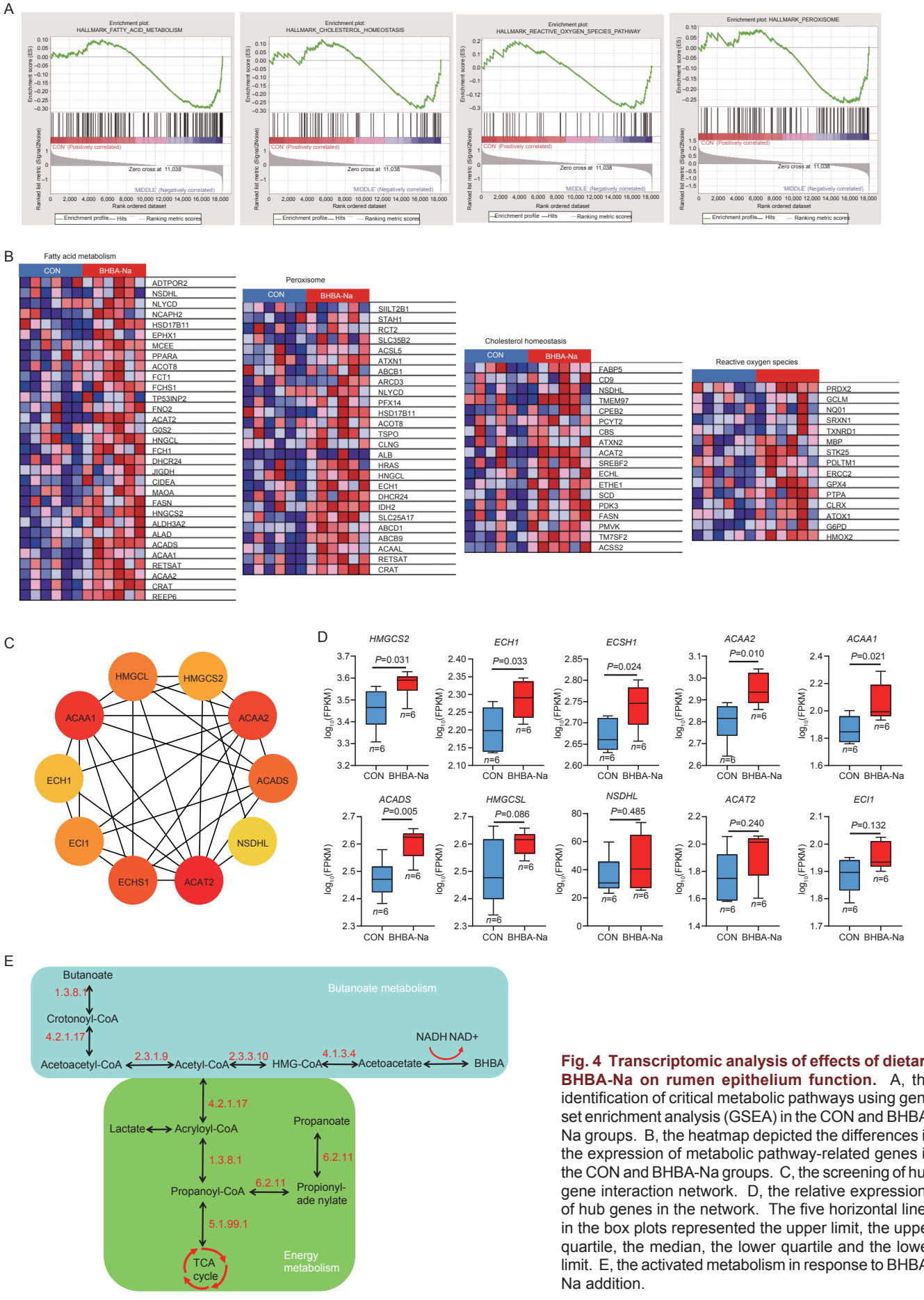


Fig. 4 Transcriptomic analysis of effects of dietary BHBA-Na on rumen epithelium function. A, the identification of critical metabolic pathways using gene set enrichment analysis (GSEA) in the CON and BHBA-Na groups. B, the heatmap depicted the differences in the expression of metabolic pathway-related genes in the CON and BHBA-Na groups. C, the screening of hub gene interaction network. D, the relative expressions of hub genes in the network. The five horizontal lines in the box plots represented the upper limit, the upper quartile, the median, the lower quartile and the lower limit. E, the activated metabolism in response to BHBA-Na addition.

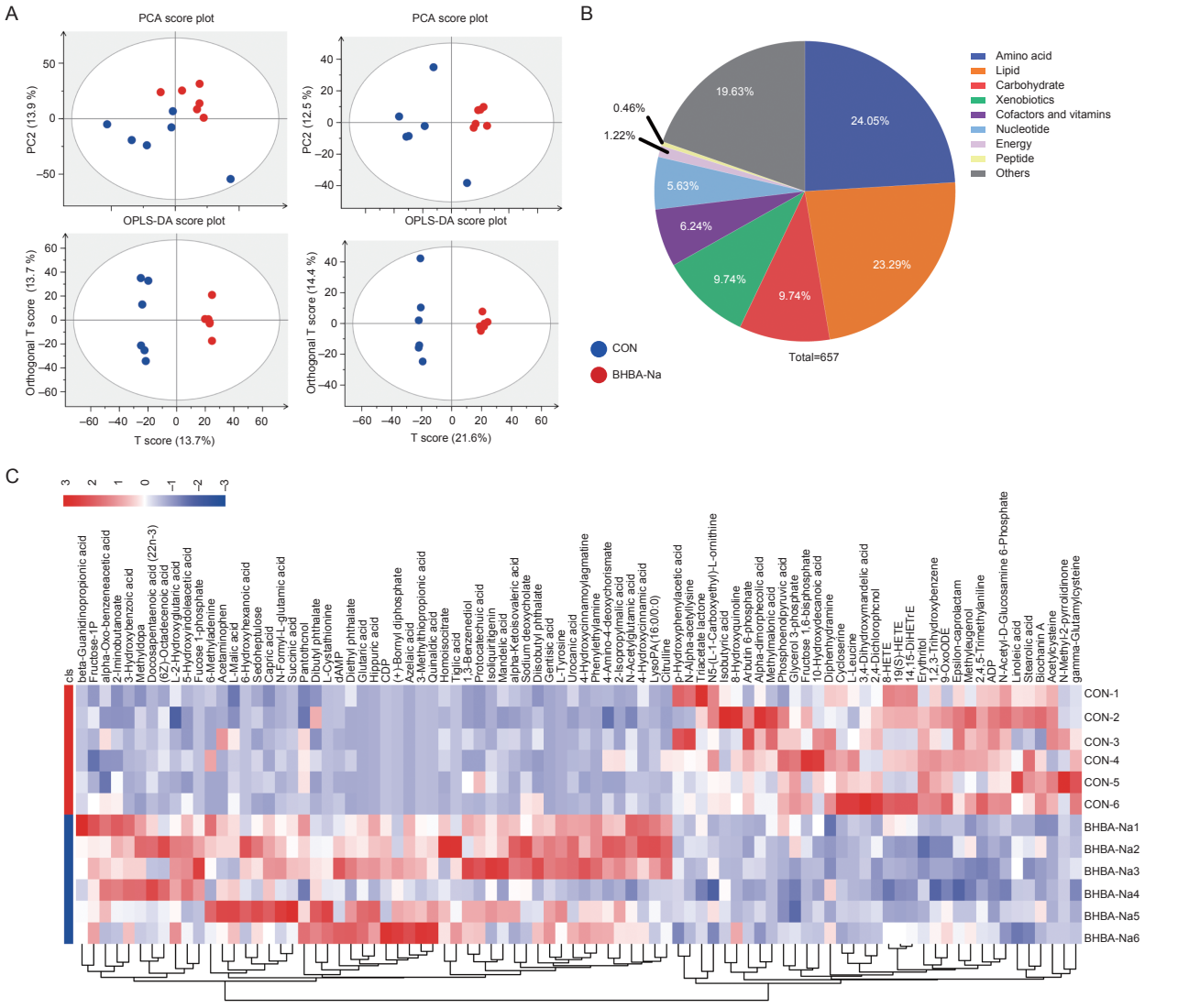


Fig. 5 Metabolomics analysis of effects of dietary β-hydroxybutyric acid (BHBA)-Na on rumen epithelium metabolism. A, the principal component analysis (PCA) of metabolic profile between the CON and BHBA-Na groups. B, the composition of rumen epithelial metabolites detected by LC-MS instrumentation. C, the heatmap depicted the 86 species of rumen epithelial metabolites with significant differences between the CON and BHBA-Na groups.

in the metabolite composition between these two groups (P<0.05) (Fig. 5-A). Across the 12 samples, 657 kinds of rumen epithelial metabolites were detected by LC-MS instrumentation. The major metabolites belonged to amino acids (24.05%), lipid (23.29%) and carbohydrate (9.74%), xenobiotics (9.74%), cofactors and vitamins (6.24%). (Fig. 5-B). Furthermore, we identified 86 metabolites (51 up-regulated and 35 down-regulated) significantly differentiating the CON and BHBA-Na groups (Fig. 5-C; Table 4).

Then, using KEGG enrichment analysis, a total of 25 pathways of these metabolites were determined, which were mainly divided into amino acid metabolism, lipid metabolism and glucose metabolism including the pathways of “phenylalanine metabolism”, “valine, leucine and isoleucine biosynthesis”, “citrate cycle”, “glycolysis/gluconeogenesis”, “pyruvate metabolism”, “butanoate metabolism” and “glycerophospholipid metabolism” (Fig. 6-A). We further quantitatively compared the representative metabolites in these pathways. Specifically, dietary BHBA-Na significantly increased the abundance of succinic acid, L-tyrosine and α-ketoisovaleric acid, and decreased the abundance of phosphoenolpyruvic acid, linoleic acid, ADP, and glycerol 3-phosphate compared with the CON group (P<0.05) (Fig. 6-B).

3.5. Associations between the goat phenotypes and rumen omics

In order to determine the relationship between goat phenotypes (i.e., growth, rumen fermentation parameters and serum biological index), rumen microbiota, epithelial transcriptomics and metabolomics, co-occurrence network according to spearman correlation analysis was conducted (P<0.05, |r|>0.5) (Fig. 7). We found that succinic acid, as a key metabolite in the tricarboxylic acid cycle, was positively correlated with rumen weight, body weight, liver weight,

Table 4 The identification of metabolites with significant difference between the CON and BHBA-Na groups

Metabolites	VIP	FC_BHBA-Na/CON	log_2(FC_BHBA-Na/CON)	*P*-value
N-Methyl-2-pyrrolidinone	1.21	0.06	–4.01	0.02
L-Leucine	1.72	0.07	–3.88	0.01
Methylmalonic acid	1.49	0.08	–3.62	0.04
Fructose 1,6-bisphosphate	1.24	0.15	–2.74	0.01
3,4-Dihydroxymandelic acid	1.21	0.18	–2.46	0.01
ADP	2.45	0.20	–2.34	0.01
Arbutin 6-phosphate	1.94	0.21	–2.23	0.03
N-alpha-acetyllysine	1.48	0.22	–2.22	0.05
Acetylcysteine	2.27	0.25	–2.02	0.01
10-Hydroxydecanoic acid	1.26	0.29	–1.78	0.03
8-Hydroxyquinoline	1.22	0.31	–1.68	0.05
Isobutyric acid	1.18	0.33	–1.61	0.05
2,4-Dichlorophenol	1.47	0.36	–1.48	0.01
gamma-Glutamylcysteine	1.54	0.37	–1.44	0.01
N-Acetyl-D-Glucosamine 6-Phosphate	1.90	0.37	–1.43	0.01
Biochanin A	1.68	0.39	–1.37	0.05
9-OxoODE	1.76	0.40	–1.34	0.03
Erythritol	1.81	0.40	–1.34	0.01
alpha-dimorphecolic acid	1.71	0.41	–1.28	0.01
Phosphoenolpyruvic acid	1.77	0.41	–1.27	0.05
Methyleugenol	1.93	0.43	–1.23	0.03
14,15-DiHETrE	1.93	0.43	–1.21	0.03
8-HETE	1.81	0.44	–1.17	0.03
Stearolic acid	2.05	0.45	–1.16	0.02
Cycloserine	1.91	0.46	–1.11	0.01
19(S)-HETE	1.82	0.47	–1.10	0.03
p-Hydroxyphenylacetic acid	1.49	0.51	–0.98	0.05
2,4,5-Trimethylaniline	2.13	0.52	–0.94	0.01
1,2,3-Trihydroxybenzene	2.29	0.53	–0.92	0.01
Triacetate lactone	1.33	0.53	–0.90	0.03
N5-(L–1-Carboxyethyl)-L-ornithine	1.93	0.55	–0.87	0.05
Linoleic acid	1.48	0.59	–0.76	0.05
Glycerol 3-phosphate	1.53	0.63	–0.66	0.02
Diphenhydramine	1.95	0.73	–0.45	0.03
Epsilon-caprolactam	2.02	0.77	–0.39	0.01
alpha-Oxo-benzeneacetic acid	1.39	1.68	0.74	0.05
L-Malic acid	1.27	1.68	0.74	0.03
Pantothenol	1.20	1.69	0.75	0.05
4-Amino–4-deoxychorismate	1.72	1.74	0.80	0.05
N-Acetylglutamic acid	1.51	1.79	0.84	0.05
Methyldopa	1.61	1.83	0.87	0.01
Dibutyl phthalate	1.69	1.96	0.97	0.03
L-2-Hydroxyglutaric acid	1.79	1.96	0.97	0.01
Mandelic acid	1.29	1.98	0.99	0.05
Acetaminophen	1.45	2.18	1.12	0.05
Fructose-1P	1.60	2.25	1.17	0.01
Citrulline	1.39	2.31	1.21	0.02
Diisobutyl phthalate	1.67	2.32	1.21	0.05
(6Z)-Octadecenoic acid	1.12	2.32	1.22	0.05
Capric acid	1.87	2.33	1.22	0.01
Sedoheptulose	1.47	2.44	1.29	0.02
2-Iminobutanoate	1.78	2.45	1.29	0.05
Docosapentaenoic acid (22n-3)	1.21	2.69	1.43	0.03
5-Hydroxyindoleacetic acid	1.58	3.11	1.64	0.02
6-Methyladenine	1.14	3.39	1.76	0.05
LysoPA (16:0/0:0)	1.41	3.41	1.77	0.01
dAMP	1.45	3.51	1.81	0.01
Protocatechuic acid	1.43	3.54	1.82	0.05
Sodium deoxycholate	1.20	3.93	1.97	0.05
Diethyl phthalate	1.88	3.98	1.99	0.05

(Continued on next page)

Table 4 (Continued from preceding page)

Metabolites	VIP	FC_BHBA-Na/CON	log_2(FC_BHBA-Na/CON)	*P*-value
Urocanic acid	1.51	4.12	2.04	0.03
1,3-Benzenediol	1.44	4.27	2.09	0.02
alpha-Ketoisovaleric acid	1.55	4.34	2.12	0.05
Homoisocitrate	1.57	4.47	2.16	0.02
3-Hydroxybenzoic acid	1.85	4.56	2.19	0.01
2-Isopropylmalic acid	1.54	4.86	2.28	0.01
Isoliquiritigenin	1.25	4.96	2.31	0.02
4-Hydroxycinnamic acid	1.24	5.59	2.48	0.05
4-Hydroxycinnamoylagmatine	2.03	5.93	2.57	0.05
beta-Guanidinopropionic acid	1.27	8.11	3.02	0.01
N-Formyl-L-glutamic acid	1.77	8.75	3.13	0.01
Succinic acid	1.25	12.74	3.67	0.01
Phenylethylamine	1.77	45.06	5.49	0.01
(+)-Bornyl diphosphate	1.36	80,336.00	16.29	0.00
CDP	1.30	100,210.00	16.61	0.00
Quinaldic acid	1.46	150,840.00	17.20	0.00
Azelaic acid	1.81	198,370.00	17.60	0.00
Fucose 1-phosphate	1.30	458,730.00	18.81	0.03
Hippuric acid	1.76	514,800.00	18.97	0.00
Gentisic acid	1.92	555,350.00	19.08	0.00
Tiglic acid	1.26	764,430.00	19.54	0.00
L-Cystathionine	1.33	980,190.00	19.90	0.00
3-Methylthiopropionic acid	1.41	1,688,200.00	20.69	0.00
6-Hydroxyhexanoic acid	1.28	3,946,300.00	21.91	0.01
L-Tyrosine	1.77	4,359,700.00	22.06	0.00
Glutaric acid	1.82	5,263,000.00	22.33	0.00

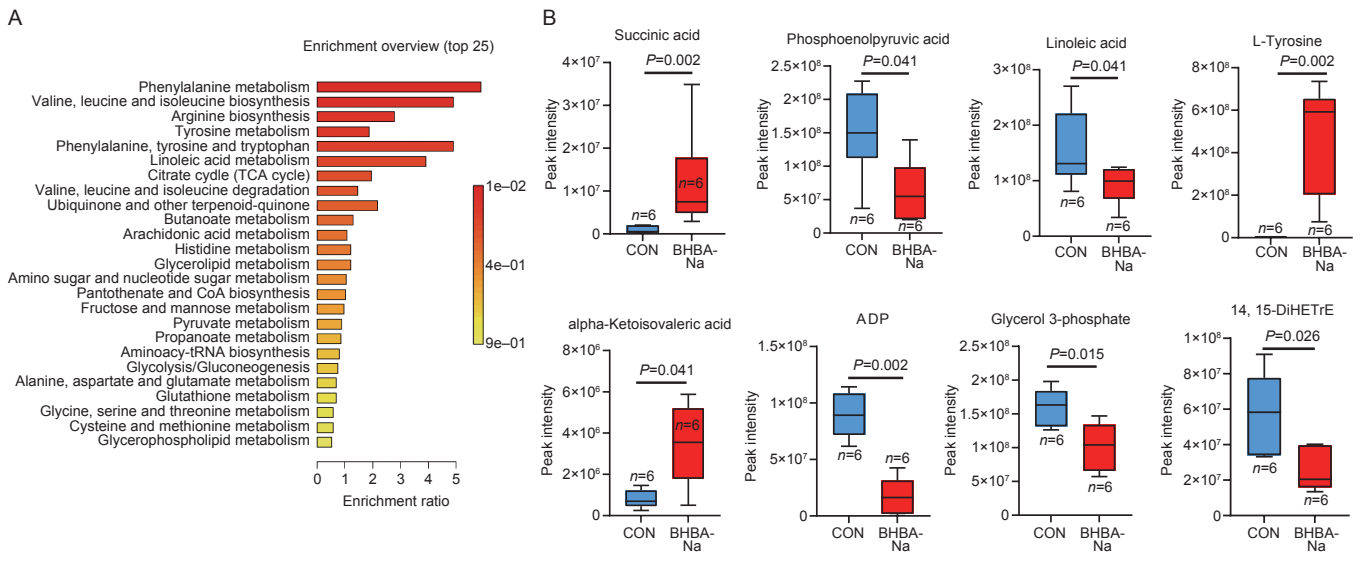


Fig. 6 The function analysis of differential metabolites associated with dietary β-hydroxybutyric acid (BHBA)-Na. A, functional enrichment analysis of differential metabolites between the CON and BHBA-Na groups. B, the relative expression levels of eight key metabolites. The five horizontal lines in the box plots represented the upper limit, the upper quartile, the median, the lower quartile and the lower limit.

ACADS, L-tyrosine, *Streptophyta* (ASV470), and negatively correlated with LPS. It could be concluded that succinic acid was directly associated with improved growth performance. In addition, L-tyrosine was positively correlated with liver weight, body weight, *ACADS*, *ACAA2*, *HMGCS2* and negatively correlated with ADP. Succinic acid, *ACADS*, *ACAA2*, ADP and L-Tyrosine have close interaction with each other.

4. Discussion

The rumen is of vital importance for the health and productivity of ruminants (Guo *et al.* 2023). The rapid development of the rumen in young ruminants can facilitate their efficient growth (Dong *et al.* 2019). As we expected, the results showed that BHBA-Na increased the rumen weight and improved the growth performance of young goats

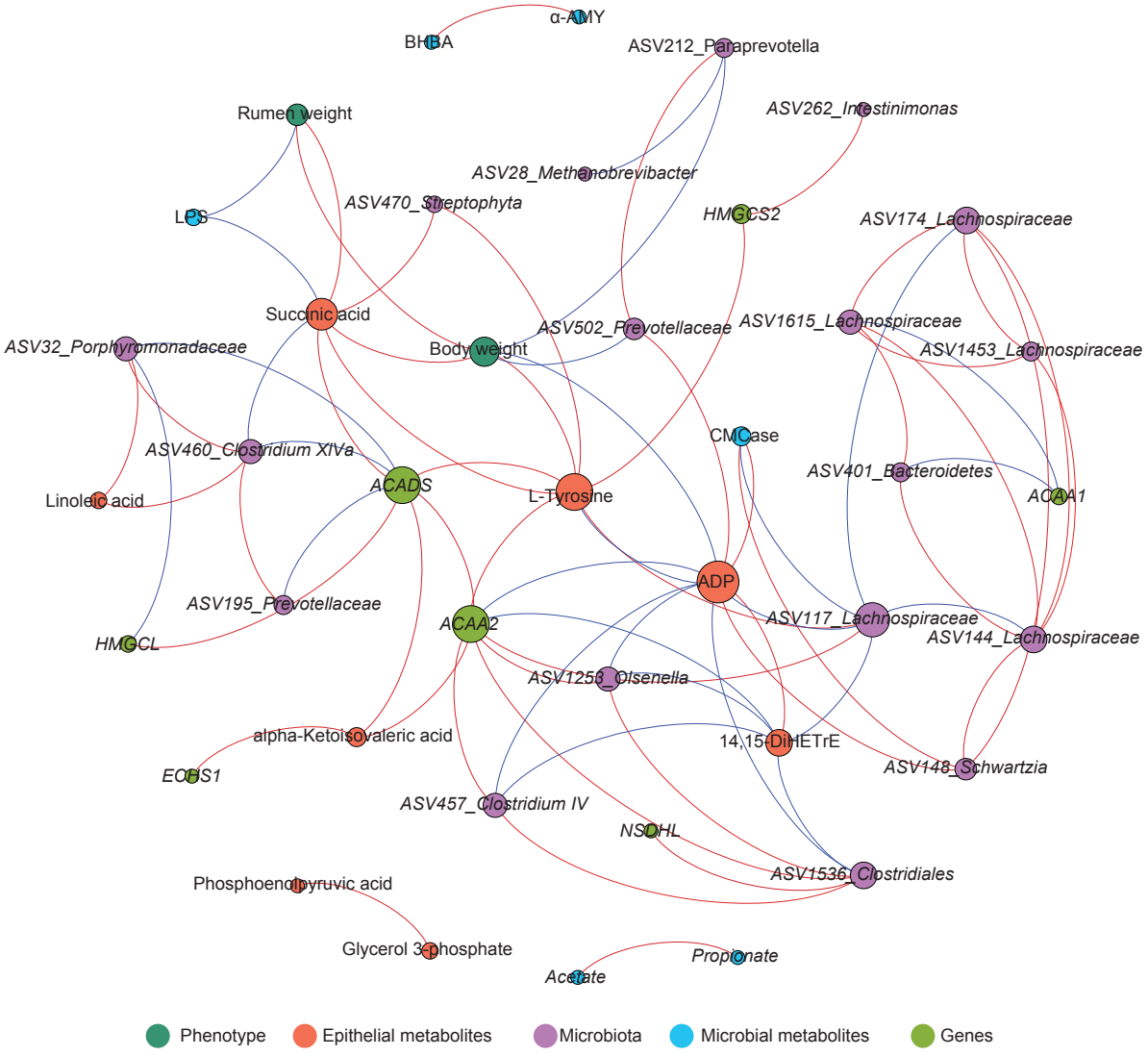


Fig. 7 Network analysis of interaction among growth performance, rumen microbiota, epithelial transcriptome and metabolomic. The size of nodes represented the degree of connection, and the color of the line represented the positive (red) or negative correlation (blue).

including body weight, ADG and DMI. The beneficial bacteria in the rumen were also increased in abundance. In addition, the genes and metabolites involved in the metabolisms of VFA and amino acid in rumen epithelium were also activated in response to BHBA-Na addition. These evidences support our hypothesis and these findings provide theoretical support for the application of BHBA-Na as a growth promoting additive for young ruminants. Furthermore, it was also found in the present study that dietary BHBA-Na addition enhanced the DMI of young goats, which is one of major factors affecting growth, rumen development and other aspects. However, specific mechanisms of BHBA-Na enhancing the DMI are unknown.

Dietary BHBA-Na has been proved to alter the gut microbiota and promote the development of intestinal epithelium. However, there was little information about its impacts on the rumen development and metabolism of pre-weaning ruminants. In this study, it was observed that dietary supplementation of BHBA-Na contributed to the enhanced development of body and rumen in young ruminants. These results are similar with the findings of Cavini *et al* (2015), who concluded that BHBA precursor could positively impact growth performance and increase DMI in lambs. The results of rumen morphology showed that the height and width of rumen epithelial papillae in young goats were more developed after BHBA-Na intervention. Microbial analysis showed BHBA-Na addition changed the microbial composition and increased the abundance of beneficial microbiota. Transcriptomic and metabolomic analysis revealed the rumen epithelial genes, metabolites and their enriched pathways related to lipid metabolism and amino acid metabolism were all activated in response to BHBA-Na addition. Through correlation network analysis, we found that the signature microbiota, hub genes and critical metabolites influenced by BHBA-Na showed complex and various interactions with animal phenotypes.

In artificial rearing systems, newborn animals are separated from their mothers, feeding on milk replacer and early weaning can restrict rumen development and negatively affect feed digestibility and growth performance (Belanche *et al.* 2020). To find a way to promote growth and improve

rumen development of ruminants under artificial rearing systems is critical. This study suggests that BHBA-Na supplementation could reach this goal. The positive effects of BHBA-Na supplementation were consistent with previous studies on butyrate supplementation, as the precursor of BHBA (Kotunia *et al.* 2004; Mazzoni *et al.* 2008), which both showed the similar ability to increase body weight and DMI in young ruminants (Cavini *et al.* 2015; Jolazadeh *et al.* 2019). Th intake of more solid feed could further provide sufficient nutrients and energy for the body growth and the rumen development in the host (Khan *et al.* 2011; Beiranvand *et al.* 2014). In addition, we also observed the improvements in rumen weight. These evidences suggested the potential and beneficial effects of BHBA-Na as a growth promoter in ruminants.

The rumen microbiota showed the strong ability to convert dietary nutrients into VFAs to meet the needs of host maintenance and growth (Zhao *et al.* 2023). This study found that dietary BHBA-Na did not change the richness and diversity of rumen microbiota. However, we observed the increased relative abundance of *Succinivibrio* and *Roseburia* in the rumen, which might be attributed to higher DMI providing abundant metabolic substrates for the proliferation of these nutrient decomposers. *Succinivibrio* showed a high efficient to produce succinate which is converted into acetate, propionate and butyrate via decarboxylation (Iqbal *et al.* 2018). *Roseburia* produces butyrate, prevents intestinal inflammation and maintain energy homeostasis by producing metabolites (Nie *et al.* 2021). This result showed positive effects of BHBA-Na on rumen microbial composition. At ASV level, *Clostridium_IV* (ASV174) and *Fibrobacter* (ASV262) were enriched in BHBA-Na group. *Clostridium_IV* is a member of the mucosal microbiota, which is highly correlated with the expression of functional lectins and helps to maintain rumen homeostasis (Shen *et al.* 2017). Microbial degraders of plant cell wall polysaccharides in the guts of herbivores, especially *Fibrobacter*, are essential for their hosts to digest food composed mainly of refractory plant fibers (Neumann and Suen 2018). These results indicated that BHBA-Na could enhanced the carbohydrate digestibility in the rumen of young goats by increasing the abundance of nutritional decomposer.

Gut microbiota influences host metabolism by regulating nutrient supply and producing large amounts of metabolites such as VFAs (Dalile *et al.* 2019). Although BHBA-Na did not significantly affect VFAs production, high concentration of BHBA accumulated in the rumen, which could be used as an energy source for the rumen, thereby altering microbial fermentation and regulating energy utilization. In addition, BHBA-Na increased the abundance of α-AMY, an important enzyme in glucose metabolism, which was beneficial to promote the digestion and utilization of abundant starch in the starter (Ezati *et al.* 2022). Moreover, LPS has been proved to induce the inflammation and damages of gut barrier function in animals (Nugent *et al.* 2023). Our results suggested that BHBA-Na also reduced inflammatory stimulation by reducing the abundance of LPS.

Rumen epithelial papillae can expand the surface area of the rumen wall, make full contact with chyme and digestive fluid, and enhance the absorption of nutrients (Malmuthuge *et al.* 2019). The length and width of rumen epithelial papillae indicated the state of rumen development (Lesmeister *et al.* 2004). In this study, dietary BHBA-Na improved the growth of rumen papillae, which could be used as an effective accelerator to promote the development of rumen epithelium. Our previous study concluded that BHBA significantly increased the DMI of goats (Abdelsattar *et al*. 2022a). Physical friction of the solid feed against the rumen wall stimulates the growth of the rumen papillae (Lv *et al.* 2019) and the abundant nutrients from the feed also provide the necessary energy for rumen development (Lin *et al.* 2019). Moreover, much more than a metabolite, BHBA has been also considered to be a critical regulator of lipid metabolism of host (Newman and Verdin 2014). Hence, we speculated that BHBA as a ligand could improve rumen development by regulating the expression of genes related to rumen metabolism indirectly.

As expected, BHBA-Na altered the profile of rumen epithelial transcriptome and metabolism profoundly. Four major gene modules were identified in response to BHBA-Na addition. Specifically, fatty acid metabolism and cholesterol homeostasis are closely related to lipid metabolism. Reactive oxygen species pathway and peroxisome are closely related to antioxidant capacity. The differential expression of CON and BHBA-Na in the four hallmarks indicates that BHBA-Na can enhance the lipid metabolism and antioxidant capacity of the body. The subnetwork identified 10 hub genes related to VFA metabolism (*HMGCS2*, *ECSH1*, *ECH1*, *ACAA2*, *ACADS*, *ACAA1*) were increased after BHBA-Na addition. *HMGCS2* is the rate-limiting enzyme involved in the generation of ketone bodies from lipids in the rumen (Wang *et al.* 2019). Recent studies have shown that the accumulation of BHBA and the increased expression of *HMGCS2* enhance the proliferation and differentiation of intestinal epithelial cells. Exogenous BHBA supplementation repaired intestinal regeneration obstruction caused by HMGCS2 loss, suggesting that BHBA might be a direct promoter of epithelial cell growth (Yu *et al.* 2019). ECH1 is recognized as a key component in mitochondrial fatty acid β-oxidation (Liu *et al.* 2021). Short-chain acyl-CoA dehydrogenase (SCAD), encoded by the *ACADS* gene, functions in the mitochondrial β-oxidation of saturated short-chain fatty acids (Yuan *et al.* 2020). In addition, *ACAA2* is a key enzyme in the fatty acid oxidation pathway, and its encoded protein catalyzes the last step of mitochondrial fatty acid β-oxidation spiral, playing an important role in fatty acid metabolism (Zhang *et al.* 2019). β-oxidation cycle is the major pathway for fatty acid degradation in most organisms and could supply a large amount of energy needed by the body (Goepfert and Poirier 2007). This was also verified in our KEGG pathway analysis, where fatty acid and amino acid metabolic pathways were enriched in the BHBA-Na group. Further analysis of important metabolites showed that BHBA-Na could promote the production of succinic acid and provide sufficient substrate for SCFAs fermentation. Microbiota-produced succinate improves glucose homeostasis via intestinal gluconeogenesis (De Vadder *et al.* 2016). In addition, succinic acid, or succinate, is involved in this process not only as one of

the intermediates of the tricarboxylic acid (TCA) cycle, but also as a signaling molecule (Prikhodko *et al.* 2021). TCA cycle is a housekeeping metabolic pathway essential for generation of energy and biosynthetic intermediates (Scagliola *et al.* 2020). The significant increase of succinic acid in BHBA-Na group can accelerate the TCA cycle to oxidize nutrients to support cellular bioenergetics, thus accelerating rumen epithelial metabolism and development (Arnold and Finley 2023). Increasing the abundance of L-tyrosine and α-ketoisovaleric acid provides raw materials for the synthesis of essential amino acids. Glucose metabolism is another important source of energy productivity in the cell. Some studies have reported that infusion of BHBA in the heart resulted in glycogen synthesis and the inhibition of glycolysis (Lund *et al.* 2015). Combined with pathway analysis, the decreased abundance of phosphoenolpyruvic acid and glycerol 3-phosphate in the BHBA-Na group was associated with inhibition of glycolysis. The dynamic interaction between rumen microbiota and metabolites could have remarkable impact on host development (Chai *et al.* 2021). In the co-expression network, *Olsenella* was positively correlated with *ACAA2*, a gene involving the lipid accumulation and metabolism, which was consistent with the previous findings that *Olsenella* was associated with the production of medium-chain fatty acids (Scarborough *et al.* 2018). Moreover, we found that succinic acid, a key metabolite in the TAC cycle (Prikhodko *et al.* 2021), was positively correlated with rumen weight, body weight, liver weight, *ACADS* and L-tyrosine and negatively correlated with LPS. Succinic acid is the precursor of VFAs (Iqbal *et al.* 2018). *ACADS*-edited proteins participate in the β-oxidation of lipid metabolism (Yuan *et al.* 2020). Therefore, a high abundance of succinic acid and its secondary metabolites might provide raw materials and energy for β-oxidation. In addition, it has a positive relationship with L-tyrosine, indicating that it is also highly involved in the synthesis of essential amino acid. According to the review, BHBA-Na could synergistically promote growth performance and rumen epithelial development of goat kids possibly by jointly enhancing DMI and regulating rumen microbiota and epithelial gene and metabolites.

5. Conclusion

In summary, this study demonstrated that dietary BHBA-Na could promote the growth performance and rumen development of young goats. BHBA-Na supplementation promoted young goats to consume more starters, which could provide abundant nutrients for the proliferation of probiotics in the rumen, and further activated the volatile fatty acid and amino acid metabolisms of the rumen epithelium to improve its rapid development (Fig. 8). This study provided new insights into beneficial effects of BHBA-Na on the young goats. The precise mechanism of how BHBA-Na promotes DMI and rumen epithelial development of young goats remains to be elucidated in further research.

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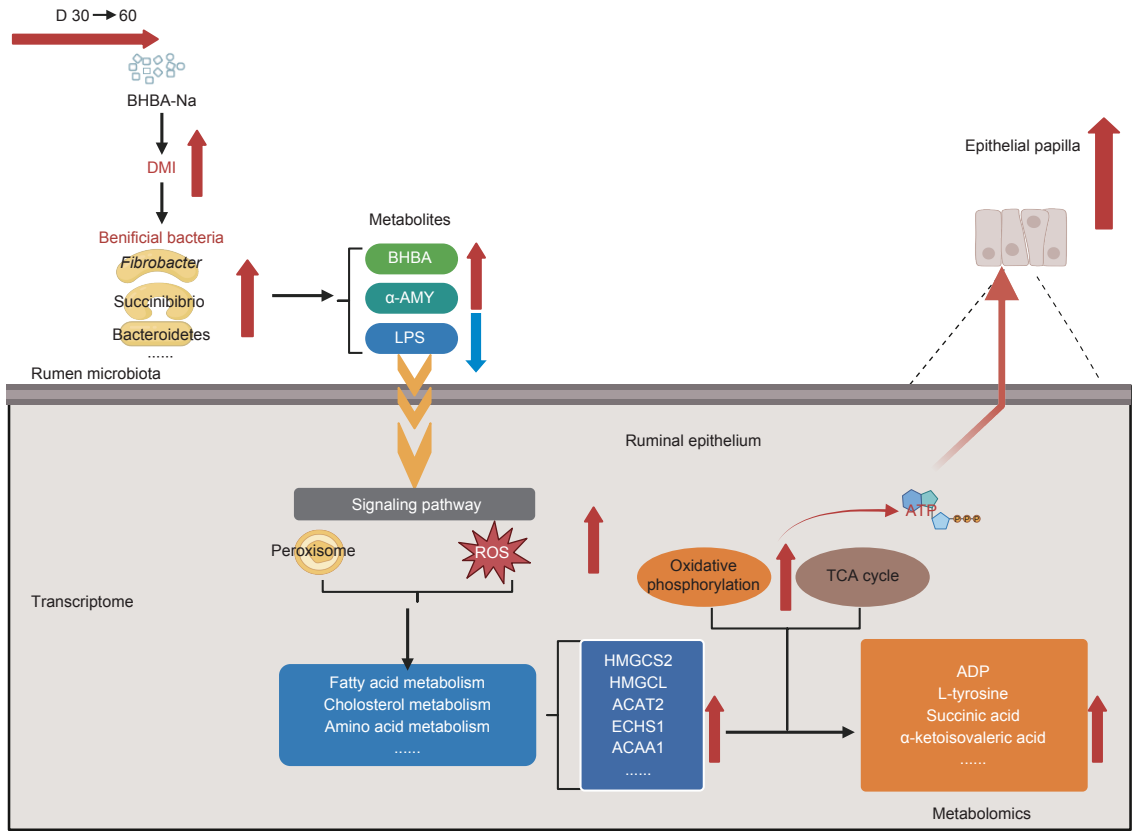


Fig. 8 β-Hydroxybutyric acid (BHBA)-Na enhanced DMI and regulated the rumen microbial community and epithelial nutrient metabolism in the young goats.

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Declaration of competing of interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical approval

The research was carried out in compliance with the guidelines set forth by the Animal Ethics Committee of the Chinese Academy of Agricultural Sciences (AEC-CAAS-20200605; Approval date: 6/9/2020).

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