Article

Dietary energy level impacts the performance of donkeys by manipulating the gut microbiome and metabolome

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Simple summary: Both dietary energy contents and gut microbiota play a pivotal role in improving the growth performance of animals. The present findings indicated that HE increased the abundance of *Prevotellaceae*, *Desulfovibrio*, and *Succinivibrio*, as well improved the content of L-aspartic acid, ornithine, L-glutamine, L-phenylalanin, L-serine, methionine, lysine, L-isoleucine, and N-acetylaspartic acid in gut content, and thus affected some growth-related metabolic pathways mainly involving the Aspartate Metabolism and Urea Cycle. Those increased bacteria and metabolites exhibited the positive correlation with the average daily gain (ADG) and feed efficiency of donkeys. Thus, HE diet can increase the richness of beneficial bacteria and thus modified the growth-related metabolic pathways, which contributed to the improved performance and feed efficiency of donkeys. These beneficial bacteria and metabolites related to dietary energy concentration are potential targets for regulating growth performance. The present findings also provided an innovative insight for developing the new growth-promoting probiotics and prebiotics.

Abstract: Little information is available regarding the impacts of dietary energy level on the gut microbiota and metabolites of donkeys. This studied aimed to explore the effects of dietary energy content on growth performance, intestinal microbiome and metabolome of Dezhou donkeys. Thirty-six 9-month-old male Dezhou donkeys were assigned to two groups fed low or high content energy diets (LE or HE). Results showed that donkeys fed HE had improved ($P < 0.05$) the average daily gain (ADG) and feed efficiency (G/F), compared with those receiving LE diet. Compared to the LE group, feeding HE specially increased the abundances of *unidentified_Prevotellaceae* ($P = 0.02$) while decreased the richness of *unidentified_Ruminococcaceae* ($P = 0.05$) of donkeys. Compared to LE group, feeding HE diet significantly ($P < 0.05$) affected the metabolic pathways involving the aspartate metabolism and urea cycle. In addition, the increased bacteria and metabolites in the HE-fed group exhibited a positive correlation with improved growth performance of donkeys. Taken together, feeding HE diet increased the richness of some specific bacteria and upregulated growth-related metabolic pathways, which contributed to the augmented growth performance of donkeys. Thus, it is a recommendable dietary strategy to feed HE diet to fattening donkey for superior production performance and feed efficiency.

Key words: digestible energy; growth performance; microbiome; metabolome; donkey

1. Introduction

Dezhou donkey (*Equus asinus*) is a monogastric herbivorous animal and a typical breed in China, known for its good performance, great body structure, and strong muscles [1]. Donkey meat has low fat content, high level of unsaturation, and a better protein and cholesterol content than those of other livestock meats [2,3]. Furthermore, milk production from young Dezhou donkey is a crucial way to increase the income of local farmers in China. In monogastric animals, dietary energy levels play an important role in growth performance and feed efficiency [4,5], with starch as the major carbohydrate source in their diets [6] and major energy provider [7]. Furthermore, short-chain fatty acid production from the microbial digestion of cellulose provides a substantial portion of the daily energy requirements of horse or donkey, with their gut microbiota being shaped predominantly by environmental factors rather than by host genetics [8], while metabolomics may act as communicators between host and microbiota [9].

Microbial degradation and fermentation of plant polymers into nutrients is a critical function of the equine intestinal ecosystems [10]. Intestinal microbiota, i.e., microorganism population inhabiting the intestine, play an important role in the nutrient digestion and absorption as well as the host health [11,12]. Tasse*, et al.* [13] found that gut bacteria produce a huge panel of carbohydrate-active enzymes to degrade dietary fibers into metabolizable monosaccharides and disaccharides. However, to the best of our knowledge, research on the gut microbiota of donkeys fed diets with different energy levels has not been reported.

Although gut microbiota allows us to look into the genetic potential of microbial communities, a great part of genes in the microbiome could not be annotated or isolate genomes and their functions remain unknown [14], thus limiting our understanding of the functional capacity of microbiota. Additionally, a single measurement of microbiota may not provide accurate information. Alternatively, metabolomics allows researchers to obtain a highly sensitive and complete description of the phenotype [15,16] and has been widely applied in human clinical trials, proving to be a powerful tool to identify potential biomarkers for the diagnosis and diseases [17,18].

Metabolomics is based on the simultaneous analysis of multiple low-molecular-weight metabolites from a given sample [19], and is a newly emerging subject based on detection techniques including gas chromatography/ mass spectrometry, nuclear magnetic resonance, liquid chromatography/ mass spectrometry, or liquid chromatography-tandem mass spectrometry (LC-MS/MS) [20,21]. Fecal metabolites can reflect the result of nutrient ingestion, digestion, and absorption by both the gut bacteria and the host tract, providing useful to the study of host-microbiota and metabolome interactions affecting growth performance [22]. Thus, metabolomics application would greatly extend our understanding of how different diet energy levels affect the metabolism. Karisa*, et al.* [23] and Weikard*, et al.* [24] stated that metabolomics can be used to predict feed efficiency (G/F), average daily gain (ADG), average daily feed intake (ADFI), and dry matter intake. Moreover, several metabolic pathways are regulated by amino acids, which are key precursors for protein and polypeptide synthesis and are mainly derived from the degradation of dietary proteins and microproteins by microbiota. However, current knowledge about the relationships between dietary levels and bacterial communities or metabolite profiles in the Dezhou donkeys is limited, and such insights are crucial to the development of technologies that support modern donkey husbandry, providing technical guidance for healthy donkey breeding.

In this study, we integrated metabolome analysis and correlation analysis of microbiota to compare the composition of rectal metabolites in donkeys that were fed a low or high digestible energy (DE) diet (LE and HE, respectively), aiming to evaluate the effect of dietary energy level on G/F, performance, gastrointestinal microbiota, and metabolome of fattening donkeys through a combination of the 16S rDNA and LC-MS/MS, and also exploring the possible relationships between microbiota and metabolites in the rectum. It was hypothesized that different dietary energy levels would influence growth performance and the microbiota and metabolites.

2. Materials and methods

All procedures involving animal care and use were in strict accordance with the animal care and use protocol approved by the Shandong Agricultural University Animal Nutrition Research Institute (Protocol No. S20200068).

2.1. Experimental design, animals, and feeding management

Thirty-six healthy 9-month-old male Dezhou donkeys with similar body weight (126.5 ± 3 kg) were randomly allocated into six pens with three donkeys in each pen (3 × 10 m). The pens were randomly assigned to two dietary treatments with three replicates per treatment. One treatment was fed control diet formulated following the feeding standard of Dezhou donkeys in China (DB 37/T 3605—2019) (denoted as LE group), and the other treatment group received a high DE diet (denoted as HE group). The diet compositions and nutritional contents are shown in Table 1.

This experiment consisted of a 20-day adaptation period and a 40-day fattening period for sample collection. Sufficient diets were made in one batch to prevent any batch effect on dietary treatments. Throughout the experimental period, all donkeys had free access to the assigned diets, which were offered two times a day (at 06:00 and 18:00 hours). Furthermore, all the animals had free access to freshwater. The proximate components of feeds were analyzed according to AOAC [25], and the content of neutral detergent fiber and acid detergent fiber was determined by the method of Soest, *et al.* [26].

Throughout the experimental period, donkeys in each pen were weighed on days 0, 20, and 40 before morning feeding to determine ADG. The daily feed offered, orts, and spillages were collected and weighed daily to determine ADFI. G/F was calculated by dividing ADG by ADFI.

Table 1. Experimental diet composition and nutrient levels.

	Treatments[1]	
Ingredients, %	**LE**	**HE**
Corn	11.00	44.40
Soybean meal	9.00	13.00
Wheat bran	13.00	6.00
Rice bran meal	13.00	6.00
Wheat flour	8.00	5.00
Rice husk	8.00	5.00
Peanut vine	18.00	9.00
Alfalfa	18.00	9.00
$CaHPO_4$	0.25	0.25

Limestone	0.60	1.2
Lysine	0.30	0.30
Met	0.05	0.05
$NaHCO_3$	0.30	0.30
NaCl	0.40	0.40
Premix[2]	0.45	0.45
Total	100	100
Nutrient content[3]		
Digestible energy (DE), MJ/kg	10.43	11.90
Crude protein, %	15.00	14.74
Ether extract	2.00	3.00
Neutral detergent fiber, %	33.13	22.92
Acid detergent fiber, %	22.14	11.92
Acid detergent lignin, %	4.43	3.06
Calcium, %	0.71	0.72
Phosphorus, %	0.49	0.41

[1] LE, low digestible energy diet; HE, high digestible energy diet.

[2] Supplied per kg of total mixed ration: Vitamins A, 5000 IU; D, 240 IU; E, 30 IU; K3, 3 mg; B1, 3 mg; B2, 8 mg; B3, 34 mg, B5, 10.8 mg; B6, 4 mg; B7, 0.13 mg; B12, 0.02 mg; Fe, 50 mg; Cu, 8 mg; Zn, 50 mg; Mn, 12.5 mg; Se, 0.20 mg; I, 1 mg.

[3] All items were measured values except DE.

2.2. Preparation of pelleted total mixed ration

The forages for the diet were ground to pass through a 3 mm screen, and all concentrates were ground to pass through a 1.5 mm screen. After mixing, the diet was pelleted at 50−60 °C (conditioning temperature) with a compression ratio of 10:1 to form a cylindrical shape (pellet diameter = 6 mm; length = 10−15 mm) using a pelleting machine [27].

2.3. Determination of microbiome and metabolome in rectum digesta

At the end of the feeding period, digesta in the rectum were collected from six donkeys within each treatment. Samples were stored in 5 mL frozen pipes and were immediately flash-frozen in liquid nitrogen until analysis. The microbiome was determined by 16S rDNA amplicon sequencing. DNA was extracted from rectum digesta samples using the CTAB/SDS method, and PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The detailed descriptions of microbe determination were stated in Supplement 1. Metabolome was determined by LC-MS/MS. First, tissues were individually grounded, incubated, and centrifuged. Then, the supernatant was injected into the LC-MS/MS system analysis, i.e., Vanquish ultra-high-performance LC system (Thermo Fisher, USA) coupled with an Orbitrap Q Exactive series mass spectrometer (Thermo Fisher). The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. Detailed descriptions of metabolome determination were stated in Supplement 2.

2.4. Statistical analyses

In this study, the pen was the experimental unit for growth performance measurements (n = 3) and microbiota analysis (n = 6). ADFI, ADG, and G/F were analyzed in SAS version 9.0 (SAS Inst. Inc., Cary, NC). Linear discriminant analysis (LDA) effect size analysis of ruminal microbiota changes was conducted using the online procedure of Galax (http://huttenhower.sph.harvard.edu/galaxy/--LEfSe). Difference was declared to be statistically significant when $P < 0.05$.

3. Results

3.1. Growth performance of donkeys

The growth performances of donkeys in different stages were presented in Table 2. No significant difference ($P = 0.194$) was observed for the initial body weight between LE and HE groups. However, the donkeys fed HE had significantly improved ($P < 0.05$) body weight (BW) and ADG throughout the experiment. In addition, feeding HE increased ($P < 0.05$) G/F of donkeys in the 40-day experimental period.

Table 2. Effects of different energy level diets on donkey production performance.

Items		Treatments[1]		SEM	*P*-value
		LE	HE		
BW[2] (kg)					
	Day 0	125.25	128.38	1.070	0.194
	Day 40	153.5	160.25	0.944	0.012
period (0 to 40 d)					
	ADG (g/d)	706	797	10.476	0.005
	ADFI (g/d)	4337	4262	19.582	0.205
	G/F	6.14	5.35	0.125	0.003

[1] LE, low digestible energy diet; HE, high digestible energy diet.

[2] BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; G/F, feed conversion ratio.

3.2. Profile and characteristics of microbiota in the rectum

The microbiota of rectum digesta were analyzed in the two dietary groups by sequencing the bacterial 16S rDNA V3+V4 region. High-throughput pyrosequencing of the samples (n = 6) generated a total of 467,846 and 434,240 raw reads in the LE and HE group, respectively. After removing low-quality sequences, 436,323 and 401,869 total tags were obtained in the rectum contents of the LE and HE group, respectively. Considering 97% sequence similarity, a total of 2,120 operational taxonomic units (OTUs) were identified in the LE group, which were assigned to 25 phyla, 39 classes, 82 orders, 141 families, and 247 genera. Meanwhile, 2,063 OTUs were obtained in the HE group, which belonged to 25 phyla, 37 classes, 70 orders, 127 families, and 239 genera. There were 2,356 OTUs shared by both experimental groups (Figure1A).

The sequencing depth almost reflected the total microbial species richness (Goods coverage > 99%). Furthermore, no significant differences were observed in the alpha-diversity indices (the Shannon, Simpson, ACE, PD_whole_tree curves, and Chao1) between groups (Table 3). The principle coordinates analysis (PCoA) based on the weight unifrac distance revealed that no completely separated samples distribution suggesting some similar structure of microbial community was between the HE and LE group (Figure1B).

The relative abundance of microbiota with over 0.5% relative abundance in rectum were determined at the phylum, family, and genus levels (Figure1C, D, E). The phyla *Firmicutes* and *Bacteroidetes* dominated the rectum microbiota in both groups, with 54.18% and 37.58% abundance in the LE group, and 47.45% and 41.61% abundance in the HE group, respectively (Figure1C). In addition, the ratio of *Firmicutes* to *Bacteroides* in the LE group was greater than that in the HE group (Figure1F). At the family level, 22 families were identified with relative abundance of more than 0.5%. The dominant families were *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, and *Rikenellaceae*, with richness of 26.44%, 15.45%, 3.97%, and 8.71% in the LE group, and 22.56%, 11.65%, 9.13%, and 9.19% in the HE group, respectively (Figure1D). Moreover, 21 genera showed relative abundance over 0.5%, and the predominant genera were *unidentified_Ruminococcaceae*, *unidentified_Bacteroidales*, *Lactobacillus*, *unidentified_Lachnospiraceae*, *Bacteroides*, and *unidentified_Prevotellaceae* (Figure1E).

Table 3. Alpha-diversity indices of the rectal microbiota of donkeys.

Group[1]	Coverage %	Richness estimator			Diversity index	
		Chao1	ACE	PD_whole_tree	Shannon	Simpson
LE	>99	1282.34 ± 103.21	1301.82 ± 98.15	89.12 ± 10.23	7.82 ± 0.35	0.99 ± 0.01
HE	>99	1251.01 ± 38.41	1271.98 ± 41.49	85.31 ± 2.68	7.61 ± 0.37	0.98 ± 0.01

[1] LE, low digestible energy diet; HE, high digestible energy diet.

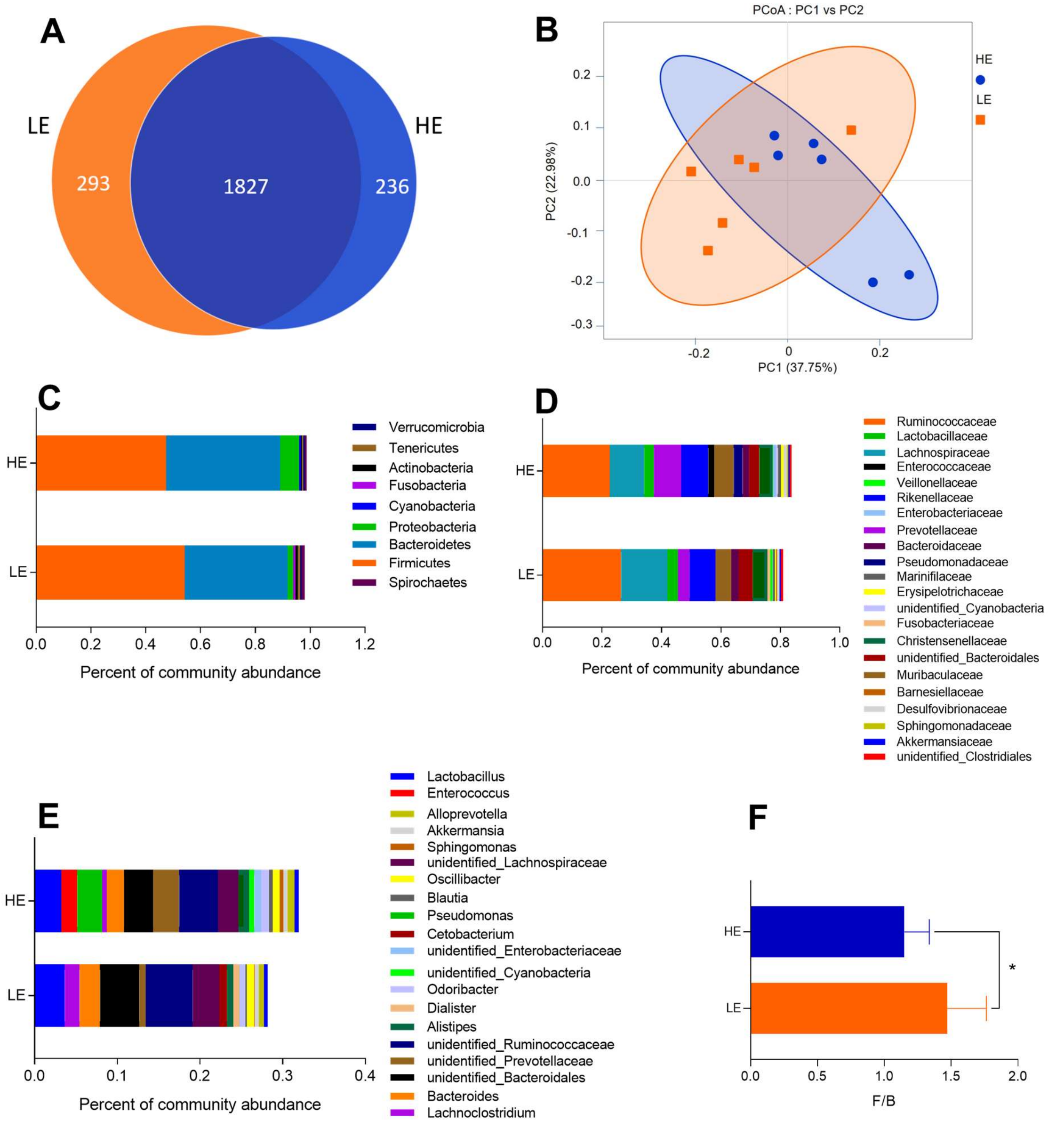

Figure 1. The OTU numbers, beta diversity (PCoA plot), and the relative abundance at the phylum, family, and genus levels of rectum microbiota of donkey fed LE or HE diet. (A) Venn diagram of OTUs in the rectal microbiota, (B) Principal coordinate analysis (PCoA). (C) The ratio of Firmicutes and Bacteroidetes. The relative abundance of bacteria on the right in each group at the phylum (D), family (E), and genus levels (F). LE and HE represent samples collected in the rectum from donkeys fed LE or HE diet. Only microbes that have a mean relative abundance of more than 0.5% are displayed.

3.3. Differences in gut microbiota between donkeys fed LE or HE

Microbial communities were different between LE and HE group at the phylum, family, and genus levels (Figure2). At phylum level (Figure2A), the relative abundances of *Firmicutes*, *Spirochaetes*, and *Elusimicrobia* in the LE group were significantly higher ($P < 0.05$) than those in the HE group, while feeding HE increased the abundance of *Proteobacteria* ($P < 0.05$). At the family level, the relative abundance of *unidentified_Clostridiales* ($P = 0.042$) and *unidentified_Elusimicrobia* ($P < 0.05$) were lower in the HE group than those in the LE group. However, the relative abundance of *Prevotellaceae* and *Succinivibrionaceae* in the HE group were higher ($P < 0.05$) than those in the LE group (Figure2B). At genus level, the abundances of *unidentified_Prevotellaceae* ($P = 0.023$), *Desulfovibrio* ($P = 0.026$), and *Succinivibrio* ($P = 0.04$) in HE group tended be higher than those in the LE group. Contrastingly, the relative abundances of *unidentified_Ruminococcaceae* and *unidentified_Elusimicrobia* ($P < 0.05$) were higher in the LE group than those in the HE group (Figure2C). Similarly, the linear discriminant analysis effect size (LEfSe) analysis indicated that feeding HE to donkeys increased the abundance in the rectum of *Prevotella_ruminicola*, *unidentified_Prevotellaceae*, *Gammaproteobacteria*, *Proteobacteria*, and *Prevotellaceae,* while decreasing the richness of *Bacteroidales_bacterium_Bact_22*, *Selenomonadales*, and *Negativicutes* compared to those in the LE group (Figure2 D, E).

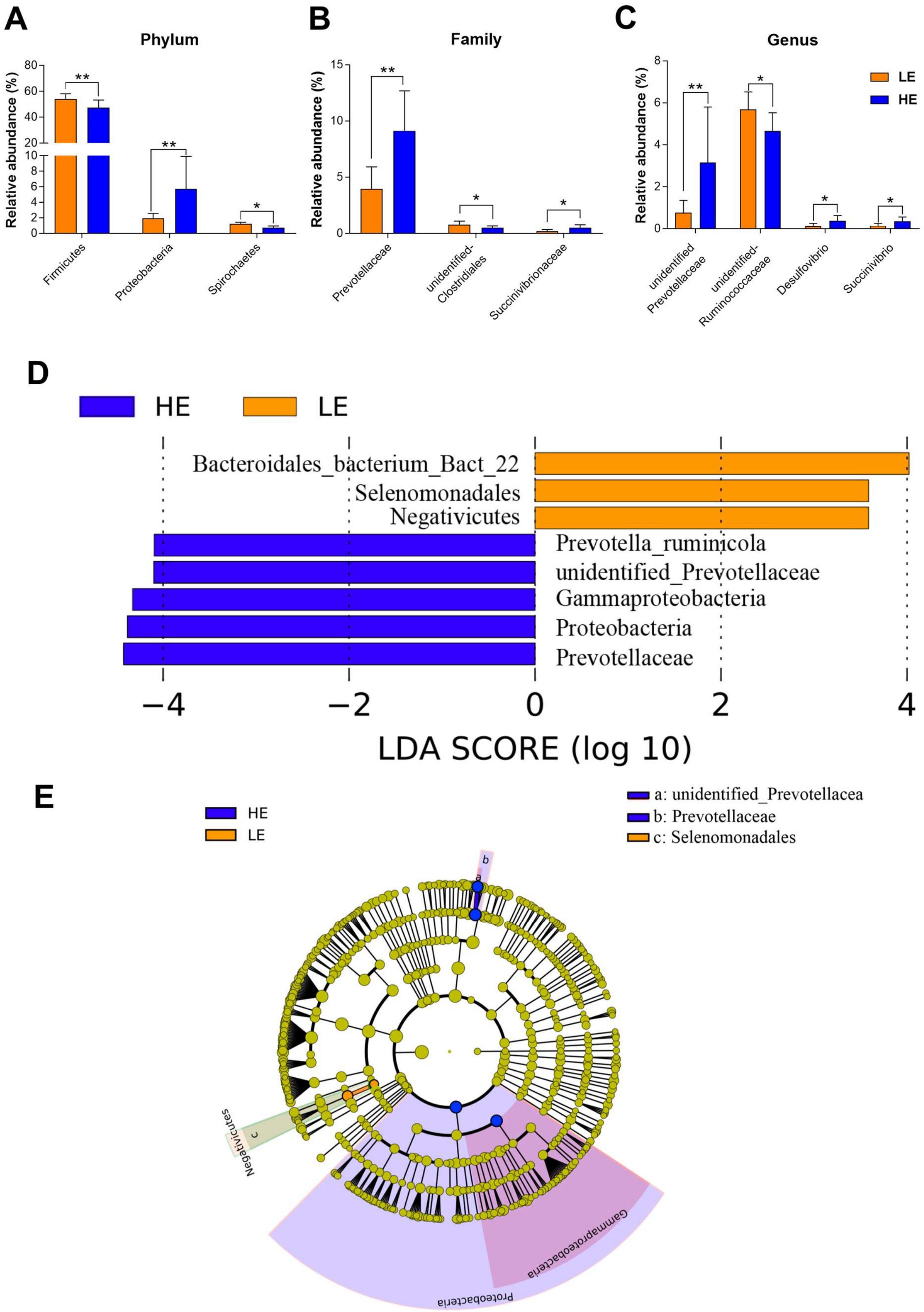
A
Phylum
Relative abundance (%)
80
60
40
20
10
8
6
4
2
0
**
**
*
Firmicutes
Proteobacteria
Spirochaetes
B
Family
Relative abundance (%)
15
10
5
0
**
*
*
Prevotellaceae
unidentified-Clostridiales
Succinivibrionaceae
C
Genus
Relative abundance (%)
8
6
4
2
0
**
*
*
*
LE
HE
unidentified Prevotellaceae
unidentified-Ruminococcaceae
Desulfovibrio
Succinivibrio
D
HE
LE
Bacteroidales_bacterium_Bact_22
Selenomonadales
Negativicutes
Prevotella_ruminicola
unidentified_Prevotellaceae
Gammaproteobacteria
Proteobacteria
Prevotellaceae
−4
−2
0
2
4
LDA SCORE (log 10)
E
HE
LE
a: unidentified_Prevotellacea
b: Prevotellaceae
c: Selenomonadales
b
a
c
Negativicutes
Gammaproteobacteria
Proteobacteria

Figure 2. The relative abundance of rectal microbiota that significantly different between LE and HE groups at the phylum (A), family (B), and genus (C) levels. Only microbes that had a relative abundance of more than 0.1% are compared. Linear discriminant analysis (LDA) value distributed histogram and cladogram of different microorganisms (LDA score > 3.5). (D) Linear discriminant analysis (LDA) value distributed histogram, (E) Cladogram performed on the microbial community relative abundance data at rectum samples between LE and HE groups.

3.4. Profile of rectum metabolites and enrichment of metabolic pathways

The rectum metabolites of donkey fed with LE and HE were analyzed by a non-targeted LC-MS/MS metabolomics platform, and 999 metabolites (positive and negative ions) were detected from two groups. Among them, 137 differentiated (log2FC >1.2， $P < 0.05$, Variable Importance in the Projection (VIP >1) metabolites were identified, (Figure3C; Supplementary Tables S4, 5.). To compare the distribution of the ruminal metabolites of the two groups, the orthogonal projections to latent structures discrimination analysis (OPLS-DA) was conducted (Fig.3. A, B). The results displayed a completely separated clustering between HE and LE group, suggesting that fecal metabolites were typically differentiated by the energy level of diets. Compared to LE group, feeding HE increased ($P < 0.05$) the concentrations of L-aspartic acid, ornithine, L-glutamine, L-phenylalanine, L-serine, methionine, lysine, L-isoleucine, and N-acetylaspartic acid, while decreased ($P < 0.05$) the concentrations of phenylpyruvic acid and argininosuccinic acid in gut content of donkey. Additionally, by matching 137 differentiated metabolites with the library of pathway-associated metabolites sets (SMPDB), 28 metabolic pathways were enriched mainly involving the growth-related essential amino acids metabolism and energy metabolism (Figure3D). Of them, arginine biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, alanine, aspartate and glutamate metabolism, phenylalanine metabolism were significantly altered by differentiated dietary energy content.

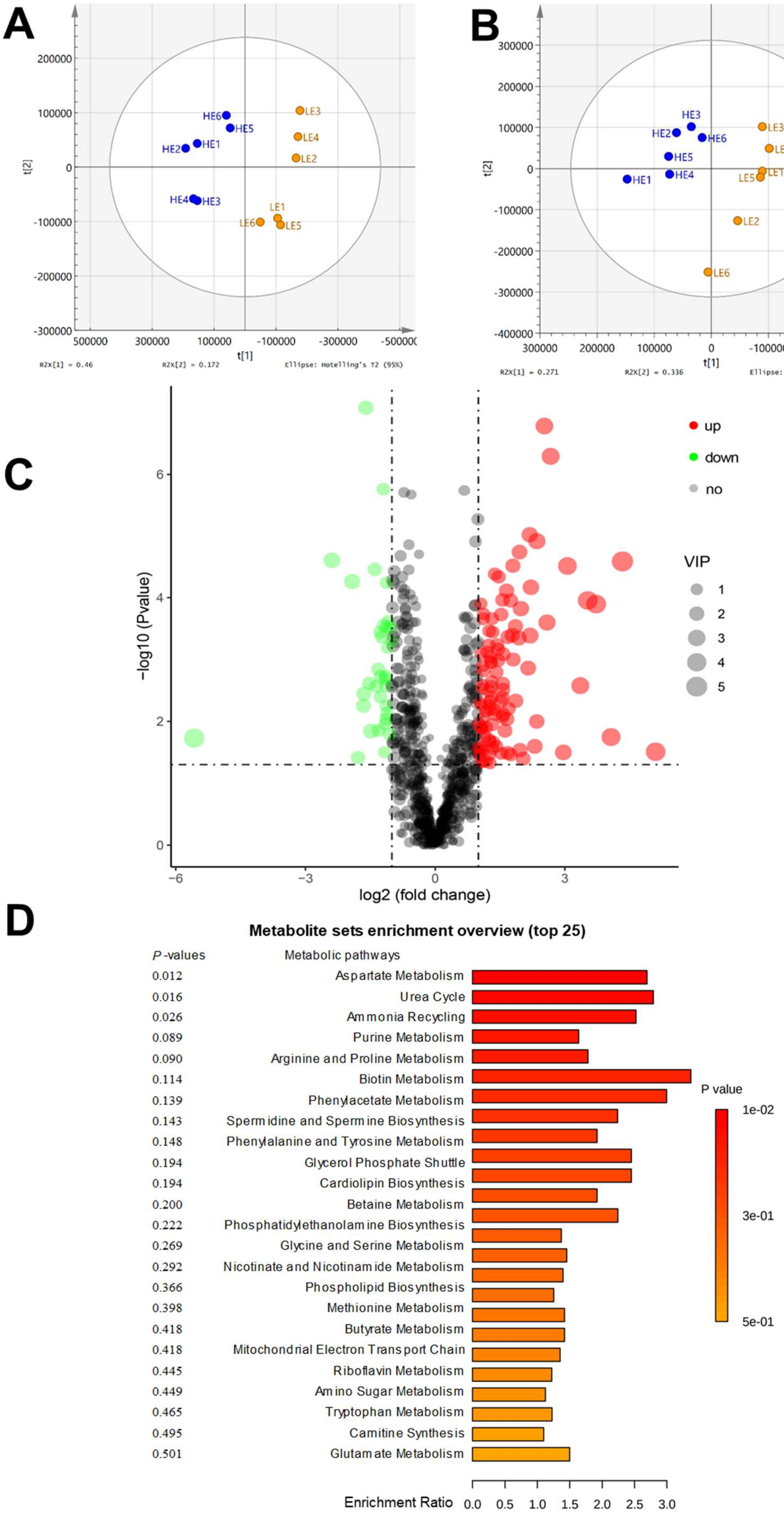
A
B
C
D
t[1]
t[2]
R2X[1] = 0.46
R2X[2] = 0.172
Ellipse: Hotelling's T2 (95%)
R2X[1] = 0.271
R2X[2] = 0.336
LE
HE
HE1
HE2
HE3
HE4
HE5
HE6
LE1
LE2
LE3
LE4
LE5
LE6
up
down
no
VIP
1
2
3
4
5
−log10 (Pvalue)
log2 (fold change)
Metabolite sets enrichment overview (top 25)
P-values
Metabolic pathways
0.012 Aspartate Metabolism
0.016 Urea Cycle
0.026 Ammonia Recycling
0.089 Purine Metabolism
0.090 Arginine and Proline Metabolism
0.114 Biotin Metabolism
0.139 Phenylacetate Metabolism
0.143 Spermidine and Spermine Biosynthesis
0.148 Phenylalanine and Tyrosine Metabolism
0.194 Glycerol Phosphate Shuttle
0.194 Cardiolipin Biosynthesis
0.200 Betaine Metabolism
0.222 Phosphatidylethanolamine Biosynthesis
0.269 Glycine and Serine Metabolism
0.292 Nicotinate and Nicotinamide Metabolism
0.366 Phospholipid Biosynthesis
0.398 Methionine Metabolism
0.418 Butyrate Metabolism
0.418 Mitochondrial Electron Transport Chain
0.445 Riboflavin Metabolism
0.449 Amino Sugar Metabolism
0.465 Tryptophan Metabolism
0.495 Carnitine Synthesis
0.501 Glutamate Metabolism
P value
1e-02
3e-01
5e-01
Enrichment Ratio
0.0
0.5
1.0
1.5
2.0
2.5
3.0

Figure 3. Orthogonal partial least squares discriminant analysis [OPLS-DA] plot of rectal metabolites in comparisons of the LE and HE groups following (A) Positive ions electrospray ionization (ESI+) and (B) negative ions electrospray ionization (ESI-). (C) Identification of the differentially metabolites between LE and HE groups. Red represents a up regulation, while green represents a down regulation. Blue represents no change. (D) Enrichment analysis of metabolic pathways.

3.5. Correlations between donkey performance, rectal differentiated bacteria, metabolites, and modified metabolic pathways

A Pearson's correlation analysis was performed to evaluate the correlation between the performance, intestinal differentiated bacteria, and metabolites (Figure4). The associations between microbes and metabolites provide a comprehensive understanding of the composition and function of microbiota.

The predominant genera of *unidentified_Prevotellaceae* was positively correlated with ADG and G/F, while *unidentified_Ruminococcaceae* was negatively correlated with the growth parameters (Figure4A). *Unidentified_Prevotellaceae,* which showed increased abundance in the HE group, was negatively associated with phenylpyruvic acid and argininosuccinic acid, while showing a positive correlation with L-aspartic acid, ornithine, L-glutamine, L-phenylalanine, L-serine, methionine, lysine, L-isoleucine, and N-acetylaspartic acid (Figure 4B). Furthermore, those metabolites showed a positive correlation with BW, ADG, and G/F (Figure 4C). The pathway analysis results for the differential metabolites revealed that several metabolite concentrations including argininosuccinic acid, citrulline, L-aspartic acid, ornithine, L-glutamine, L-phenylalanine, L-serine, methionine, lysine, L-isoleucine, phenylpyruvic acid, and N-acetylaspartic acid were related to arginine biosynthesis, aminoacyl-tRNA biosynthesis, phenylalanine, tyrosine, and tryptophan biosynthesis and alanine, aspartate and glutamate metabolism (Figure4D). In contrast, *unidentified_Ruminococcaceae,* which showed decreased richness in the HE group compared to that in the LE group, was negatively associated with L-aspartic acid, L-glutamine, methionine, lysine, and N-acetylaspartic (those metabolites were positively correlated with ADG and G/F), while showing a positive association with argininosuccinic acid and phenylpyruvic acid, which negatively correlated to ADG and G/F (Figure4C). Additionally, *Alistipes* and *unidentified_Enterobacteriaceae* were positively associated with citrulline, L-serine, L-glutamine, L-phenylalanine, methionine, and L-isoleucine. These metabolites including citrulline, L-aspartic acid, L-glutamine, L-phenylalanine, L-serine, methionine, lysine, L-isoleucine, and N-acetylaspartic acid were related to aminoacyl-tRNA biosynthesis, phenylalanine, tyrosine, and tryptophan biosynthesis, and alanine, aspartate, and glutamate metabolism (Figure4D).

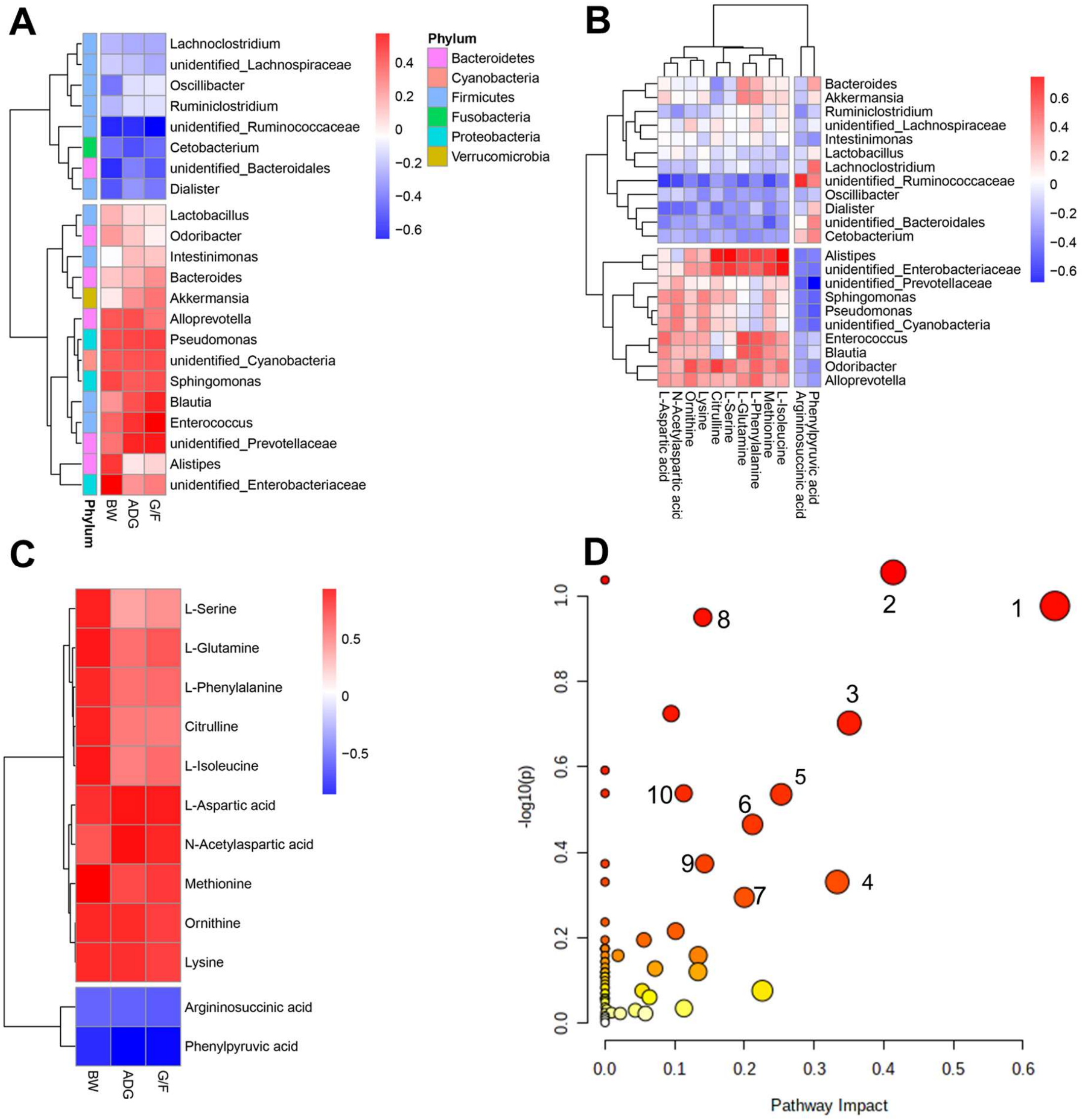

Figure 4. Correlations between bacteria in which the relative abundance more than 0.5% and performance parameters (A), differential metabolites and bacteria (B), and differential metabolites on pathways differed significantly ($P < 0.05$) and performance parameters (C). Each row in the graph represents a metabolite, each column represents a performance parameter, and each lattice represents a Pearson correlation coefficient. Red represents a positive correlation, while blue represents a negative correlation. Pathway analysis of differential metabolites (D). the manipulated metabolic pathways based on the analysis of differentiated ruminal metabolites of donkeys fed HE or LE diets following the Bos Taurus KEGG pathway database. The metabolome view shows all matched pathways according to the p-values from the pathway enrichment analysis and impact values from the topology analysis. The node colors varied from yellow to red means the metabolites are in the data with different levels of significance. 1. Aspartate Metabolism, 2. Urea Cycle, 3. Cardiolipin Biosynthesis, 4. Glycerol Phosphate Shuttle, 5. Glycerolipid Metabolism, 6. Arginine and Proline Metabolism, 7. Malate-Aspartate Shuttle, 8. Ammonia Recycling, 9. Amino Sugar Metabolism, 10. Histidine Metabolism.

4. Discussion

The results of this study demonstrated that feeding HE to donkeys enhanced their growth performance in terms of ADG and G/F, which was consistent with previous studies. Ge, Wang, Ying, Zhang, Su, Cheng, Feng, Zhou, Zhang and Wang [4] demonstrated that broilers fed HE had greater G/F than the basal energy groups, while Fang, *et al.* [28] indicated that G/F decreased as dietary metabolic energy level decreased over the entire experimental period. Moreover, Ahmad, *et al.* [29] found that G/F was significantly higher ($P < 0.001$) with an increased dietary energy level. Our results suggested that the dietary energy content changed the composition and abundance of gut microbial community, especially specially increased the abundance of *Prevotellaceae*, and thus modulated some growth-related metabolism pathways manipulated the metabolic pathways, which contributed to the improvement of donkey production performance.

The composition (structure and diversity) and function (metabolic mechanism) of the intestinal microbiome are crucial to animal health and metabolism [30] in the highly dynamic symbiotic relationship. Visconti, *et al.* [31] indicated an intense interplay between the gut microbiome and its host. In this study, alpha-diversity analysis showed no significant ($P > 0.05$) difference in the rectal bacterial community between two dietary energy levels. Our previous studies characterized the special host bacterial community, which might have played an important role in this study [27].

More than 90% of the species in the bacteria community belong to *Firmicutes* and *Bacteroidetes* [32]. In our study, *Firmicutes* and *Bacteroidetes* were the dominant bacteria, agreeing with the observations from previous studies on microbial communities of monogastric herbivorous animals. For example, Su, Zhao, Liu, Liu, Du, Wu, Bai, Li, Bou, Zhang and Dugarjaviin [30] found that the phyla with greatest abundances were the *Firmicutes* (55.01%) and *Bacteroidetes* (24.76%) in horse, while Liu, *et al.* [33] pointed out that they are abundant (both accounting for >40%) in the hindgut of Dezhou donkey. Moreover, Zhao, *et al.* [34] demonstrated that *Firmicutes* and *Bacteroidetes* were the most abundant and predominant phyla in fecal samples of horse. In this study, the proportion of *Firmicutes* and *Bacteroidetes* were similar in the gut microbiomes between the HE and LE group and played an important role in the rectum. *Firmicutes* are involved in the degradation of structure carbohydrates, such as cellulose and hemicellulose [30,35]. Meanwhile, *Bacteroidetes* can break down protein and fast-fermentable carbohydrates [36,37]. *Ruminococcaceae* is a family in the phylum *Firmicutes*, and *Prevotellaceae* is the dominant family in the phylum *Bacteroidetes*. In this study, *unidentified_Prevotellaceae* and *unidentified_Ruminococcaceae* in the HE group had significantly higher and lower relative abundance, respectively, than those in the LE group. This could be explained by HE having more starch, as the proportion of corn was higher than that in LE. Sanchez-Tapia, *et al.* [38] stated that *Prevotella* is associated with the intake of carbohydrates and simple sugars. In addition, cellulolytic *Clostridia*, which are ubiquitous in cellulosic anaerobic environments, represent a major paradigm for efficient biological degradation of cellulosic biomass [39]. In our study, *Lachnospiraceae unidentified_Clostridiales* were more abundant in LE- than in HE-fed donkeys, which may be explained by LE having more fiber sources of peanut vine and alfalfa than HE. *Lachnospiraceae* is the dominant family in the order *Clostridiales*, within the phylum *Firmicutes* [40]. It belongs to the *Fibrolytic* communities and has been associated with the production of butyrate necessary for the health of colonic epithelial tissue [41]. *Clostridia* are known as major producers of short-chained fatty acids, which are important energy sources for enterocytes and also exert immunoregulatory functions [42], and presenting a large proportion of *Clostridia* is likely to be important for cellulolytic capabilities [43].

The gut microbiota interacts with the diet [37] and impacts host physiology and metabolism [32]. When the diet provides adequate amounts of protein, the increment in energy levels produced by adding carbohydrate can improve protein synthesis [44]. In this study, we found that feed with different energy levels significantly altered the concentration of most metabolites associated with protein digestion and absorption as well as biosynthesis of amino acids. Aminoacyl-tRNA synthetases are an essential and universally distributed family of enzymes that play a critical role in protein synthesis, pairing tRNAs with their cognate amino acids for decoding mRNAs [45,46]. In our study, L-phenylalanine, L-Glutamine, L-Aspartate, L-Serine, L-Methionine, L-Lysine, and L-isoleucine were upregulated in the HE group, which indicated that HE can promote the synthesis of protein. In the HE group, we observed increased levels of L-phenylalanine, which has specific characteristics that make it useful as marker of protein metabolism. Glutamate is the largest contributor to tricarboxylic acid cycle intermediate fluxes, and its dietary composition alters L-glutamate catabolism [47]. Despite cysteine and methionine metabolism having no impact (only 2 hits), the level of L-Methionine in HE-fed donkeys was higher ($P < 0.05$) than that in LE-fed donkeys. L-Methionine is a precursor to other sulfur-containing amino acids and the essential and limiting amino acid for donkey growth and production [48]. In addition, L-Methionine also plays important role in intestinal bacteria protein synthesis.

The abundance and composition of intestinal microbiota was believed to be correlated with energy harvesting and performance[49]. In this study, changes in intestinal microbial abundance induced by diet resulted in a shifted metabolome of intestinal microbiota, as shown by Pearson's correlation analysis. *Unidentified_Ruminococcaceae* was negatively associated with ornithine, L-phenylalanine, and L-Arginine, while showing a positive correlation with argininosuccinic acid and phenylpyruvic acid. Phenylpyruvic acid and argininosuccinic acid had a negative correlation with donkey performance, possibly owing to LE altering the arginine biosynthesis pathway and arginine synthesis from argininosuccinic acid, which could cause excessive ammonia in the body that is harmful to donkey growth and health. Moreover, the phenylalanine, tyrosine, and tryptophan biosynthesis pathway were altered and presented phenylalanine synthesis. *Unidentified_Prevotellaceae* was negatively correlated with phenylpyruvic acid and argininosuccinic acid, while showing a positive correlation with ornithine, L-phenylalanine, and L-Arginine. This suggested that higher production of microbiota-derived amino acids may be positively correlated with higher performance in donkeys. Phenylpyruvate is an intermediate product of the conversion from phenylalanine to valine. The accumulation in body means that the transformation pathway is blocked, which often leads to phenylpyruvuria and the deficiency of valine. Valine is a precursor to the synthesis of a range of neurotransmitters and hormones in animals, and its lack can lead to poor growth performance. Argininosuccinic acid is an important component involved in the ornithine cycle in the body, and its enrichment (high content) generally indicates that the excretion of ammonia was blocked. High concentration of Arginine succinate and ammonia will cause cytotoxicity and reduced energy utilization, and causally resulted in the decreased healthy condition and productivity[50].

Several studies have found that both positive and negative associations between specific gut bacteria and metabolism and intestinal microbiota are associated with various metabolic pathways, such as lipid metabolism and amino acid synthesis [51,52]. Our metabolome data revealed that different energy diets alter the concentrations of metabolites in the rectum, and indicate that rectal metabolism might be linked with microbial activities. Additionally, Koh and Backhed [53] demonstrated that an absence of microbial compositional changes does not necessarily mean an absence of microbial contributions to host metabolism. However, there is not enough evidence to indicate the microbe species and counts relation with certain metabolic products in gastrointestinal samples. Thus, additional efforts have to be directed toward increasing our knowledge in terms of causality and mechanisms.

Taken together, compared to LE group, feeding HE diet to donkeys characterized the gut microbiome with a low diversity but improved richness of certain specific efficient bacteria. Moreover, there were tight correlation between the abundance of microbes, contents of metabolites, and performance in the HE group. This revealed the interaction of dietary energy content and gut microbiota reshaped the exclusive configuration of microbial community, which underlay the alteration of the growth-promoting metabolites and metabolic pathways and thus resulted in the increased production performance of lambs. It has been suggested that increased enrichment of specific microbes and metabolic pathways rather than the greater diversity contributed to the better energy harvest and improved production performance of animals [49,54]. This was consistent with our current results.

The present results supported our hypothesis that feeding HE diet altered the gut microbiome and metabolome, and upregulated the growth-related metabolic pathways, which underlay the increased production performance and feed efficiency. Additionally, this study provided a new viewpoint in understanding the underlying mechanism of diet energy content impacting the growth performance of animals by intervening the axis of 'microbiome-metabolome-phenotypes' to achieve the superior productivities of animals. The findings also suggested that the specific growth-related microbes and metabolites may be the potential targets for modifying the production performance of animals by specific diet consumption, which provided the novel perspective for developing a dietary strategy to improve the production performance of donkeys reared in the intensive feeding system.

5. Conclusions

In conclusion, feeding HE diet improved the growth performance and feed efficiency of donkeys. The donkey fed HE or LE diet had the similar microbial community, but feeding HE diet to donkeys specially increased the relative abundance of *unidentified_Prevotellaceae*, while decreasd the relative abundance of *Unidentified_Ruminococcaceae* in the rectum. Meanwhile, HE diet improved the content of L-aspartic acid, ornithine, L-glutamine, L-phenylalanin, L-serine, methionine, lysine, L-isoleucine, and N-acetylaspartic acid in gut content of donkeys, and thus affected some growth-related metabolic pathways mainly involving the Aspartate Metabolism and Urea Cycle. Those increased bacteria and metabolites exhibited the positive correlation with the average daily gain (ADG) and feed efficiency of donkeys. Thus, HE diet can increase the richness of beneficial bacteria and thus modified the growth-related metabolic pathways, which contributed to the improved performance and feed efficiency of donkeys. These beneficial bacteria and metabolites related to dietary energy concentration are potential targets for regulating growth performance. The present findings also provided an innovative insight for developing the new growth-promoting probiotics and prebiotics.

Conflict of interest statement

The authors declare that they have no competing interests.

Author contributions

GZ and and YL designed the experiments. CZ and YPW, MYD conducted the experiments. YPW and MYD performed the determination of samples. CYZ and CZ analyzed the data. CZ, GZ, and YL wrote and revised the manuscript. All authors read and approved the final manuscript.

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