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Research Article

Shifts in the microbial community and metabolome in rumen ecological niches during antler growth

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ABSTRACT

Antlers are hallmark organ of deer, exhibiting a relatively high growth rate among mammals, and requiring large amounts of nutrients to meet its development. The rumen microbiota plays key roles in nutrient metabolism. However, changes in the microbiota and metabolome in the rumen during antler growth are largely unknown. We investigated rumen microbiota (liquid, solid, ventral epithelium, and dorsal epithelium) and metabolic profiles of sika deer at the early (EG), metaphase (MG) and fast growth (FG) stages. Our data showed greater concentrations of acetate and propionate in the rumens of sika deer from the MG and FG groups than in those of the EG group. However, microbial diversity decreased during antler growth, and was negatively correlated with short-chain fatty acid (SCFA) levels. *Prevotella*, *Ruminococcus*, *Schaedlerella* and *Stenotrophomonas* were the dominant bacteria in the liquid, solid, ventral epithelium, and dorsal epithelium fractions. The proportions of *Stomatobaculum*, *Succiniclasticum*, *Comamonas* and *Anaerotruncus* increased significantly in the liquid or dorsal epithelium fractions. Untargeted metabolomics analysis revealed that the metabolites also changed significantly, revealing 237 significantly different metabolites, among which the concentrations of γ-aminobutyrate and creatine increased during antler growth. Arginine and proline metabolism and alanine, aspartate and glutamate metabolism were enhanced. The co-occurrence network results showed that the associations between the rumen microbiota and metabolites different among the three groups. Our results revealed that the different rumen ecological niches were characterized by distinct microbiota compositions, and the production of SCFAs and the metabolism of specific amino acids were significantly changed during antler growth.

1. Introduction

A unique hallmark of ruminant animals belonging to the family Cervidae, that distinguishes these species from other ruminants, is the presence of a renewable organ, the antler [1]. It is well recognized that the growth cycle of antlers has a certain periodicity, including antler casting in early spring, rapid longitudinal bone and cartilage growth in late spring and summer, and calcification in autumn [2]. Antlers also exhibit marked similarities to human limbs, containing bone, cartilage, skin, blood vessels and nerves [3]. Importantly, antlers are the fastest growing bones in the animal kingdom, reaching a maximum growth rate of 2.75 cm/day and a mineral apposition rate of 3.2 μm/day [4]. These findings suggest that bone is the fundamental component in antler growth. Understanding the mechanisms underlying the rapid growth of

Abbreviations: SCFAs, short-chain fatty acids; ASVs, amplicon sequence variants; PCoA, principal coordinates analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; PLS-DA, partial least squares-discriminant analysis; VIP, variable importance in the projection; DHMA, 3,4-dihydroxymandelic acid; GABA, γ-aminobutyrate; NANA, N-acetyl-D-neuraminic acid; IAA, indoleacetic acid; H_2S, hydrogen sulfide; SRB, sulfate-reducing bacteria.

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deer antlers is not only fascinating for bone biologists but will also improve the production of velvet antlers, which are used in traditional Chinese medicine.

The rapid growth of antlers appears to be determined by genetic factors and influenced by environmental factors. Our comparative transcriptomic analyses revealed the important roles of the Wnt pathway and epidermal growth factor receptor signaling pathway in antler growth [5]. Single-cell RNA-sequencing analyses have further revealed that antler blastema progenitor cells serve as a stem cell pool for antler growth [6]. However, with continuous and fast growth, antlers must obtain enough nutrients to support their elongation and full development. It is reported that the voluntary feed intake and growth rate of Cervidae peak in summer in summer, the period of antler growth, and reaches a minimum in winter [7]. The rumen tissue weight and moisture of the rumen digesta of wild sika deer were found to be greater in both summer and autumn, than in winter [8]. Moreover, Asano et al. reported that the number of protozoa in the rumen was higher in summer than in spring, and the molar proportions of propionate and butyrate in the rumen changed significantly according to season [9]. Furthermore, antler composition and quality are strongly influenced by dietary quantity and quality during antler growth [10,11]. These findings indicate that the microbial community in the digestive tract might also change during antler growth.

The rumen houses a complex microbial community that enables the host to utilize nutrients in feed through microbe-mediated fermentation, with the production of short-chain fatty acids (SCFAs), having a critical impact on host energy metabolism and physiology [12]. By comparing the rumen microbiota and metabolites among sika deer, elk, and their hybrids, we previously identified that critical microbial genera (e.g., *Prevotella*, *Succinivibrio*, *Quinella,* and *Fibrobacter*), metabolites (butyrate), and the associated pathways (alanine, arginine, proline and phenylalanine metabolism pathways) in the rumen are associated with the weight of deer antlers [13]. We also revealed that the production of butyrate is affected by the rumen microbiota, including *Prevotella* and *Succinivibrionaceae*, under different dietary compositions [14]. These results suggested a possible association between the rumen microbiota and antler growth. Results for other mammals showed that butyrate exerts regulatory control over bone development by activating the Wnt pathway in osteoblasts [15]. These findings led us to hypothesize that the rumen microbiota and its associated metabolites change during antler growth. Growing evidence indicates that the rumen microbiota also varies internally among ecological niches, including the solid, liquid, ventral epithelium, and dorsal epithelium microbiota [16]. The solid and liquid microbiota are involved in the degradation of plant cellulose and metabolism of soluble nutrients, respectively [16], while the epithelial microbiota (ventral and dorsal fractions) mainly play roles in oxygen scavenging [17] and energy utilization [18]. However, the changes of the microbial community in different rumen ecological niches during antler growth remain unclear.

In the present study, we used captive sika deer as a model and applied 16S rRNA gene sequencing and metabolomic analysis to reveal changes in the rumen microbial community in different ecological niches and the changes in metabolites during antler growth.

2. Materials and methods

2.1. Animals and sample collection

A total of 15 healthy, 4 year old, male, sika deer were used in this study, consisting of 5 sika deer at velvet antler casting (0-day, early antler growth, EG group), 5 sika deer at velvet antler grow 30 days since the casting (metaphase antler growth, MG group), and 5 sika deer at velvet antler grow 45 days after the casting (fast antler growth, FG group). Each sika deer was raised in an independent pen, and was fed twice daily at 7:00 a.m. and 16:00 p.m., with free access to clean drinking water. The animals were fed the same total mixed ration (TMR) based on roughage and concentrate (45:55, dry matter basis, Table S1) during antler growth. Animals were cared for in accordance with the guidelines for animal research of the Animal Ethics Committee of Jilin Agricultural University (Approval No: 20210314002).

The animals were slaughtered 3 h after the morning feed by intravenous injection of sodium thiopental (0.125 mg/kg BW). The whole rumen contents were mixed and homogenized, and then filtered with four layers of cheesecloth to separate the rumen liquid and rumen solid fractions. The dorsal and ventral rumen wall (approximately 8 cm^2) were collected and washed three times with a cold sterile saline solution. The epithelium associated microbes were scraped with a sterilized slide. All samples were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. DNA extraction, 16S rRNA gene amplification, sequencing and bioinformatic analysis

Total genomic DNA from all fractions were extracted using the bead-beating method [19]. The V3-V4 region of the bacterial 16S rRNA genes was amplified using the primer pair 338F and 806R. The resultant amplicons were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, CA, USA) and quantified using a Nanodrop 2000 (Thermo Fisher Scientific, Madison, USA). The pooled PCR products were used to construct Illumina MiSeq platform (Biozeron Biological Technology Co. Ltd., Shanghai, China). The raw sequences were quality controlled used FASTP [20], and assembled into contigs using FLASH [21]. The sequences were used to cluster amplicon sequence variants (ASVs) with 100 % similarity cutoff by DADA2 [22] and UCHIME [23] was used to identify and remove chimeric sequences. The representative sequences of each ASVs were analyzed by uclust algorithm against the SILVA database (SSU138) using a confidence threshold of 0.8 [24]. The alpha diversity including Shannon and Simpson indices was calculated by the microeco package. The principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity, Weighted UniFrac distance, and Unweighted UniFrac distance were used to evaluate the bacterial community. PERMANOVA was used to indicate group differences and P values were determined based on 999 permutations.

2.3. Measuring of metabolites in rumen liquid and analysis

The concentration of SCFAs in rumen liquid was determined using a gas chromatograph (7890B, Agilent, UK) [25]. The ammonia concentration was measured with a UV-1201 spectrophotometer (Shimadzu, Kyoto, Japan) [26]. In addition, frozen rumen liquid (20 mg) was weighted, mixed with 1 mL 40 % methanol and then centrifuged at 12, 000 rpm for 15 min at 4 °C. The supernatants and quality control (QC) sample mixed were analyzed by the ultra-high performance liquid chromatography (UHPLC) system. The Orbitrap Exploris 120 mass spectrometer was used to acquire MS/MS spectra on information-dependent acquisition (IDA) mode in the control of the Xealibur software (Thermo Fischer Scientific, Waltham, MA). The raw data were processed by XCMS software, filtered for deviations, and normalized based on public databases, including online KEGG and HDMB [27]. The concentration of metabolites was applied to log transformation and anto scaling using MetaboAnalyst 5.0 [28], and then used to conduct the partial least squares-discriminant analysis (PLS-DA). The significantly changed metabolites (MG vs EG, FG vs MG, and FG vs EG) were identified based on variable importance in the projection (VIP) values that exceeded 1.0 and $P < 0.05$ (independent t-test), which was used to conduct the enrichment analysis based on KEGG databases ($P < 0.05$).

2.4. Co-occurrence network and statistical analysis

The Spearman's correlation coefficient between metabolites and microbiota within the EG, MG and FG group was analyzed using the

Hmisc package [29]. The significant correlations ($P \leq 0.05$, $|rho| > 0.8$) were visualized using Gephi [30]. The differences for the concentrations of SCFAs and ammonia, alpha diversity indices, and the relative abundances of taxonomy and KEGG pathways among the three groups were determined by one-way analysis of variance (ANOVA). The P values were corrected using the false discovery rate of the Benjamini-Hochberg method, and a P value < 0.05 was regarded as statistically significant. If the ANOVA comparison indicated significance, we also applied the Wilcoxon rank sum (WRS) test to determine the significance of each comparison between two groups. The values are expressed as the mean ± standard error of mean (SEM) unless otherwise indicated.

3. Results

3.1. Significantly increased production of rumen SCFAs during antler growth

We first aimed to determine whether the rumen metabolic profiles changed during antler growth. The results showed that that the concentrations of total SCFAs were significantly greater in the MG and FG groups than that in the EG group, and the concentration of ammonia in the FG group was also greater than that in the MG group ($P < 0.05$, Table 1). Moreover, the concentrations of beneficial SCFAs, such as acetate and propionate, in the rumens of the FG and MG groups were significantly greater than those in the rumen of the EG group ($P < 0.05$). However, the molar proportions of isobutyrate and isovalerate in the MG and FG groups were lower than those in the EG group ($P < 0.05$). These results indicate that nutrient metabolism in the rumen differs during antler growth, which is likely due to perturbations in the rumen microbiota.

3.2. Characteristics of the rumen microbiota in solid, liquid, and dorsal and ventral epithelium fractions during antler growth

16 S rRNA high-throughput sequencing generated a total of 2,254,213 high-quality reads from the four sample fractions from the EG, MG and FG groups, with an average of 37,570 reads per sample (17,336 to 52,931, Table S2). Taxonomic classification showed that Bacteroidota, Bacillota, Pseudomonadota, Actinomycetota and Fibrobacterota were the most prevalent phyla in all groups (Fig. 1A, Tables S3–S6). In the dorsal epithelium, *Prevotellaceae* (EG = 32.47 ± 5.00 %; MG = 37.21 ± 5.14 %; FG = 26.64 ± 6.08 %), *Lachnospiraceae* (EG = 18.63 ± 2.83 %; MG = 16.93 ± 1.05 %; FG = 21.08 ± 3.14 %) and *Oscillospiraceae* (EG = 9.06 ± 1.08 %; MG = 9.83 ± 2.73 %; FG = 8.85 ± 1.14 %) were the prevalent families, and *Prevotella* (EG = 30.81 ± 4.56 %; MG = 35.92 ± 5.08 %; FG = 25.76 ± 5.86 %), *Ruminococcus* (EG = 5.75 ± 0.94 %; MG = 8.52 ± 2.51 %; FG = 7.12 ± 0.87 %) and *Schaedlerella* (EG = 5.69 ± 2.09 %; MG = 5.74 ± 0.90 %; FG = 6.58 ± 1.54 %) were the most prevalent genera. In the rumen liquid, *Prevotellaceae* (EG = 39.54 ± 1.51 %; MG = 37.99 ± 2.83 %; FG = 28.82 ± 7.14 %), *Lachnospiraceae* (EG = 9.66 ± 0.94 %; MG = 11.46 ± 0.46 %; FG = 12.85 ± 1.56 %) and *Oscillospiraceae* (EG = 9.04 ± 0.90 %; MG = 13.23 ± 3.30 %; FG = 12.23 ± 1.82 %) were the prevalent families, and *Prevotella* (EG = 36.82 ± 1.06 %; MG = 36.22 ± 2.62 %; FG = 26.84 ± 6.63 %), *Ruminococcus* (EG = 5.52 ± 0.98 %; MG = 11.57 ± 3.05 %; FG = 9.11 ± 1.26 %) and *Schaedlerella* (EG = 1.61 ± 0.35 %; MG = 2.88 ± 0.72 %; FG = 3.47 ± 0.93 %) were the most prevalent genera. In the rumen solids, *Prevotellaceae* (EG = 35.43 ± 3.62 %; MG = 38.99 ± 4.89 %; FG = 40.02 ± 1.51 %), *Lachnospiraceae* (EG = 16.09 ± 1.82 %; MG = 16.74 ± 1.61%; FG = 15.79 ± 1.46%) and *Oscillospiraceae* (EG = 9.20 ± 1.02%; MG = 8.19 ± 1.72 %; FG = 9.62 ± 0.88 %) were the prevalent families, and *Prevotella* (EG = 33.89 ± 3.65%; MG = 37.45 ± 4.66 %; FG = 37.88 ± 1.15 %), *Ruminococcus* (EG = 6.02 ± 0.60 %; MG = 6.54 ± 1.52 %; FG = 7.64 ± 1.14%) and *Schaedlerella* (EG = 3.27 ± 0.79 %; MG = 5.78 ± 1.29 %; FG = 4.66 ± 1.16 %) were the most prevalent genera. In the ventral epithelium, *Prevotellaceae* (EG = 37.13 ± 5.05 %; MG = 30.49 ± 5.36 %; FG = 29.65 ± 4.80 %), *Lachnospiraceae* (EG = 13.28 ± 1.38 %; MG = 16.32 ± 1.24 %; FG = 18.14 ± 3.41 %) and *Oscillospiraceae* (EG = 7.38 ± 1.80 %; MG = 8.60 ± 2.46 %; FG = 7.55 ± 0.87 %) were the prevalent families, while *Prevotella* (EG = 35.73 ± 4.93 %; MG = 29.37 ± 5.36 %; FG = 28.21 ± 4.36 %), *Ruminococcus* (EG = 5.03 ± 1.44%; MG = 7.28 ± 2.22 %; FG = 6.08 ± 0.74 %) and *Schaedlerella* (EG = 2.84 ± 0.77 %; MG = 5.21 ± 1.09 %; FG = 5.23 ± 1.97 %) were the most prevalent genera (Tables S3–S6).

When we compared the microbial diversity, the EG group had higher microbial diversity than those in the MG and FG groups, as indicated by the significant differences in alpha diversity measured by the Chao1 index, Shannon index and phylogenetic diversity ($P < 0.05$, Fig. S1A). The alpha diversity indices of the rumen solid from the FG group were significantly higher than those in the liquid fraction (Fig. 1C). The Shannon index and phylogenetic diversity of the rumen liquid from the MG and FG groups were significantly lower than those in the rumen liquid of the EG group ($P < 0.05$, Fig. 1D). Given the significant differences in alpha diversity and SCFAs in rumen liquid, we explored the correlation between the alpha diversity indices and the concentrations of acetate, propionate, and butyrate, and found significant negative correlations ($P < 0.01$, Figs. 1E and S1B).

Table 1
The fermentation parameters in rumen liquid of sika deer among EG, MG and FG groups.

Item	EG	MG	FG	*P*-values
Total SCFAs, mM	$123.98^{b} \pm 7.38$	$157.17^{a} \pm 14.67$	$168.34^{a} \pm 8.03$	< 0.05
Ammonia, mg/100 mL	$30.53^{ab} \pm 4.69$	$27.57^{b} \pm 6.13$	$44.35^{a} \pm 2.80$	< 0.05
Acetate, mM	$76.15^{b} \pm 4.04$	$95.03^{a} \pm 7.55$	$100.29^{a} \pm 4.51$	< 0.05
Propionate, mM	$25.53^{b} \pm 2.32$	$34.45^{a} \pm 3.11$	$35.41^{a} \pm 0.94$	< 0.05
Butyrate, mM	$17.81^{b} \pm 1.70$	$23.76^{ab} \pm 3.92$	$28.08^{a} \pm 2.74$	< 0.01
Valerate, mM	1.94 ± 0.11	2.30 ± 0.49	2.65 ± 0.16	0.28
Isobutyrate, mM	1.20 ± 0.17	0.76 ± 0.12	0.97 ± 0.15	0.15
Isovalerate, mM	1.34 ± 0.20	0.88 ± 0.15	0.93 ± 0.17	0.17
Acetate, (mol/%)	61.53 ± 0.56	60.74 ± 0.84	59.62 ± 0.43	0.14
Propionate, (mol/%)	20.46 ± 0.85	22.04 ± 0.87	21.18 ± 0.91	0.47
Butyrate, (mol/%)	14.28 ± 0.75	14.75 ± 1.15	16.53 ± 1.03	0.28
Valerate, (mol/%)	1.57 ± 0.08	1.43 ± 0.23	1.57 ± 0.03	0.70
Isobutyrate, (mol/%)	$1.02^{a} \pm 0.21$	$0.49^{b} \pm 0.07$	$0.56^{b} \pm 0.07$	< 0.05
Isovalerate, (mol/%)	$1.13^{a} \pm 0.23$	$0.56^{b} \pm 0.08$	$0.54^{b} \pm 0.09$	< 0.05

Note: [a, b,] in the same row indicate the significant differences from each other ($P < 0.05$).

3.3. Changes in the rumen microbiota during antler growth

The PCoA results showed significant qualitative (unweighted UniFrac distance, $P = 0.001$) and quantitative (Bray—Curtis dissimilarity, $P = 0.001$; weighted UniFrac distance, $P = 0.005$; Fig. S2A) differences among the EG, MG and FG groups. Significant differences in microbial community membership and structure were also observed in the rumen solids, liquid, ventral epithelium, and dorsal epithelium fractions for the EG, MG and FG groups, respectively ($P = 0.001$, Fig. S2B). We then examined changes in the rumen microbiota in the different ecological niches for the EG, MG and FG groups, respectively (Fig. 2). Qualitative and quantitative differences were also detected in the MG group (unweighted UniFrac distance, $P = 0.025$; weighted UniFrac distance, $P = 0.015$; Fig. 2B) and FG group (unweighted UniFrac distance, $P = 0.001$; weighted UniFrac distance, $P = 0.022$; Fig. 2C). However, only the microbial community composition in the rumen liquid differed significantly among the EG, MG and FG groups (Bray—Curtis

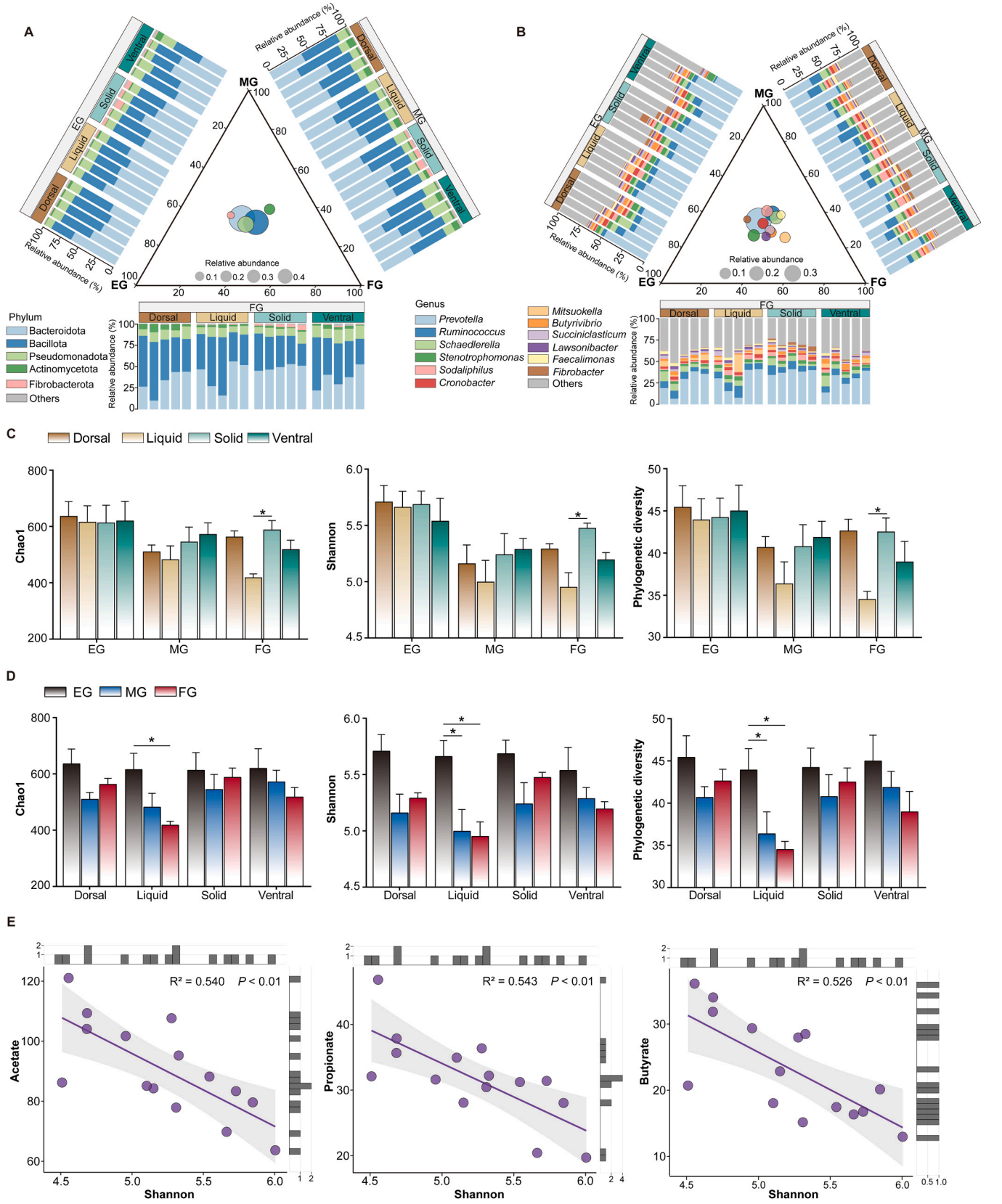

Fig. 1. Microbial community composition and membership in different rumen ecological niches during growth stages. The ternary plot and bar plot showing the microbial composition in the rumen solids, liquid, ventral epithelium, and dorsal epithelium at the phylum (A) and genus (B) levels. Comparison of diversity indices among solid, liquid, and dorsal and ventral epithelium at the same antler growth (C). Comparison of diversity indices among EG, MG and FG group at the same ecology niche (D). Correlation between SCFAs and Shannon index in the rumen liquid (**E**). Significance is designated by using an F-test for testing linear regression coefficients. * indicate $P < 0.05$.

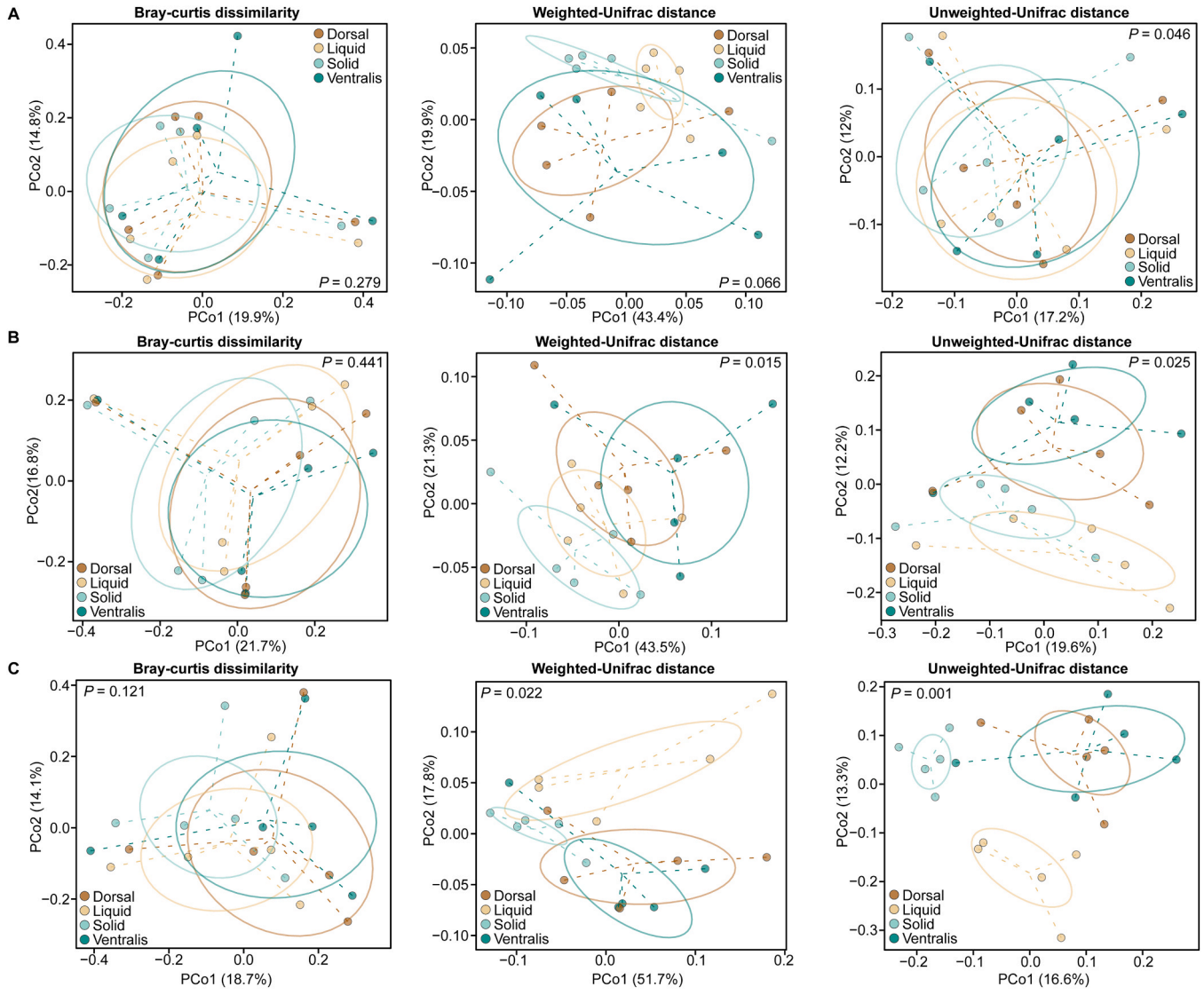

Fig. 2. The variation of rumen microbiota in different rumen ecological niches. PCoA results revealing the change of EG (A), MG (B) and FG (C) groups in dorsal epithelium, liquid, solids, and ventral epithelium samples, respectively, based on Bray-Curtis dissimilarity, weighted UniFrac distance and unweighted UniFrac distance at ASV level.

dissimilarity, $P = 0.007$), although the microbiota in the rumen solid, ventral epithelium, and dorsal epithelium clustered separately (Fig. S3).

We further explored the differences of the microbiota in the rumen dorsal epithelium, liquid, solids, and ventral epithelium among the EG, MG and FG groups, respectively ($P < 0.05$, Fig. 3 and Tables S7–S10). In the rumen dorsal epithelium, the relative abundances of *Succiniclasticum*, *Comamona*, *Olsenella* and *Eisenbergiella* were significantly greater in the FG group (2.52 ± 0.36 %, 0.22 ± 0.08 %, 0.77 ± 0.18 %, 0.21 ± 0.05 %, respectively), than in the EG (1.55 ± 0.33 %, 0.07 ± 0.04 %, 0.23 ± 0.03 %, 0.22 ± 0.06 %, respectively), or MG (1.66 ± 0.13 %, 0.11 ± 0.05 %, 0.43 ± 0.11 %, 0.09 ± 0.02 %, respectively) group. In the rumen ventral epithelium, the relative abundances of *Stomatobaculum* (1.33 ± 0.21 %) and *Lactonifactor* (0.66 ± 0.17 %) were significantly higher in the FG group, than in the EG group (0.75 ± 0.15% and 0.30 ± 0.07 %). In the rumen liquid, the relative abundances of *Stomatobaculum* (1.72 ± 0.32 %) and *Anaerotruncus* (0.29 ± 0.04 %) were significantly higher in the FG group, than in the MG (1.54 ± 0.30% and 0.17 ± 0.06 %), or EG group (1.10 ± 0.25 % and 0.19 ± 0.05 %). In the rumen solid, the relative abundances of *Stomatobaculum* (1.48 ± 0.19 %) and *Olsenella* (0.51 ± 0.08 %) were significantly greater in the FG group, than in the EG group (0.88 ± 0.12 % and 0.22 ± 0.05 %).

We also examined the differences in the microbiota in the different ecological niches for the EG, MG and FG groups, respectively ($P < 0.05$, Fig. S4 and Tables S11–S13). In the EG group, the relative abundances of *Fibrobacter*, *Coprococcus* and *Treponema* were significantly higher in the rumen solid (4.30 ± 1.05 %, 0.80 ± 0.08 %, 1.19 ± 0.28 %, respectively), than in the dorsal epithelium (0.71 ± 0.08 %, 0.47 ± 0.12 %, 0.15 ± 0.02 %, respectively), liquid (0.59 ± 0.26 %, 0.17 ± 0.02 %, 0.04 ± 0.01 %, respectively) and ventral epithelium fractions (0.95 ± 0.20 %, 0.32 ± 0.04 %, 0.35 ± 0.20 %, respectively). Bacteria belonging to the genera *Desulfobulbus* (0.33 ± 0.10 %), *Butyrivibrio* (2.69 ± 0.46 %) and *Enterocloster* (0.91 ± 0.15 %) were significantly enriched in the rumen dorsal epithelium, while *Succiniclasticum* (2.02 ± 0.18 %), *Olsenella* (0.29 ± 0.05 %) and *Anaerovibrio* (0.43 ± 0.08 %) bacteria were significantly enriched in the rumen liquid. In the MG group, the proportions of *Fibrobacter*, *Pseudobutyrivibrio*, *Saccharofermentans* and *Herbinix* were significantly higher in the rumen solids (4.14 ± 0.89 %, 0.81 ± 0.11 %, 0.65 ± 0.11 %, 0.40 ± 0.05 %, respectively) than in the liquid (0.41 ± 0.15 %, 0.49 ± 0.08 %, 0.25 ± 0.05 %, 0.23 ± 0.04 %, respectively), dorsal epithelium (0.74 ± 0.12 %, 0.37 ± 0.06 %, 0.27 ± 0.06 %, 0.28 ± 0.05%, respectively) and ventral epithelium fractions (0.64 ± 0.12 %, 0.26 ± 0.01%, 0.32

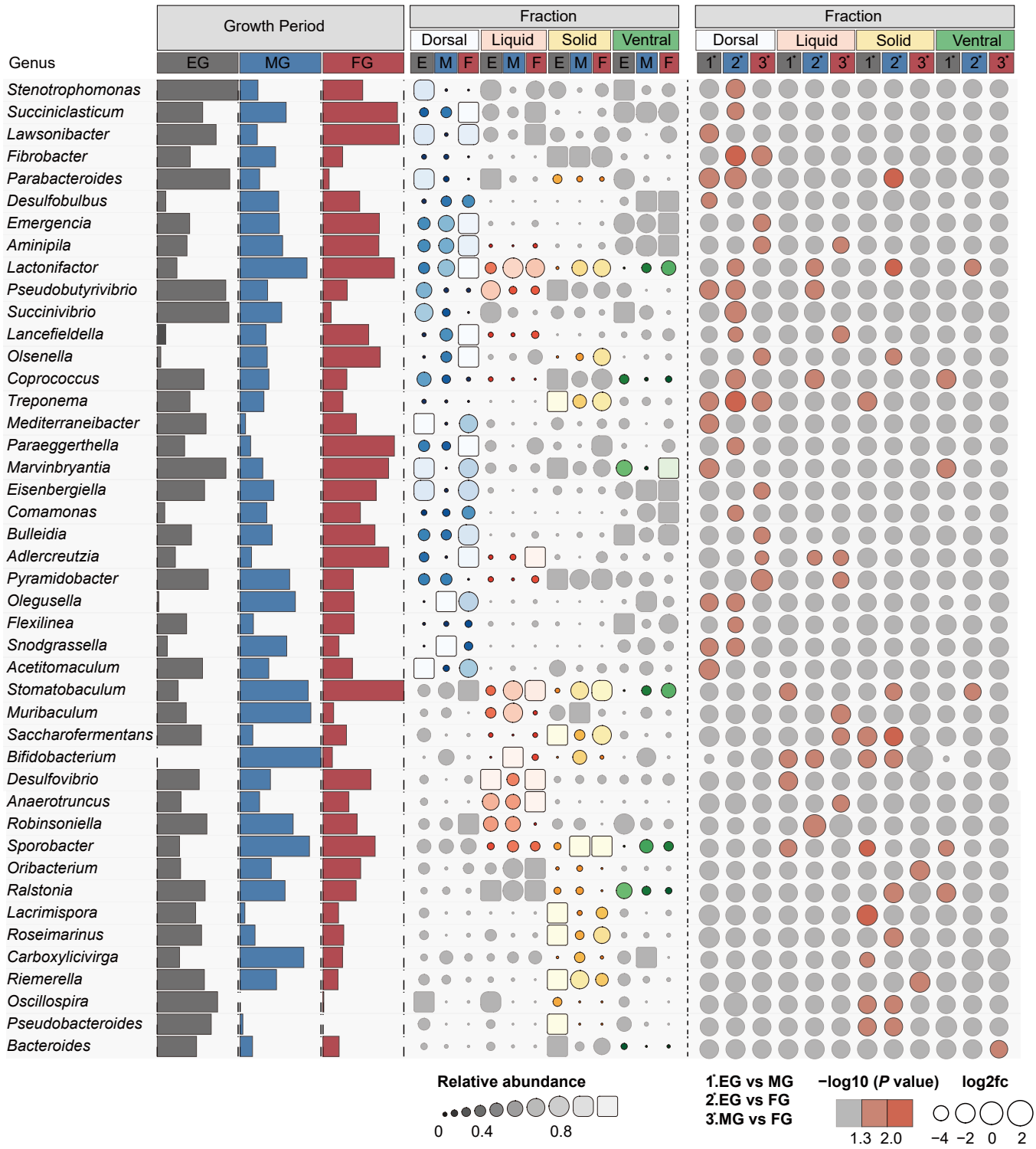

Fig. 3. Differences of rumen microbiota among three groups. The significantly changed bacterial genera. Bar graphs showing the average relative abundance of bacteria at the genus level in the EG (gray), MG (blue) and FG (red) groups. Bubble graphs showing significantly changed bacterial genera, significance, and fold change for three stages in the dorsal rumen epithelium (light blue), liquid (pink), solid (yellow) and ventral epithelium (green), respectively.

± 0.05 %, 0.25 ± 0.02 %, respectively), while the relative abundances of *Desulfobulbus* (1.11 ± 0.25 %) and *Enterocloster* (0.84 ± 0.14 %) were significantly higher in the rumen dorsal epithelium than in the liquid (0.01 ± 0.01 %, 0.42 ± 0.07 %, respectively). In the FG group, the relative abundances of *Fibrobacter* (3.01 ± 0.78 %), *Saccharofermentans* (0.90 ± 0.14 %) and *Treponema* (0.71 ± 0.22 %) were significantly higher in the rumen solids, than in the rumen liquid (0.24 ± 0.13 %, 0.39 ± 0.03 %, 0.03 ± 0.02 %, respectively) and ventral epithelium fractions (0.53 ± 0.23 %, 0.41 ± 0.06 %, 0.26 ± 0.09 %, respectively). Bacteria belonging to the genera *Anaerobutyricum* (1.37 ± 0.17 %), *Enterocloster* (0.98 ± 0.12 %) and *Mogibacterium* (1.80 ± 0.42 %) were enriched in the rumen dorsal epithelium, while *Ruminococcus* (9.11 ± 1.26 %) and *Oribacterium* (0.47 ± 0.12 %) bacteria were enriched in the rumen liquid.

3.4. Metabolome of rumen liquid during antler growth

An untargeted metabolomic analysis was conducted to identify the changes in metabolites in the rumen liquid. A total of 667 and 239 compounds were identified in positive and negative ion modes, respectively. PLS-DA revealed that the metabolites in the MG and FG groups were clearly distinct from those in the EG group in negative (Fig. 4A) and positive (Fig. 4B) ion modes, respectively. In negative ion mode, we found that a total of 33, 12, and 26 metabolites were significantly different in the EG, MG, and FG groups, respectively (VIP > 1 and $P < 0.05$, Table S14), and these were enriched in a total of 6, 12 and 5 metabolic pathways, respectively ($P < 0.05$, Fig. 4C). The concentrations of ketoleucine, dopamine, 3,4-dihydroxymandelic acid (DHMA), pyruvate, γ-aminobutyrate (GABA) and α-hydroxybutyrate were greater in the FG group than in the EG or MG group ($P < 0.05$, Fig. 4E). The butanoate metabolism, arginine and proline metabolism and alanine, aspartate and glutamate metabolism pathways were significantly enriched in FG vs. MG and MG vs. EG, while valine, leucine and isoleucine biosynthesis and degradation were significantly enriched in FG vs. EG and FG vs. MG. Specifically, tyrosine metabolism was significantly enriched during antler growth (FG vs. EG, FG vs. MG, MG vs. EG).

In positive ion mode, we identified a total of 107, 33, and 87 differentially abundant metabolites in the EG, MG, and FG groups, respectively (VIP > 1 and $P < 0.05$, Table S15). The concentrations of creatine, N-acetyl-D-neuraminic acid (NANA), dimethylethanolamine, aminoacetone, epimelibiose, L-DOPA, norepinephrine, β-alanyl-L-lysine and 4-guanidinobutanoic acid in the FG or MG group were significantly higher than those in the EG group ($P < 0.05$, Fig. 4F), and were enriched in a total of 12, 2 and 12 metabolic pathways, respectively ($P < 0.05$, Fig. 4D). Specifically, galactose metabolism and amino sugar and nucleotide sugar metabolism were significantly enriched in the FG group compared with the EG group, while tyrosine metabolism, tryptophan metabolism, β-alanine metabolism, arginine and proline metabolism and glycine, serine and threonine metabolism were significantly enriched in FG vs. EG and MG vs. EG.

3.5. Co-occurrence network of the rumen liquid microbiota and metabolites

Correlations and co-occurrence network of the rumen liquid microbiota and metabolites were established for the EG, MG and FG groups (Figs. 5 and S5). In the EG group, the relative abundances of *Butyrivibrio*, *Lawsonibacter*, *Paraprevotella*, *Stenotrophomonas*, *Saccharofermentans* and *Pseudobutyrivibrio* were negatively correlated with the concentrations of acetate, propionate or butyrate (Fig. 5A). In the MG group, the proportion of *Olsenella* was positively correlated with the concentrations of epimelibiose, ketoleucine, NANA, propionate, and the proportions of *Faecalimonas*, *Centipeda* and *Robinsoniella* (Fig. 5B). The concentration of GABA was positively correlated with the proportions of *Ruminococcus*, *Selenomonas* and *Faecalibacterium* and the concentrations of creatine, taurine, acetate and butyrate. In the FG group, the relative abundance of *Olsenella* was positively correlated with the concentrations of β-alanyl-L-lysine, dopamine, α-hydroxybutyrate, L-kynurenine and norepinephrine (Fig. 5C). The relative abundance of *Prevotella* was positively correlated with the concentration of indoleacetic acid (IAA).

4. Discussion

We explored the rumen microbiota in the liquid, solid, ventral epithelium and dorsal epithelium and the metabolome in the rumen of sika deer and aimed to understand metabolic adaptation during antler growth. The results showed metabolites were significantly changed in the rumen during antler growth. We also observed increased concentrations of acetate, propionate and butyrate, resulting in an increase in total SCFA levels. This finding is consistent with previous findings showing that total SCFAs levels were significantly greater in summer than in winter [8], and that the energy requirements of sika deer increased significantly during antler development [31]. SCFAs are important sources of energy, both for the microorganisms themselves and for the host, providing 70 % of the energy required by ruminants [12]. In the liver, propionate is converted to glucose, and acetate serves as a precursor for fatty acid synthesis and can be converted to ketone bodies, while butyrate is converted to ketone bodies in the rumen epithelium and liver [32]. We have also demonstrated that the concentration of butyrate in the rumen increases with antler weight [13]. However, previous studies have shown that the voluntary feed intake of Cervidae peaks in summer [7], that the moisture content of the rumen digesta of wild sika deer is greater in summer than in winter [8], and that the total mean retention time of feed in the digestive tract of sika deer during summer was numerically longer than that in spring [9]. The latter could prolong the microbial fermentation time and enzymatic digestion time, resulting in increased SCFA production. We also observed a decrease in diversity indices, which were negatively correlated with the concentrations of propionate and butyrate in rumen liquid. Previously, lower richness of the microbiome gene content and taxa in the rumen was shown to be tightly linked to higher feed efficiency, resulting in better energy and carbon channeling in cows [33]. These findings implied that enhanced rumen fermentation toward the production of propionate and butyrate is closely associated with antler growth. This was further supported by the increased relative abundances of butyrate- and propionate-producing bacteria such as *Stomatobaculum* [34] and *Anaerotruncus* [35]. Emerging evidence indicates that SCFAs are key metabolites that regulate bone formation [36], and increased SCFA generation also increases bone mineral density [37]. It has been reported that the increased production of butyrate stimulates bone formation by activating Wnt signaling in osteoblasts [15]. These findings underscore the importance of propionate and butyrate during antler growth.

Our results also revealed significant enrichment of metabolic pathways for amino acids, such as arginine and proline metabolism and alanine, aspartate and glutamate metabolism, during antler growth. This finding is consistent with the previous observation that the enrichment of arginine and proline metabolism was associated with increased antler weight [38]. The increased intake of alanine might be beneficial for bone mineral density in women [39], and the concentrations of GABA and glutamate in serum were found to be significantly associated with bone mineral density in young and middle-aged women [40]. Several findings have shown that GABA upregulates bone formation genes by activating the GABA receptor to stimulate osteoblastogenesis in rats [41], and glutamate signaling regulates both bone formation and resorption [42]. Moreover, glutamate and GABA could serve as major substrates for butyrate formation, and the fermentation of alanine and aspartate contributes to the production of propionate through pyruvate [43]. Given the increased concentrations of GABA and pyruvate, these findings suggested that a potential fermentation pathway from amino acids to SCFAs changed during antler growth.

The dominance of bacteria belonging to the phyla Bacteroidota and Bacillota in the rumen during antler growth is consistent with previous findings for the gastrointestinal tract of ruminants [16,44]. The microbiota in the rumen solids was dominated by cellulolytic bacteria, including *Fibrobacter* [45], *Saccharofermentans* [46] and *Treponema* [46], while the liquid fractions were represented by bacteria mainly involved in soluble nutrient breakdown, including *Olsenella* [47], *Anaerovibrio* [48] and *Ruminococcus* [49]. This finding is consistent with observations in yak [16], sheep [50], and cows [51]. Interestingly, *Desulfobulbus*, a genus of sulfate-reducing bacteria (SRB), was abundant in the dorsal epithelium [52]. Hydrogen sulfide (H_2S) is produced mainly through cysteine catabolism, and to a lesser extent, by SRB, including *Desulfovibrio*, *Desulfobacter*, *Desulfobulbus*, and *Desulfotomaculum*. H_2S is regarded as a bone-regulating molecule that promotes bone formation by activating Wnt signaling via increased Wnt10b production and

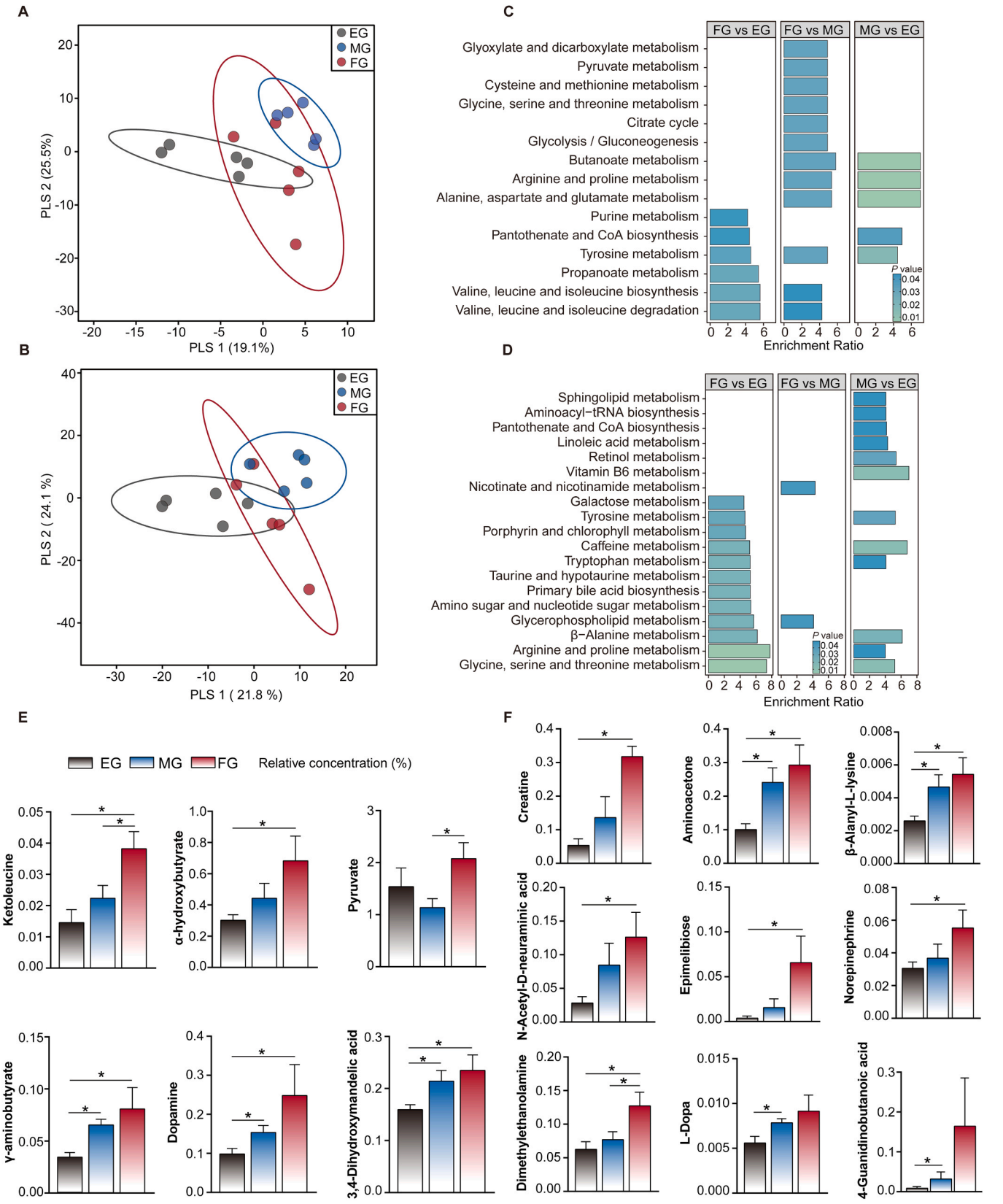

Fig. 4. The metabolic profiles of rumen liquid. PLS-DA showing the variation of metabolites in the rumen liquid among the EG, MG and FG groups at the negative (A) and positive (B) ion modes. The significantly enriched KEGG pathways based on the significantly changed metabolites during antler growth at negative (C) and positive (D) ion modes. Comparison of the concentrations of metabolites that are enriched KEGG pathways at negative (E) and positive (F) ion modes. (VIP > 1.0 and $P < 0.05$). *, indicate $P < 0.05$.

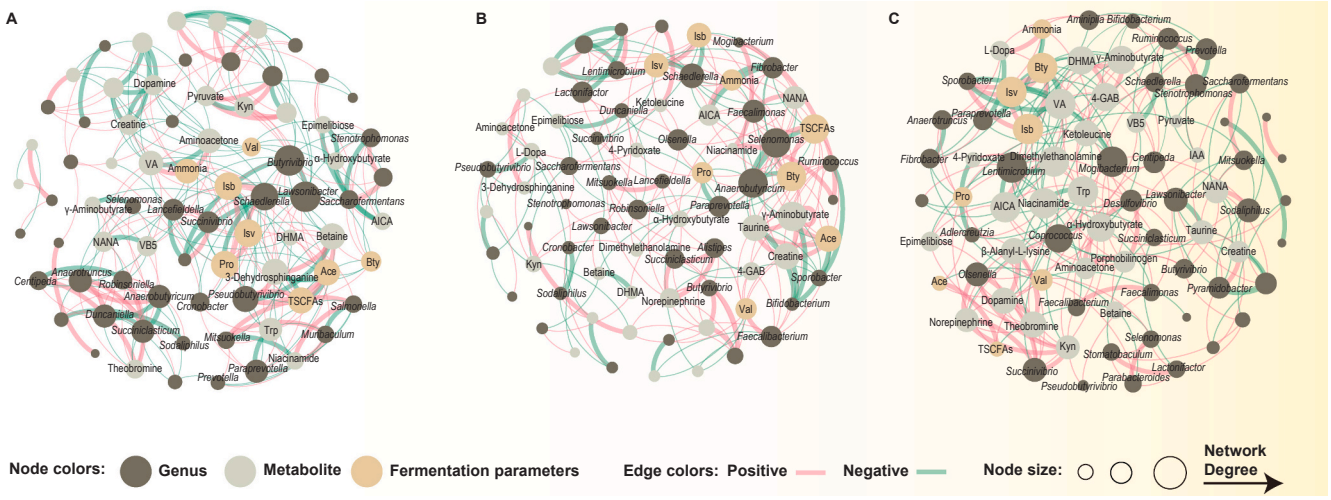

Fig. 5. The co-occurrence network of microbiota and metabolites in the rumen liquid. The network of the Spearman's correlations among significantly different genera, metabolites, and fermentation parameters in EG (A), MG (B) and FG (C) groups. Ace: Acetate, Pro: Propionate, Bty: Butyrate, Val: Valerate, Isv: Isovalerate, Isb: Isobutyrate, NH_3: Ammonia, VB_5: D-Pantothenic acid, NANA: N-Acetylneuraminic acid, Trp: L-Tryptophan, VA: Vitamin A, AICA: 5-Aminoimidazole-4-carboxamide, 4-GAB: 4-Guanidinobutanoic acid, IAA: Indoleacetic acid, DHMA: 3,4-Dihydroxymandelic acid.

preventing bone loss [53]. Moreover, butyrate-producing bacteria were enriched, such as *Butyrivibrio* [54] in the dorsal epithelium and *Anaerobutyricum* [55] in the ventral epithelium. Previous studies have shown that butyrate regulates intestinal epithelial cell proliferation [56]. These results indicated the potentially specific changes of the epithelial microbiota during antler growth.

Significant differences in the microbial community were also observed in the different rumen ecological niches. The relative abundances of *Stomatobaculum*, *Succiniclasticum* and *Comamonas* increased in the dorsal or ventral epithelium of the rumen during antler growth. The genus *Comamonas* includes oxygen-scavenging bacteria that maintain an anaerobic environment in the epithelium and are involved in nitrogen metabolism [57]. A meta-analysis revealed that *Succiniclasticum* is a core member of the ruminant rumen epithelium [58]. Bacteria belonging to the genera *Succiniclasticum* [59] and *Stomatobaculum* [60] also produce SCFAs. These findings indicate the possibility that the SCFA production potential of the rumen epithelial microbiota is improved during antler growth. Interestingly, it has been reported that *Stomatobaculum* was significantly more abundant in oral swab samples from control patients than in those from patients with oral cancer [61]. Horvath et al. (2019) reported that the detection of common oral microbes in the intestine (oralization of the intestine) might indicate increased intestinal permeability [62]. These findings indicated tight adhesion and close interaction between *Stomatobaculum* and the rumen epithelium during antler growth. It is speculated that these microorganisms might improve the permeability of the rumen epithelium, facilitating the absorption of SCFAs.

Our results also revealed significant differences in the associations between the rumen microbiota and metabolites during antler growth. Negative correlations between SCFAs and *Butyrivibrio*, *Paraprevotella*, *Saccharofermentans* or *Pseudobutyrivibrio* were observed in the EG group, indicating that the catabolic activity of the fiber in the rumen likely decreases during the early stage of antler growth. In the MG group, GABA was positively correlated with *Ruminococcus*, *Selenomonas*, *Faecalibacterium*, and creatine. This finding is consistent with previous reports showing that *Ruminococcus*, *Selenomonas* and *Faecalibacterium* promote the production of GABA [63,64]. In the FG group, *Olsenella* was positively correlated with the intermediates of tyrosine metabolism, β-alanine metabolism and propanoate metabolism, and *Prevotella* was positively correlated with IAA. GABA and its receptors serve as novel factors that promote osteogenesis [65]. Creatine supplementation enhances the accumulation of bone minerals, influences bone remodeling processes in both pediatric and adult populations [66], and promotes the differentiation or survival of GABAergic neurons [67]. β-alanine metabolism is involved in regulating bone mass in rats [68]. Tyrosine metabolism plays an important role in the sympathetic nervous system in animals [69]. In mammals, IAA is an important indole derivative derived via catabolism of dietary tryptophan by the intestinal microbiota [70]. An increase in the proportion of *Prevotella* can lead to the intestinal production of indole derivatives [71]. These results indicated the role of the interaction between the rumen microbiota and amino acid metabolism during the antler growth.

5. Conclusions

In the present study, we characterized the microbiota and metabolites in the rumen of sika deer to understand the change in rumen metabolic profiles during antler growth. The results showed that the production of SCFAs was enhanced, which was accompanied by decreased microbial diversity indices in the rumen. The different rumen ecological niches were characterized by distinct microbiota compositions. The rumen microbiota and metabolites varied during antler growth. An obvious shift in the microbiota in the rumen epithelium was observed, suggesting a potentially specific changes of the epithelial microbiota during antler growth. Although differences in the microbiota and metabolites were observed during antler growth, our study is limited by the small number of animals and the use of a 16S rRNA gene sequencing approach due to its resolution and sensitivity. Therefore, examinations of the metagenome are needed to further elucidate the changes in the rumen microbiome in many animals during the antler growth period. Moreover, the integrated analysis of the microbiota and metabolites suggested the role of amino acid metabolism during antler growth.

Ethics approval and consent to participate

All animal-specific procedures were approved and authorized by the Ethical Committee of Jilin Agricultural University (Approval No: 20210314002).

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CRediT authorship contribution statement

Songze Li: Formal analysis, Investigation, Visualization, Writing – original draft. **Ruina Mu:** Investigation, Writing – original draft. **Yuhang Zhu:** Investigation. **Fei Zhao:** Investigation. **Qiang Qiu:** Investigation. **Huazhe Si:** Investigation, Writing – original draft, Writing – review & editing. **Zhipeng Li:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing. **André-Denis G. Wright:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2024.04.018.

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