Garlic Skin Induced Shifts in the Rumen Microbiome and Metabolome of Fattening Sheep

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Abstract

Background: Garlic and its constituents exhibit activities on modifying rumen fermentation and improving growth performance. As a by-product of garlic processing, garlic skin contains similar bioactive components as garlic bulb. However, studies in ruminants using garlic skin are scarce. This experiment was conducted to investigate the effects of garlic skin supplementation on rumen fermentation characterizes, growth performance, and involved mechanism in ruminants. Twelve Hu lambs were randomly assigned into one of two treatments: basal diet (CON) or basal diet supplemented with 80 g/kg DM of gallic skin (GAS). The experiment lasted for10 weeks, with the first 2 weeks for adaptation.

Results: The results revealed that the average daily gain and volatile fatty acid concentration were higher ($P < 0.05$) in lambs fed GAS than that in the control group. Garlic skin supplementation did not significantly ($P > 0.10$) affect the α-diversity indices. Increased ($P < 0.05$) abundances of *Prevotella*, *Bulleidia*, *Howardella*, *Methanosphaera* but a decreased ($P < 0.05$) abundance of *Fretibacterium* were observed in GAS-fed lambs. Besides, the garlic skin supplementation favorably regulated ($P < 0.05$) pyrimidine metabolism, purine metabolism, vitamin B6 and B1 metabolism. Moreover, high correlations were observed between fluctuant rumen microbiota and metabolites.

Conclusions: Supplementation of garlic skin improved the growth performance of sheep by modifying rumen fermentation through inducing shifts in the rumen microbiome and metabolome.

Introduction

In recent years, plant-derived bioactive compounds have been concerned for its potential alternatives to growth promoting antibiotics in ruminant production [1–2]. Garlic (*Allium sativum* L.), which has been widely used as a foodstuff in the world, contains numerous active metabolites such as sulfur compounds (allicin, alliin, diallyl sulfide, and diallyl trisulfide) [3–4]. These components are known to possess antimicrobial, antibacterial, antioxidant, anti-inflammatory, and anticancer [5–7].Due to its antimicrobial properties, garlic power [8] and garlic oil [9] exhibit activities on modifying rumen fermentation parameters, improving nutrient digestibility, decreasing rumen protozoa numbers, and reducing methane emissions.

The annual production of garlic is approximately 20 million tons in the world, with China, India, and Korea being the main producers [10]. As a by-product of garlic processing, garlic skin contained similar bioactive components as garlic bulb [11]. Garlic bulbs yield approximately 760 g cloves, and 240 g garlic skin per kilogram [12]. Therefore, garlic skin could be of importance due to its abundance and possible utilization as ruminant feed stuff. However, to the best of our knowledge, no previous studies investigated the effects of garlic skin on the rumen fermentation characteristics, growth performance, and involved mechanism in ruminants.

Rumen harbors a wide variety of microorganisms, and rumen microbiome can directly and indirectly affect performance, health, and immune system of the host [13–14].During diet digestion, the rumen microbiota coproduces a large array of small molecules, which play critical roles in shuttling information between the microbial symbionts and their host's cells [15]. Therefore, a better understanding of the rumen microbiota composition and the metabolome is crucial to investigate the mechanism involved in feedstuff affecting rumen fermentation. We hypothesized that the rumen microbiota and rumen metabolites might be affected by garlic skin, which would demonstrate a regulatory role of garlic skin on rumen metabolism. The aim of this study was to investigate the effects of garlic skin on the dynamic changes in the ruminal microbiome and metabolome in growing sheep.

Material And Method

Animals and experimental design

All the experimental protocols were approved by the Animal Care Committee of Anhui Agricultural University. Twelve Hu lambs (local breed, 23 ± 2.3 kg initial body weight) divided into two groups (n = 6), were fed basal diet (CON) or basal diet supplemented with 80 g/kg DM of gallic skin (GAS). The experimental period lasted for 10 weeks, with the first 2 weeks for adaptation. The basal diets were formulated to meet the Feeding Standards of Meat-producing Sheep and Goats in order to achieve a daily gain of 0.250 kg (Ministry of Agriculture of P. R. China, 2004; Table 1). All the animals were housed in individual pen with free access to water and were fed twice daily at 07:00 and 19:00 h, with approximately 10% feed refusal.

Table 1

Feed ingredients and chemical compositions of the experimental diets[1].

Item	CON	GAS
Ingredient (%, DM)		
Ground corn grain	28.00	25.76
Soybean meal, 43.5% crude protein	15.00	13.80
Distollers Dried Grains with Solubles	9.00	8.28
Wheat bran	4.00	3.68
Sodium bicarbonate	1.00	0.92
Salt	1.00	0.92
Dicalcium phosphate	0.50	0.46
Calcium carbonate	0.50	0.46
Premix[2]	1.00	0.92
Garlic skin	0	8.00
Peanut straw	15.00	13.80
Soybean straw	25.00	23.00
Chemical composition (%, DM)		
Organic matter	91.30	91.4
Crude protein	15.10	14.49
Neutral detergent fiber	38.70	39.62
Acid detergent fiber	23.20	25.31
Ether extract	3.10	2.96
Calcium	0.75	0.74
Phosphorus	0.43	0.42
Metabolizable energy[3], MJ/Kg	9.83	9.74

[1]CON= the control diet; GAS = the gallic skin diet.

[2]Formulated to provide (per kilogram of premix) 600 KIU of Vitamin A, 80 KIU of vitamin D_3, 5000 IU of Vitamin E, 8000 mg of Zn, 60 mg of Se, 200 mg of I, 9400 mg of Fe, 72 mg of Co, 10400 mg of Mn, and 1600 mg of Cu.

[3]Calculated according to Ministry of Agriculture of P. R. China, 2004.

Sample Collection And Measurement

Diet and ort samples were collected daily for 3 consecutive days (1st to 3rd day) every week. Samples were composited per lamb for the whole experimental period, subsampled, and were dried in a baking oven at 65℃ for 48 h, and were milled through a 1-mm screen for the analysis of dry matter (DM, method 924.05; AOAC,1990), crude protein (method 988.05; AOAC, 1990), ash (method 942.05; AOAC, 1990), neutral detergent fiber (NDF, Van Soest et al., 1991) and acid detergent fiber (ADF, Van Soest et al., 1991) [16–17].Body weight was measured on d 0, 28, and 56 before morning feeding and average daily gain (ADG) was calculated.

Ruminal fluid (approximately 25 mL) was collected using an oral stomach tube immediately prior to feeding on the last day of the trial as described by Shen et al. (2018) [18].The pH of the ruminal fluid sample was measured immediately after collection using a portable pH meter (Ecoscan pH 5, Eutech Instruments, Singapore). Rumen fluid collections were squeezed through 4 layers of cheese cloth. 1.0 mL subsample of each rumen filtrate sample was acidified by adding 0.2 mL of 25% HPO_3 for analysis of volatile fatty acids (VFAs) according to the method described by Hu et al. (2005) [19]. Another 2.0 mL subsample was used to determine the ammonia-nitrogen by colorimetric method, as described by Chaney and Marbach (1962) [20]. Another two 5 mL subsamples of were infused into two10 mL spiral centrifuge tubes and immediately placed into a liquid nitrogen container and then transported to the laboratory, stored at -80 °C for further analysis of microbiota and metabolites.

Rumen Microbiota: 16s Rdna Sequencing

Microbial genomic DNA was extracted from rumen fluid (4 mL) using the E.Z.N.A. stool DNA kit (Omega Bio-tek, Norcross, GA, United States) according to the manufacturer's instructions and assessed by 1% (w/v) agarose gel electrophoresis. The concentration of the extracted DNA was measured by a Qubit 2.0 (Life Technologies, Carlsbad, CA, USA). The V3-V4 hypervariable region of 16S ribosomal DNA (rDNA) gene was PCR amplified (first 5 cycles of denaturing at 95 °C for 30 s, annealing at 45 °C for 30 s, elongation at 72 °C for 30 s, then 20 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s and a final extension at 72 °C for 5 min) using the forward primer 515F (5’-CCCTACACGACGCTCTTCCGATCTN-3’) and the reverse primer 806R (5’-GACTGGAGTTCCTTGGCACCCGAGAATTCCA-3’) with dual-index barcodes. PCRs were performed in a triplicate 30 μL mixture containing 20 ng of template DNA, 15 μL 2 × Taq master Mix (Invitrogen, Carlsbad, CA), and 1.5 μL of each primer (10 μM).

The amplicons were run on a 1% (w/v) agarose gel, and the DNA were extracted from the bands using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer’s instructions. The concentration and quality of the eluted amplicons were measured using a Qubit 2.0 (Life Technologies, Carlsbad, CA, USA). Purified amplicons were pooled in equal amounts

(10 ng) and sequenced on the Illumina MiSeq platform (Sangon, Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA648813).

Bioinformatics

The raw reads containing ambiguous bases and any longer than 480 base pairs (bp) were dislodged and those with a maximum homopolymer length of 6 bp were allowed, and sequence short than 200 bp were removed [21].Paired-end raw reads were merged as raw tags using FLSAH (v 1.2.3) with a minimum overlap of 10 bp [22].Noisy sequences of raw tags were filtered by the Pre.cluster tool to obtain clean tags. Chimeras were detected by using Chimera UCHIME. The effective reads were clustered into operational taxonomic units (OTUs) of ≥ 97% similarity using the UPARSE pipeline [23], and the representative sequence of each OUT was classified as organisms (phylum, class, order, family, and genus) by Naïve Bayesian assignment using an RDP classifier (version 2.12) [24].Species richness and diversity statistics including coverage, rarefaction, Chao1, ACE, Simpson, and Shannon were calculated using Mothur (version 1.30.1). A weighted and unweighted UniFrac distance matrix was also generated by Mothur (version 1.30.1), and a principal coordinate analysis (PCoA) was conducted based on the unweighted Unifrac distance method [25].

Metabolomics profiling: ultra-high-performance liquid chromatography-tandem quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS)

Rumen samples were prepared according to the previously described procedures [26].UPLC-TOF/MS analysis was performed using an Agilent 1290 (Agilent, USA) gas chromatograph system equipped with a UPLC BEH Amide column (2.1 * 100 mm, 1.7 μm, Waters) and coupled with TripleTOF 6600 mass spectrometry (AB Sciex) with an electrospray ionization (ESI) source. The mobile phase consisted of 25 mM ammonium acetate and 25 mM ammonia hydroxide in water(pH = 9.75, solvent A) and acetonitrile (solvent B). The analysis was carried with elution gradient as follows: 0 ~ 0.5 min, 95% B; 0.5 ~ 7.0 min, 95%~65% B; 7.0 ~ 8.0 min, 65%~40% B; 8.0 ~ 9.0 min, 40% B; 9.0 ~ 9.1 min, 40%~95% B; 9.1 ~ 12.0 min, 95% B. The flow rate was 0.5 mL/min. The column temperature was 25 °C. The auto-sampler temperature was 4 °C. The ESI-Q-TOF-MS was applied on an information-dependent basis in both positive and negative ion modes with the conditions as follows: Gas 1 as 60 psi, Gas 2 as 60 psi, Curtain Gas as 35 psi, Source Temperature as 600 °C, Decluttering potential as 60 V, Ion Spray Voltage Floating as 5000 V or -4000 V in positive or negative modes, respectively.

Metabolomics Data Analysis

The injection volume was 1 μL (positive) or 1 μL (negative), respectively. During the operation, the acquisition software (Analyst TF 1.7, AB Sciex) continuously evaluates the full scan survey MS data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. In each cycle, the most intensive 12 precursor ions with intensity above 100 were chosen for MS/MS at collision energy

(CE) of 30 eV. The cycle time was 0.56 s. MS raw data were converted to the mz XML format by Proteo Wizard and processed by R package XCMS (version 3.2). The process includes peak deconvolution, alignment and integration. Minfrac and cut off are set as 0.5 and 0.3 respectively. In-house MS2 database was applied for metabolites identification. A total of 1812 resulting peaks with the peak numbers, sample names, and normalized peak areas were import to the SIMCA software package (V15.0.2, Sartorius Stedim Data Analytics AB, Umea, Sweden) for principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA). Additionally, 7-fold permutation tests in the OPLS-DA model were used to verify model validity and robustness. Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) was utilized for metabolite identification and confirmation, and MetaboAnalyst 4.0 was used for the pathway topology analysis.

Statistical analysis

The matter intake (DMI), growth performance, and rumen fermentation characteristics were analyzed as a randomized block design using PROC MIXED of SAS software (version 9.3, SAS Institute Inc., Cary, NC, United States). Dietary treatment was included as a fixed effect, and lamb was included as a random effect. Means were separated using the PDIFF option in the LSMEANS statement.

Statistical calculations rumen bacterial community, metabolomic, and their correlation data were carried out by conducting tests using the SPSS software package (SPSS version 23.0; SPSS Inc., Chicago, IL, United States). The Kruskal-Wallis sum-rank test was used to select and demonstrate differentially abundant taxa between the groups. For metabolite identification, the first principal component with a variable importance in the projection (VIP) value > 1.0 and a P value < 0.05 in Student's t test were considered significantly different.

The correlations between different rumen microbial genera ($P < 0.05$ and relative abundance > 0.05% in at least one of the samples) and varied altered rumen metabolites (VIP > 1.0 and $P < 0.05$) were assessed by Spearman's correlation test. Significance was declared at $P < 0.05$, and a tendency was declared at $0.05 \leq P < 0.10$.

Results

Dry matter intake and growth performance

As shown in Table 2, dry matter intake was not significantly affected by the dietary treatments ($P > 0.05$). Lambs consuming the GAS diet had significantly higher ADG than lambs in the control group ($P < 0.05$).

Table 2
Dry matter intake (DMI) and growth performance for the dietary treatments (n = 6 lambs per treatment).

Item[1]	Treament[2]		SEM[3]	*P-value*
	CON	GAS		
Dry matter intake, kg/d	1.32	1.35	0.036	0.35
Initial body weight, kg	23.25	23.55	1.150	0.77
Final body weight, kg	34.44	36.68	0.881	0.22
Average daily gain, g/d	199.9[b]	234.4[a]	8.73	0.04
[1]ADG= average daily gain.				
[2]CON= the control diet; GAS = the gallic skin diet.				
[3]SEM= standard error of mean.				
[a,b]Means in same row with different superscripts are different from each other ($P < 0.05$), the same as below.				

Rumen Fermentation Parameters

Rumen pH was similar ($P > 0.05$) between GAS and the control group (Table 3). The ammonia-nitrogen decreased significantly in GAS compared with CON ($P < 0.05$). Total concentrations of VFA and concentrations of acetate demonstrated significant ($P < 0.05$) or trend ($P < 0.10$) responded to GAS compared with CON fermenters. Concentrations of propionate, butyrate, valerate, and acetate-to-propionate ratio did not differ ($P > 0.05$) between the two dietary groups.

Table 3
Rumen fermentation characteristics for the dietary treatments (n = 6 lambs per treatment).

Item	Treament[1]		SEM[3]	*P-value*
	CON	GAS		
pH	6.61	6.59	0.035	0.56
Ammonia-nitrogen, mg/dL	8.96[a]	6.21[b]	0.518	0.02
Total VFA[2], m*M*	66.1[b]	73.5[a]	1.85	0.04
Acetate (A), m*M*	45.2	51.2	1.69	0.07
Propionate (P), m*M*	12.2	13.5	0.45	0.19
Butyrate, m*M*	7.72	7.85	0.332	0.85
Valerate, m*M*	0.81	0.78	0.027	0.66
A:P	3.71	3.84	0.108	0.60
[1]CON= the control diet; GAS = the gallic skin diet.				
[2]VFA= volatile fatty acid.				
[3]SEM= standard error of mean.				

Table 4
Alpha-diversity indices of the rumen microbiota for the dietary treatments (n = 6 lambs per treatment).

Item[1]	Treament[2]		SEM[3]	*P-value*
	CON	GAS		
OTUs no	1450	1459	90.1	0.96
ACE	6.61	6.59	0.035	0.56
Chao1 value	1865	1906	74.8	0.79
ACE value	1966	1942	81.2	0.89
Shannon indices	4.60	4.39	0.115	0.38
Simpson indices	0.0431	0.0429	0.0018	0.96
[1]OTU = operational taxonomic unit; ACE = abundance-based coverage estimator.				
[2]CON= the control diet; GAS = the gallic skin diet.				
[3]SEM= standard error of mean.				

Bacterial Analyses Of Rumen Samples

In total, 1,233,294 raw reads were obtained for the bacterial 16S rRNA genes in the two groups. After screening, 1,217,625 effective tags were obtained, accounting for 98.7% of the raw reads. The Good's coverage values for all samples were greater than 99.5%. The flattened rarefaction demonstrated that most of the diversity was captured (Fig. S1). The α-diversity indices showed no significantly difference ($P > 0.05$) between GAS and CON. Based on the unweighted UniFrac distances, the PCoA revealed that the microbiota structure of the GAS group was separated from that of the control group (Fig. 1).

The rumen-microbiota taxonomic distributions at the phylum, family, and genus levels are shown in Fig. 2. In total, 20 bacterial phyla were identified in the rumen samples. The relative abundances revealed that *Bacteroidetes* (58.7 ± 7.72;mean ± standard deviation) was the most dominant phylum, followed by *Firmicutes* (31.3 ± 8.00) (Fig. 2A, B). The relative abundances of *Synergistetes*were significantly decreased ($P < 0.05$) in GSA compared with CON. However, the relative abundance of *Firmicutes* tend to increase ($0.05 < P < 0.10$) in GSA compared with CON.

There were 260 bacterial taxa identified at the genus level (Fig. 2C, D), *Prevotella* (32.8 ± 8.27%), *Methanobrevibacter* (5.35 ± 2.72%), *Succiniclasticum* (4.06 ± 2.65%), *Selenomonas* (1.68 ± 1.48%), *Ruminococcus* (1.44 ± 2.03%), and *Clostridium IV* (1.29 ± 1.15%) were considered as high-abundance taxa. Table 5 compares the ruminal bacteria relative abundance between the diets. Among these, 5 genera bacteria were identified in comparisons between GAS and CON. The percentages of *Prevotella* (*P*

< 0.05), *Bulleidia* (*P* < 0.05), *Howardella* (*P* < 0.01), and *Methanosphaera* (*P* < 0.01) were significantly increased in GAS compared with CON. In contrast, the proportions of *Fretibacterium* were significantly decreased (*P* < 0.05) in GAS compared with CON.

Table 5
Main microbiota (accounting for ≥ 0.05% of the total sequences in at least one of the samples) that significantly changed between the dietary treatments (abundance of the genera is expressed as a percentage) (n = 6 lambs per treatment).

Phylum	Genus	Treament[1]		SEM[2]	*P-value*
		CON	GAS		
Bacteroidetes	*Prevotella*	28.29[b]	37.23[a]	3.066	0.02
Synergistetes	*Fretibacterium*	0.32[a]	0.01[b]	0.124	0.04
Firmicutes	*Bulleidia*	0.02[b]	0.56[a]	0.294	0.03
	Howardella	0.02[b]	0.04[a]	0.004	< 0.01
Euryarchaeota	*Methanosphaera*	5.08[b]	5.63[a]	0.013	< 0.01
[1]CON= the control diet; GAS = the gallic skin diet.					
[2]SEM= standard error of mean.					

Metabolomic Analyses Of Rumen Samples

Our untargeted LC-MS approach led to the deification of 1004 metabolites out of all 12 sheep samples. The PCA plot was conducted to visualize the trends ad outliers, showing noticeable separations between GAS and CON (Fig. 3A, B). In order to further examine the metabolic changes, OPLS-DA analysis was performed to compare the two groups' rumen fluid samples (Fig. 3C, D). R2Y (cum) and Q2 (cum) were respectively of 0.956 and 0.608 in negative ion mode and 0.959 and 0.678 in positive ion mode, which indicated a stable and accurate prediction of the both models. In addition, a random-permutations test was carried out to prevent overfit of the models (Fig. 3E, F). The intercept of Q2 on Y axis was − 0.63 in negative ion mode and − 0.80 in positive ion mode (less than 0.05), showing that the models had good predictability and did not overfit. All the samples in the score plots of the rumen fluid inside the 95% Hotelling's T-squared ellipse. Clear separation and discrimination were found between GAS and CON both in the PCA and OPLS-DA plots.

The potential biomarkers were screened according to the VIP value (> 1.0) from OPLS-DA modeling and statistical tests (*P* < 0.05) in Student's *t* test. Compared with those in the control group, a total of 139 metabolites (40 in the negative mode and 99 in the positive mode) changed significantly in the rumen

fluid of the GAS sheep. Among these metabolites, 93 metabolites in GAS were up-regulated, and 46 metabolites were down-regulated (Table S1). These metabolites involved in protein digestion and absorption (including amino acids, peptides, and phenols), carbohydrate metabolism (organic acids and derivatives), lipids metabolism (VFA, linoleic acid, and α-linolenic acid), vitamin metabolism (B1, B2 and B6), purine metabolism, and pyrimidine metabolism. Generally, most of the carbohydrates, fatty acids, amino acids, dipeptide, pyrimidine, and purine were higher for the GAS group than for the control group.

Different metabolites were used in hierarchical clustering with Cluster 3.0 software. Rumen metabolites from the GAS group clustered separately from the CON group (Fig. 4A). As shown in Table S2, 22 metabolic pathways were generated in the GAS group compared with in the CON group. The significantly different metabolites between the two groups were mainly involved in pyrimidine metabolism, purine metabolism, vitamin B6 metabolism, thiamine metabolism, riboflavin metabolism, and alpha-linolenic metabolism (Fig. 4B).

Correlation Between The Rumen Microbiome And Metabolome

The functional correlations between the different microbial genera and the altered rumen metabolites was obtained through a correlation analysis based on Spearman's correlation coefficient (Fig. S2). High connections with a *P*-value less than 0.05 and $r > 0.70$ were showed in Fig. 5. In the growing lambs, *Prevotella* was positively correlated with Tyr-Asp, tropic acid, trimethoprim, thiamine, Pro-Trp, Pro-Thr, pelletierine, pantothenol, oxyquinoline, pyridoxic acid and negatively correlated with thioetheramid-PC, phenylacetic acid, and PC (16:0/16:0). *Methanosphaera* was positively correlated with hydroxyacetone, D-mannitol, 7,8-dihydrobiopterin, and citronellic acid and negatively correlated with resorcinol, Arg-Tyr. *Howardella* was positively correlated with xanthurenic acid, valproic acid, uridine, Tyr-Val, Tyr-Asp, Tropic acid, thiamine, thiamine, 7,8-dihydrobiopterin, 5-hydroxyhexanoic acid and negatively correlated with alapha-linolenic acid. *Fretibacterium* was positively correlated with alpha-linolenic acid, phenylacetic acid, Phe-Pro, PC (16:0/16:0), cyclopamine and negatively correlated with xanthurenic acid, uridine, tropic acid, trimethoprim. *Bulleidia* was positively correlated with 2-dehydro-3-deoxy-D-gluconate, and negatively correlated with Arg-Tyr, oxindole, and prednisolone.

Discussion

Recent findings showed that the garlic (for example garlic powder), garlic extracts (for example garlic oil) and garlic by-products (for example garlic husk and garlic leaf) had potential to modify rumen fermentation, improve animal performance, and might be an alternative for growth promoting feed antibiotics [27–29].In the present study, an increase of ADG was observed in the GAS group compared with that in the control group, which is consistent with previous studies which reported that ADG was increased by garlic powder and garlic leaf supplementing in growing lambs [29]. Ma *et al*. (2016) reported that supplementation with garlic extraction improved nutrient utilization by stimulating cellulolytic

bacterial activity in the rumen [30]. Bioactive components present in garlic skin are polyphenolic in nature [31] and induce a positive effect on energy metabolism [32]. Therefore, improved growth rates in the GAS group could be attributed to lower energy loss, and higher nutrient utilization.

The average pH value was not affected by garlic skin in the present study. The results are consistent with other studies where rumen pH values did not differ on addition of garlic components [30], garlic oil [33],or garlic leaf [34].Supplementary garlic skin increased the ruminal concentration of total VFA, but decreased that of ammonia, which is similar to the results reported by Busquet et al. (2005) [35]and Klevenhusen et al (2011) [36]who supplemented various garlic components in *vitro* and in growing sheep diet. The increased total VFA concentrations with the garlic skin supplementation indicated stimulated rumen microbial fermentation activities and help support the improved ADG as was observed in the present study. The increased population of R. *flavefaciens* with garlic extracts supplementation was observed by Ma et al. (2016) [30]. Increased concentrations of VFA and acetate production in our study could be attributed to an increased fiber-digesting bacteria population. The ratio of acetate to propionate was not affected by garlic skin suggested that rumen fermentation pattern was unchanged. The increased of branched-chain volatile fatty acids, for example 2,2-dimethyl succinic acid, 3,3-dimethylglutaric acid, and 2-methylglutaric acid might be due to the fact that more branched-chain amino acids were degraded as less energy (metabolizable energy: 9.74 MJ/Kg vs 9.83 MJ/Kg) and carbon skeleton (crude protein:14.49% vs 15.10%) were available in the GAS diets than that in the CON.

The taxonomic assignment of ruminal bacteria in our study revealed that the most abundant phyla were *Bacteroidetes* and *Firmicutes*, which was in accordance with the results of previous studies [37, 38].The *Firmicutes* phylum are the main bacteria that degrade fibers, including a large number of bacteria which can promote decomposition of cellulose and fermentation of polysaccharides [39]. It was reported that *Bulleidia* has the capability to utilize saccharides and starch as energy source [40]. It has been reported that *Prevotella* accounting for 60–70% of rumen microorganisms [41], and several *Prevotella* were known to degrade oligosaccharides and hemicellulose [42]. It was also reported that *Prevotella* has the ability to utilize starch, xylan and pectin [43].Thus, the greater relative abundance of the phylum *Firmicutes*, the genus *Bulleidia* and *Prevotella* in the GAS group indicated that garlic skin supplementation effectively improved carbohydrate metabolism, especially the cellulose digestion. In addition, allicin is one of the active components of garlic [44]. Ma et al. (2016) found that supplementing a basal diet with allicin effectively increased the apparent digestibility of neutral detergent fiber and acid detergent fiber in growing lambs [30].

Methanosphaera, a methanogen capable to utilize methylated substrates like the *Methanomassiliicoccaceae*, increased in the GAS group. This agrees with Martinez-Fernandez et al. (2015) who observed that the abundances of *Methanosphaera* were increased in anti-methanogenic organosulphur supplementation lambs [45]. Saro et al. (2018) reported that greater abundance of *Methanosphaera* and lower abundance of *Methanomassiliicoccaceae* in the rumen of low methane-emitting lambs treated with a combination of garlic essential oil and linseed oil, which partially agrees with our results [46]. It has also been reported that the genus *Methanosphaera* was H2 utilizing species

within the rumen [47]. The genus *Fretibacterium* was reported to participate in hydrogenation of long-chain fatty acid [38]. Thus, the decreased relative abundance of *Fretibacterium* in the GAS group could be partially attributed to the increased abundance of *Methanosphaera*.

Rumen is an important organ where microbes ferment the plant biomass into proteins, VFAs, and other nutrients that can be used by the host. In total, 139 metabolites were changed as a result of garlic skin supplementation, 93 out of 139 significantly different metabolites had higher concentration including amino acids, dipeptides, fatty acids, and carbohydrates in GAS-fed lambs compared with CON-fed lambs. These amino acids and dipeptides can be used as precursors for the synthesis of microbial protein, while carbohydrates and fatty acids can provide energy for the synthesis of microbial protein [48].Additionally, our study also demonstrated pyrimidine and purine metabolism pathways were changed in GAS-fed lambs. Purine and pyrimidine are basic components of DNA and RNA. The levels of uridine, deoxycytidine, deoxyuridine, thymidine, and uracil in the pyrimidine metabolism as well as xanthine, deoxyadenosine, deoxyinosine, deoxyguanosine, and guanosine in the pyrimidine metabolism were all significantly higher for the GAS group than for the control group. In the rumen, xanthine and hypoxanthine are the nucleic acid degradation products of rumen microbial nucleic acids; thus, xanthine and hypoxanthine have been used as biomarkers of microbial protein synthesis [49]. Our findings suggest that lambs may obtain more nutrients from GAS than from CON to produce more microbial protein.

Pyridoxal is the main active form of vitamin B6, and 4-pyridoxic acid is the major urinary catabolite of vitamin B6 [50].In our study, garlic skin supplementation increased the levels of pyridoxal, 4-pyridoxic acid, and thiamine, which indicated the upregulation of thiamine (vitamin B1) and vitamin B6 metabolisms. By serving as coenzymes, including pyruvate dehydrogenase and α-ketoneglutaric acid dehydrogenase, thiamine plays a critical role in carbohydrate metabolism [51].Besides, thiamine could participate in the production of acetyl-CoA via pyruvate-ferredoxin oxidoreductase in *Megasphaera* [52]. A positive correlation between thiamine *Megasphaera* was observed in our study, and the higher concentration of ruminal acetate in GAS-fed lambs might contribute from the higher thiamine and *Megasphaera*. Vitamin B6 were reported to involve in amino acid, glucose and lipid metabolism [53].It is well known that B vitamins can be synthesized by the ruminal microbiota, which is the main source for ruminants [48].Thus, in our study, the up-regulated levels of B vitamins and their metabolites indicated that the utilization of carbohydrates and proteins might be enhanced.

Poly-unsaturated fatty acids are thought to be more toxic than saturated fatty acid, therefore, biohydrogenation of poly-unsaturated fatty acid into fatty acid is one of the important microbial processes in the rumen [54].Biohydrogenation process provide a variety of biohydrogenation intermediates including vaccenic acid, conjugated linoleic acid, and conjugated linolenic acid [55].In our study, garlic skin supplementation decreased the level of alpha-linolenic acid and trans-vaccenic acid, which indicated the downregulation of alpha-linolenic acid metabolism. Spearman's correlation analysis revealed that *Howardella* was negatively correlated with alpha-linolenic acid, indicating that garlic skin probably affected alpha-linolenic acid metabolism through enriching the abundance of *Howardella*,

which was consistent with previous study reported that higher abundances of *Howardella* in the gut inversely contributed to the enrichment of alpha-linolenic acid metabolism [56].

Conclusion

In conclusion, garlic skin supplementation improved the rumen fermentation, increased the average daily gain. We further investigated the effects of garlic skin supplementation on the rumen microbiome structure by 16S compositional sequencing and on the ruminal metabolome using untargeted metabolomics approach. Garlic skin supplementation did not significantly affect the α-diversity indices. Increased abundances of *Prevotella*, *Bulleidia*, *Howardella*, *Methanosphaera* but a decreased abundance of *Fretibacterium* were observed in GAS-fed sheep. Besides, the garlic skin supplementation favorably regulated pyrimidine metabolism, purine metabolism, vitamin B6 and B1 metabolism. These findings suggested that garlic skin supplementation not only changed the community of the rumen microbiome but also altered ruminal fluid metabolism in fattening sheep.

Declarations

Ethics approval and consent to participate

The procedures of this study were approved by the Animal Care and Use Committee of Anhui Agricultural University (Hefei, China) and were in accordance with the university's guidelines for animal research (SYXK(Wan)2016-007).

Consent for publication

Not applicable.

Availability of data and materials

Raw unprocessed sequence data in this study are available from the National Centre for Biotechnology Information Sequence Read Archive (SRA) database under accession number: PRJNA648813.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Wen Zhu and Hongguo Cao proposed the project, and designed the experiments; Zhen Su, Wei Xu, and Hanxue Sun performed the experiments; Wen Zhu wrote the manuscript. Jinfeng Gao, Danfeng Tu, and Hongguo Cao revised and edited the manuscript.

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Figures

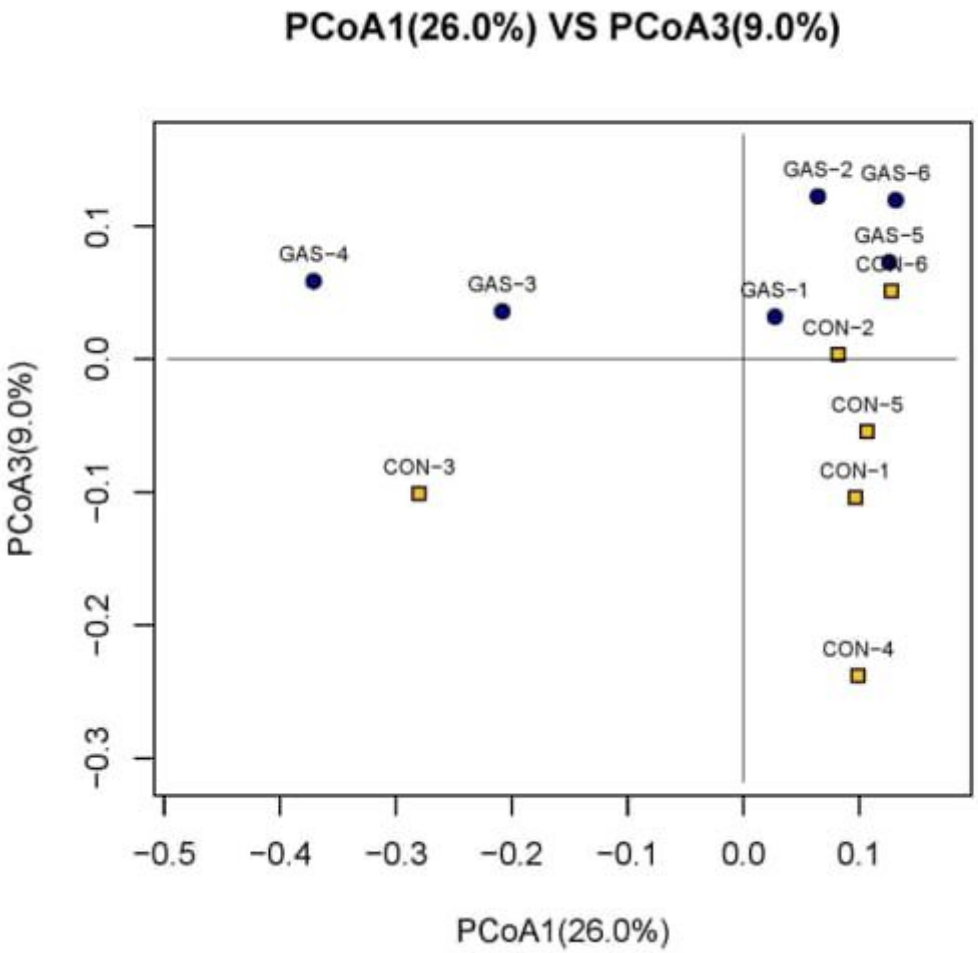

Figure 1

Unweighted Principal coordinates analysis (PCoA) analysis of taxonomical classifications of rumen bacterial communities in the GAS (the garlic skin diet) and the CON (the control diet) groups.

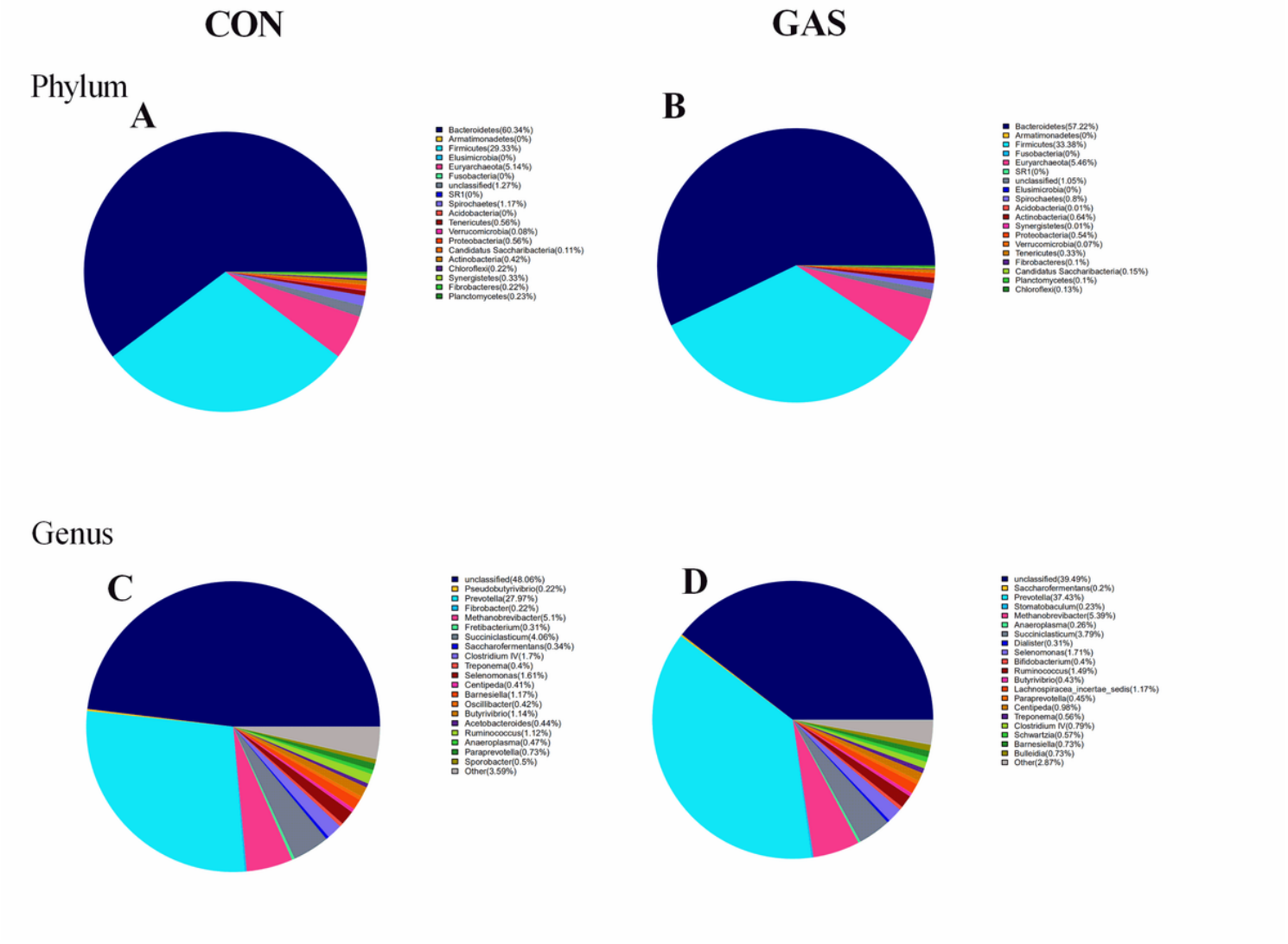

Figure 2

Distribution of bacterial taxa averaged under phyla (A, B) and genera (C, D) level between the dietary treatments (as a percentage of the total sequence).

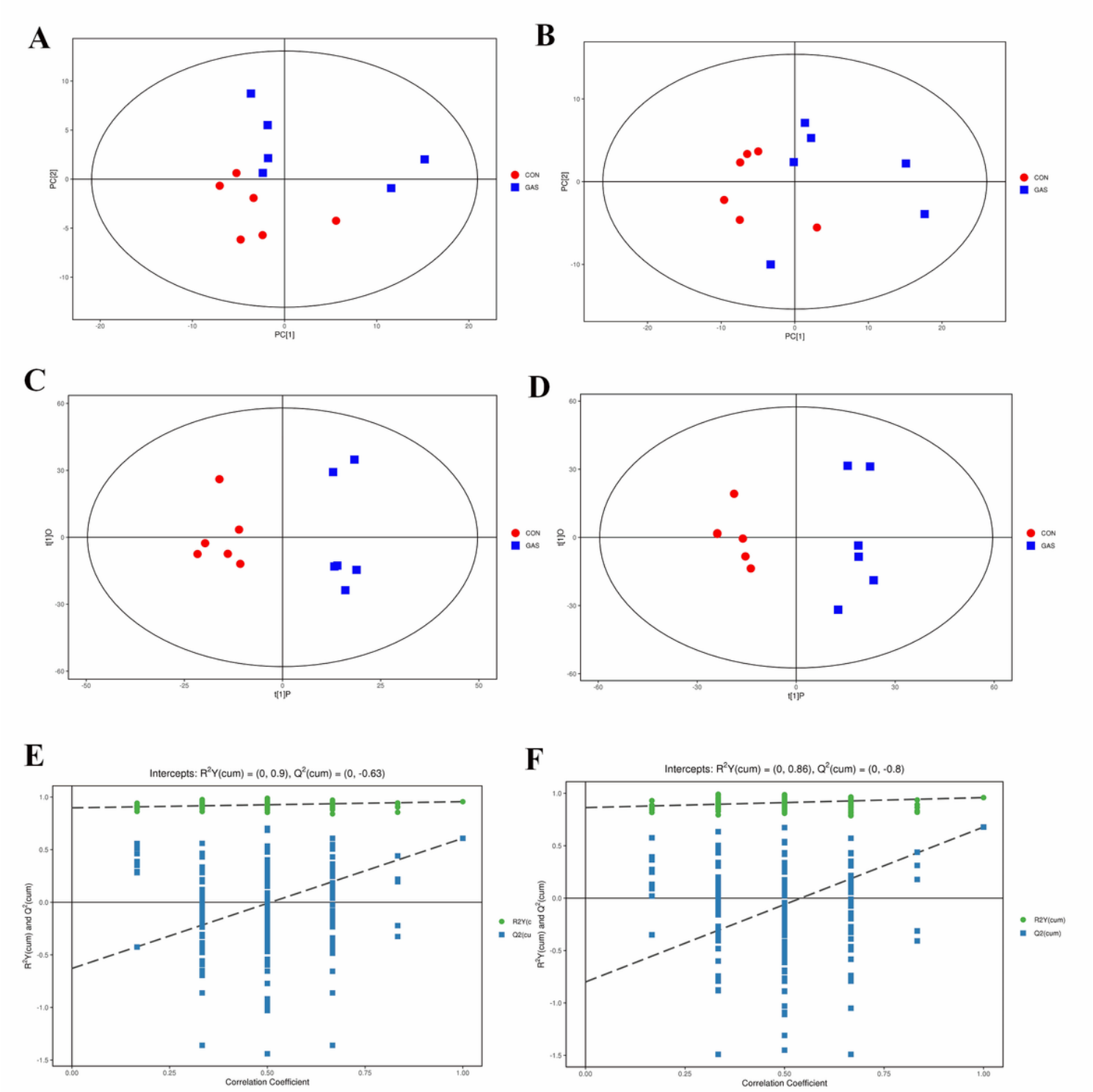

Figure 3

Identification of discriminating biomarkers by metabolomics analysis between the GAS (the garlic skin diet) and the CON (the control diet) groups. The PCA (A) and OPLS-DA(C) score plot are in negative ion mode. PCA (B) and OPLS-DA (D) score plot are in positive ion mode. Permutation tests conducted with 200 random permutations in the OPLS-DA model is built for negative (E) and positive ion mode (F).

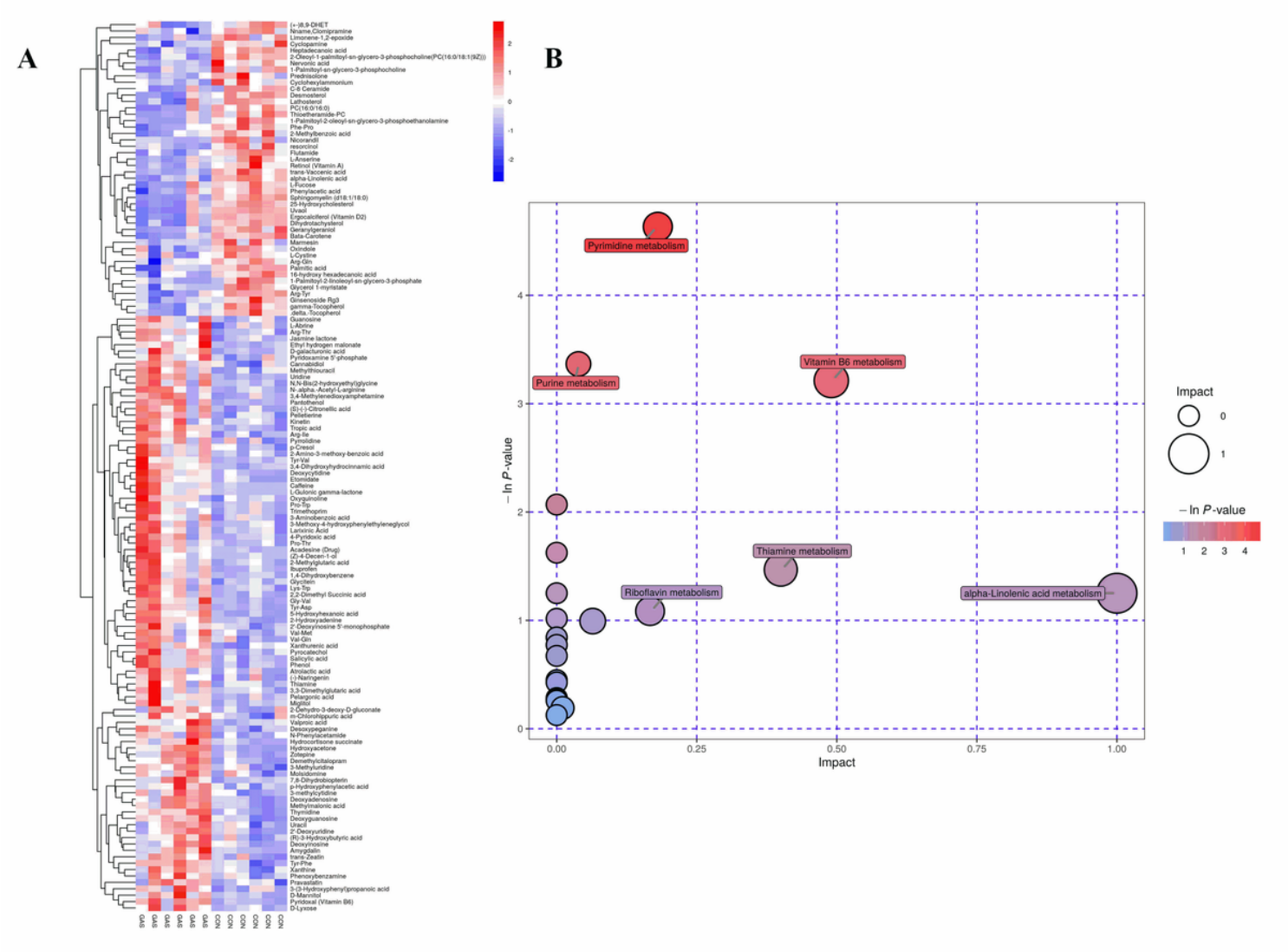

Figure 4

(A) Heatmap of the 139 significantly altered metabolites in the GAS (the garlic skin diet) and the CON (the control diet) groups. Individual samples (horizontal axis) and metabolites (vertical axis) are separated using hierarchical clustering. (B) Ruminal metabolomics pathway analysis on the lambs with the garlic skin treated compared to the control diet. The colors and sizes of the shapes represent the effects of the garlic skin treatments on sample metabolism relative to the control treatments; larger red shapes indicate a greater effect on the pathway.

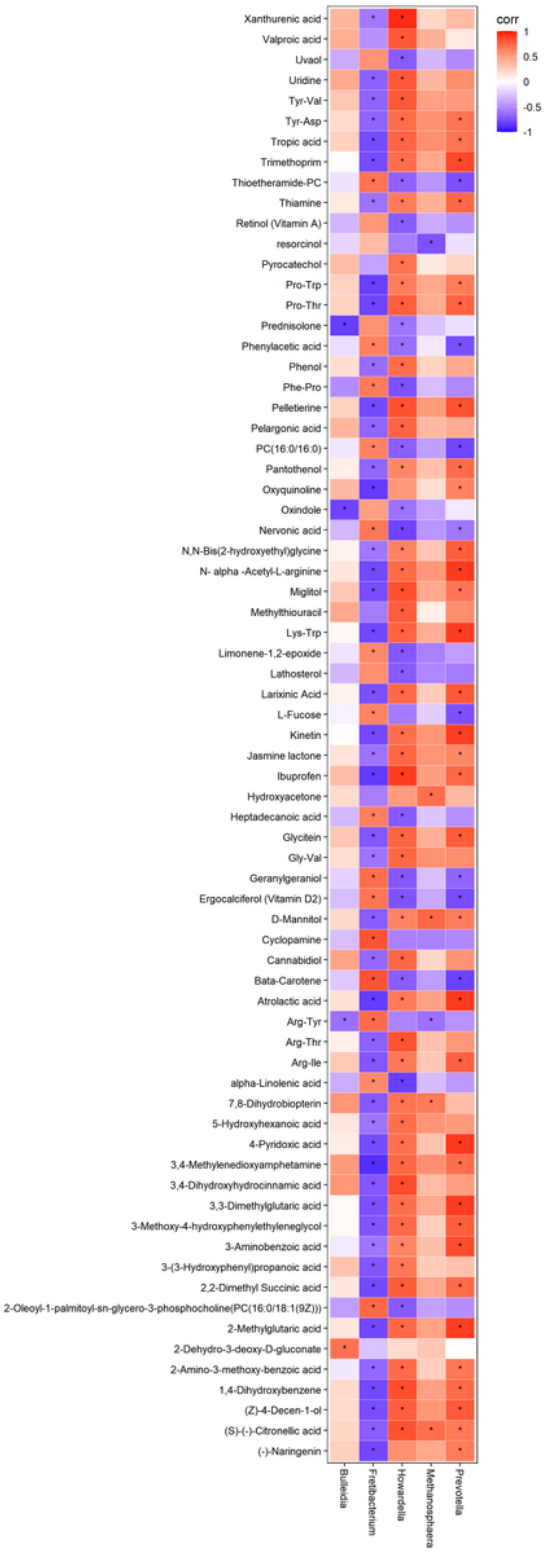

Figure 5

Correlation matrix between the ruminal differential metabolites affected by the garlic skin treatment and the differential microbiota at the genus level (only absolute Spearman rank correlation above 0.70 was showed). Positive correlations are shown in red and negative correlations in blue. *means P <0.05.

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