



# Diet Supplementation With Sulfur Amino Acids Modulated Fermentation Metabolome and Gut Microbiome in Goats

Tsegay Teklebrhan<sup>1,2,3\*</sup> and Zhiliang Tan<sup>1</sup>

<sup>1</sup> CAS Key Laboratory for Agro-Ecological Processes in Subtropical Region, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, China, <sup>2</sup> University of Chinese Academy of Sciences, Beijing, China, <sup>3</sup> School of Animal and Range Sciences, Haramaya University, Dire Dawa, Ethiopia

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### \*Correspondence:

Tsegay Teklebrhan  
ttmamy06@gmail.com

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Dietary amino acids shift hydrogen metabolism to an alternative hydrogen sink consisting of dissolved hydrogen sulfur (dH<sub>2</sub>S) rather than methanogenesis; and influences the fermentation metabolome and microbiome associated with particles and liquid fractions in gut regions (foregut, small intestine, and hindgut) of goats. A completely randomized block design with a total of 20 goats (5 goats per treatment) was used to conduct the trial. The goats were fed on a diet that consisted of a concentrated mixture with maize stover roughage (50:50, on a dry matter basis) and randomly assigned to one of the four treatments: without amino acid supplementation (a basal diet), a basal diet supplemented with methionine (Met), a basal diet supplemented with lysine (Lys), and a basal diet supplemented with methionine and lysine (ML). Goats fed Met alone or in combination had less acetate, acetate to propionate ratio, and greater propionate ( $p < 0.05$ ) in the foregut and hindgut than those fed control or Lys. Nonetheless, the goats fed on the amino acid supplements had higher levels of branched-chain VFA ( $p < 0.05$ ) in the foregut and hindgut than the control goats. Goats fed on ML had the highest ammonia ( $p < 0.01$ ), followed by Met or Lys, both in the foregut and hindgut, compared with the control. Those fed on Met alone or in combination, had lower dH<sub>2</sub>, dCH<sub>4</sub> ( $p < 0.01$ ), and higher dH<sub>2</sub>S ( $p < 0.01$ ) in the foregut and hindgut than the control or Lys. The goats that were fed on Met alone or in combination, had higher 16S rRNA gene copies of total bacteria, methanogens, and 18S rRNA gene copies of protozoa, fungi, and fiber-utilizing bacterial species ( $p < 0.01$ ) associated with particles vs. liquid, both in the foregut and hindgut than the control goats. This study gives insights into the use of sulfur-containing amino acids, as an alternative dietary mitigation strategy of methanogenesis in ruminants and highlights the need for further research in this direction.

**Keywords:** amino acids, hydrogen, metabolites, metagenomics, microbiome

## INTRODUCTION

Microbial fermentation of amino acids produces ammonia, volatile fatty acids (VFA), carbon dioxide (CO<sub>2</sub>), methane (CH<sub>4</sub>), and molecular hydrogen (H<sub>2</sub>) in the gut (foregut, small intestine, and hindgut) of ruminants. It is crucial to reduce the level of crude protein (CP) in ruminant diets with the supplementation of limiting amino acids such as methionine and lysine, to improve the number of metabolizable amino acids; and decrease nitrogen losses, feed costs, and greenhouse gas emissions without adverse effect on animal performance (Sinclair, 2014; Guyader et al., 2016). It has been shown that dietary supplements with high sulfate could shift H<sub>2</sub> toward energetically advantageous pathways away from methanogenesis and reduce methane emissions and yield (Judy et al., 2019; Lan and Yang, 2019; Teklebrhan et al., 2020). For instance, a sulfur-rich corn gluten diet decreased dissolved hydrogen (dH<sub>2</sub>) and methane (dCH<sub>4</sub>), while increasing dissolved hydrogen sulfur (dH<sub>2</sub>S) which was associated with reduced methanogenesis in goats fed on corn meal (Teklebrhan et al., 2020). Hydrogen has been shown to be involved in amino acid fermentation in several ways. In some cases, hydrogen or reducing equivalents required for hydrogenation reactions can be obtained by the uptake of molecular hydrogen or may be generated from one amino acid for the reduction of another (Nisman, 1954; Barker, 1961). This suggests that amino acid biosynthesis can either release or consume H<sub>2</sub>, which could affect gut fermentation pathways and gaseous production. Biosynthesis of sulfur-containing amino acids like methionine involves H<sub>2</sub> being reduced to H<sub>2</sub>S, suggesting that methionine biosynthesis could facilitate uptake of H<sub>2</sub> by sulfidogenic bacteria rather than methanogens.

Research on diet supplementation with amino acids is limited to using amino acids in microbial fermentation and microbiome patterns in ruminants, though few *in vitro* trials Abbasi et al. (2019) and Hassan et al. (2021) reported inconsistent results for lysine and methionine in fermentation and microbiota. Hence, it is crucial to investigate and compare the effects of supplementation of dietary methionine or lysine, either alone or in combination with a low protein diet in modulating microbial fermentation and the microbial ecosystem in the gut (foregut, small intestine, and hindgut) of ruminants. We hypothesized that methionine supplementation, either alone or combination in corn stover based diet could shift hydrogen metabolism toward an alternative electron sink and modulates fermentation metabolome and microbiome in goats. As a result, this study investigated that sulfur amino acids shift hydrogen to H<sub>2</sub>S than methanogenesis and altered the microbiome associated with solid and liquid fractions in the gut regions of goats.

## MATERIALS AND METHODS

### Animals Feeding and Management

The study used twenty Liuyang black male goats with an average age of 10 ± 0.2 months old and an initial body weight of

18.2 ± 2.5 kg. The experiment was conducted using a completely randomized block design with a total of 20 goats (5 goats per treatment). All of the goats were kept in stainless steel metabolic cages (150 cm × 60 cm × 80 cm) with free access to clean water. The metabolic room's temperature was set to 22 ± 1°C. The diet was designed to meet 140% of the metabolic energy maintenance needs (Lu and Zhang, 1996). The ingredients and nutrient composition of a basic diet are given in **Table 1**. In total, twenty goats were randomly divided into four groups. Each group of five goats was randomly assigned to one of four diets: a basal diet with no amino acid supplementation (control), a control diet supplemented with methionine (Met), a control diet supplemented with lysine (Lys), and a control diet supplemented with both methionine and lysine (ML). Goats were adapted to treatment diets through step-wise increments for 14 days until they all reached their stable dry matter (DM) intake according to the standard of metabolic body weight. The experimental period lasted for another 12 d. Goats were fed *ad libitum*, targeting less than 5% refusal. Daily meals were offered twice equally at 8:00 am and 4:00 am. The amounts of methionine and lysine supplement in Met, Lys, and ML treatments were 1.27 g, 1.96 g, and 1.27 plus 1.96 g of concentrate on a DM basis, respectively, according to Sun et al. (2007). Briefly, the amounts of supplements were computed from the intestinal degradability of amino acids and the pattern in muscle protein of goats in order to balance the supply of amino acids to the duodenum. To balance the extra effect of nitrogen from the amino acid supplements, control, Met, and Lys were supplemented with 1.08, 0.81, and 0.25 g urea per 100 g of concentrate, respectively. The duodenal and ileal flows, as well as ileal apparent digestibility

**TABLE 1** | Ingredients<sup>a</sup> and nutrient composition of the diet.

Item	Basal diet
Ingredient (g/kg DM)	
Corn	224
Wheat bran	179
Soybean meal	60.0
Rapeseed meal	1.00
Maize straw	500
Urea	10.8
Salt	6.00
Vitamin/mineral premix <sup>b</sup>	20.0
Nutrient composition (g/kg DM)	
Crude protein	126.9
Acid detergent fiber	231.8
Neutral detergent fiber	493.5
Calcium	2.00
Phosphorus	4.00
Metabolizable energy (MJ/kg)	2.26

<sup>a</sup>Ingredients composition (% DM), contained 22.4% of corn, 17.9% wheat bran, 6.0% of soybean meal, 0.1% of rape seed meal, 50% of maize stover, 1.08% of urea, 0.6% of salt, and 2.0% of premix.

<sup>b</sup>Premix formulated (per kg of dietary DM): 119 g of MgSO<sub>4</sub>·H<sub>2</sub>O, 1.53 g of FeSO<sub>4</sub>·H<sub>2</sub>O, 0.8 g of CuSO<sub>4</sub>·5H<sub>2</sub>O, 3 g of MnSO<sub>4</sub>·H<sub>2</sub>O, 5 g of ZnSO<sub>4</sub>·H<sub>2</sub>O, 10 mg of Na<sub>2</sub>SeO<sub>3</sub>, 40 mg of KI, 30 mg of CoCl<sub>2</sub>·6H<sub>2</sub>O, 95,000 IU of vitamin A, 17,500 IU of vitamin D, and 18,000 IU of vitamin E.

of amino acids, were calculated according to the following equations:

$$Q_d = \frac{C_t}{C_d} \quad (1)$$

$$Q_i = \frac{C_t}{C_i} \quad (2)$$

$$DFAA_i = CDAA_i \times Q_d \quad (3)$$

$$IFAA_i = CIAA_i \times Q_i \quad (4)$$

$$DAA_i = \frac{DFAA_i - IFAA_i}{DFAA_i} \times 100\% \quad (5)$$

where  $Q_d$  is the DM flow in the duodenum,  $C_t$  the total amount of the administrated  $Cr_2O_3$  in the rumen per day,  $C_d$  the  $Cr_2O_3$  content in the dried duodenal digesta,  $Q_i$  the DM flow at the ileum,  $C_i$  the  $Cr_2O_3$  content in the dried ileal digesta,  $DFAA_i$  the  $AA_i$  flow at the duodenum,  $CDAA_i$  the  $AA_i$  content in the dried duodenal digesta,  $IFAA_i$  the  $AA_i$  flow at the ileum,  $CIAA_i$  the  $AA_i$  content in the dried ileal digesta, and  $DAA_i$  is the ileal apparent digestibility of  $AA_i$ . In addition, the amounts of Met and Lys infused into the lumen of the duodenum were calculated by the following equation as follows:

$$\frac{DAA_i}{R_i} \Delta X_i - \sum_{i=1}^n DAA_i \times \Delta X_i = D_t Q_t - \frac{D_i \times DFAA_i}{R_i} \quad (6)$$

where  $D_t$  is the ileal digestibility of total amino acids,  $Q_t$  is the duodenal flow of total amino acids,  $\Delta X_i$  is the computed amount of  $AA_i$  infused into the duodenum and  $R_i$  is the  $AA_i$  proportion of total amino acids in the muscle.

## Sampling and Processing

After the morning feed on d 12, 5 goats from each treatment were euthanized according to the ethical procedure of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (procedure number: ISA-W-201802). The abdomen was opened, and the gut was immediately separated from the carcass. To avoid mixing of digesta for sampling, the gut regions (foregut, small intestine, and hindgut), including reticulo-rumen (foregut), duodenum, ileum, jejunum (small intestine), cecum, colon, and rectum (hindgut), were tied with a sterile thread at the start and end of each region. Each gut region was longitudinally incised along the dorsal line using sterile equipment. The contents in each gut region were first homogenized and then mixed thoroughly to reduce the localized effect.

Representative samples of the foregut (~100 g), small intestine (~60 g), and hindgut (~60 g) were collected in sterile anoxic tubes. A schematized diagram of the sampling regions is given in **Figure 1**. Approximately, 10 g of a subsample from each gut region was used for immediate measurement of  $dH_2$ ,  $dH_2S$ , and pH. Another subsample of each gut region was diluted with 1:5 (m/v) iced sterile anaerobic phosphate-buffered saline (PBS; pH 6.8). Samples were then homogenized and filtered through four layers of sterile cheesecloth to obtain approximately

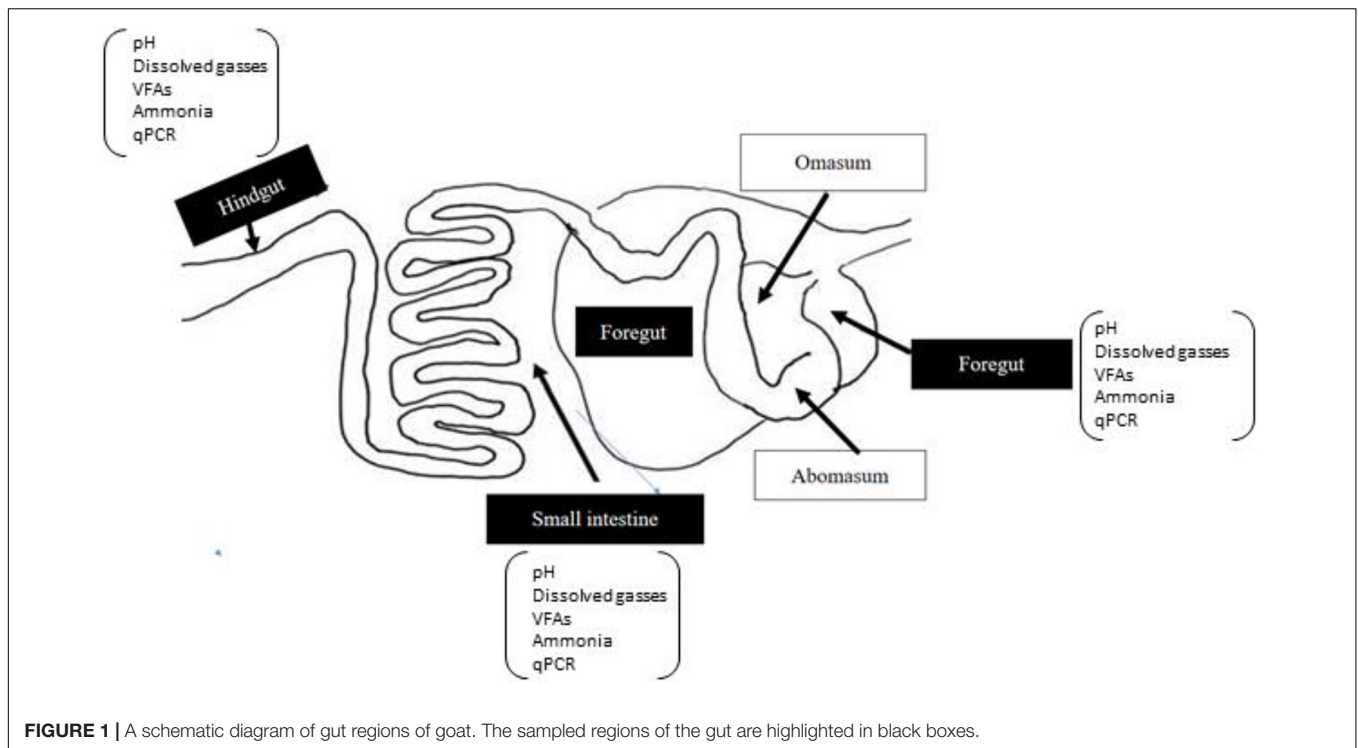
100 ml of liquid and remaining particle-associated samples from each region, respectively, for the liquid and particle-associated samples. Then samples were immediately snap-frozen using liquid nitrogen at  $-80^\circ C$  for genomic DNA isolation. The remaining liquid from each region was used for the analysis of VFA, ammonia, and  $dCH_4$ .

## Sample Analysis

All samples of the feed offered, and refusals were dried at  $105^\circ C$  for 24 h for DM determination and then ground, using a hammer mill to pass through a 1-mm sieve. Crude protein (CP) ( $N \times 6.25$ ) was determined using the Kjeldahl method (AOAC, 1995). Neutral detergent fiber (NDF) with the addition of  $\alpha$ -amylase and sodium sulfite and acid detergent fiber (ADF), both expressed inclusive of residual ash, were analyzed according to Van Soest et al. (1991). Metabolizable energy (ME) was calculated, according to Lu and Zhang (1996). Phosphorus and Ca concentrations were determined, following the procedure of AOAC International (2006).

The pH of gut samples was measured using a portable pH meter (Starter 300; Ohaus Instruments Co., Ltd., Shanghai, China). The  $dH_2$  and  $dH_2S$  of gut samples were determined by micro-sensor, using  $H_2$  and  $H_2S$  electrodes, respectively, according to protocols of the manufacturer's manual (Unisense, Aarhus, Denmark). Dissolved methane ( $dCH_4$ ) was extracted from the liquid phase of gut samples into the gas phase using the procedure of Wang et al. (2016a) with slight modification. A 20-ml syringe containing 10 ml of  $N_2$  gas (>99.99%) was transferred into a 50-ml plastic syringe containing 35 ml of gut samples *via* polyurethane tubing. The gas dissolved in the liquid phase was released into the gas phase, by shaking at 200 revolutions per second for 5 min in an orbital shaker (WSZ-100A, Shanghai Yiheng Scientific Instruments Co., Ltd., Shanghai, China). Gas samples were collected, using evacuated tubes for analysis, using GLC (Agilent 7890A, Agilent Inc., Palo Alto, CA, United States). Then, the concentration of  $dCH_4$  in the original liquid fraction was computed using the equation of Wang et al. (2016a). Frozen liquids were thawed and centrifuged at  $12,000 \times g$  for 10 min at  $4^\circ C$ , and supernatants were used to determine the VFA profile according to Wang et al. (2016a) using gas chromatography (Agilent 7890A, Agilent Inc., Palo Alto, CA, United States). Ammonia concentrations were measured according to Weatherburn (1967).

One ml liquid and 1 g particle-associated samples from each gut region were used for the isolation of genomic DNA following the procedures of Yu and Morrison (2004). The quality of the DNA was determined, using agarose gel electrophoresis (0.8%). The quantity of DNA was measured, using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, United States). The qPCR analysis of target microbes was performed, using an ABI 7900HT, Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, United States) using SYBR Premix Ex Taq (Perfect Real Time, Takara, Shiga, Japan). A standard curve was generated for each bacterial species and total bacteria, methanogens, protozoa, and fungi, using plasmid DNA containing the insert for the primers as shown in **Table 2**. The ten-fold serial dilutions of each standard were



**TABLE 2** | Primers are used for quantitative PCR (qPCR).

Target species	Primer	Primer sequence (5'-3')	Size (bp)	Reference	E <sup>1</sup> (%)
Bacteria	Forward	CGGCAACGAGCGCAACCC	146	Denman and McSweeney, 2006	100.4
	Reverse	CCATTGTAGCACGTGTGTAGCC			
Protozoa	Forward	GCTTTCGWTGGTAGTGTATT	223	Sylvester et al., 2004	96.3
	Reverse	CTTGCCCTCYAATCGTWCT			
Methanogens	Forward	GGATTAGATACCCSGGTAGT	192	Hook et al., 2009	101.9
	Reverse	GTTGARTCCAATTAACCGCA			
Fungi	Forward	GAGGAAGTAAAAGTCGTAACAAGGTTTC	120	Denman and McSweeney, 2006	97.2
	Reverse	CAAATTCACAAGGGTAGGATGATT			
<i>Prevotella ruminicola</i>	Forward	GAAAGTCGGATTAATGCTCTATGTTG	74	Stevenson and Weimer, 2007	99.8
	Reverse	CATCCTATAGCGGTAACCTTTGG			
<i>Selenomonas ruminantium</i>	Forward	CAATAAGCATTCCGCCTGGG	138	Stevenson and Weimer, 2007	102.2
	Reverse	TTCACTCAATGTCAAGCCCTGG			
<i>Ruminococcus albus</i>	Forward	CCCTAAAAGCAGTCTTAGTTTCG	176	Koike and Kobayashi, 2001	101.3
	Reverse	CCTCCTTGCGGTTAGAACA			
<i>Ruminococcus flavefaciens</i>	Forward	CGAACGGAGATAATTTGAGTTTACTTAGG	132	Denman and McSweeney, 2006	102.2
	Reverse	CGGTCTCTGTATGTTATGAGGTATTACC			
<i>Fibrobacter succinogenes</i>	Forward	GTTCGGAATTACTGGGCGTAAA	121	Denman and McSweeney, 2006	100.7
	Reverse	CGCCTGCCCTGAACTATC			
<i>Ruminobacter amylophilus</i>	Forward	CTGGGGAGCTGCCTGAATG	102	Stevenson and Weimer, 2007	100.3
	Reverse	GCATCTGAATGCGACTGGTTG			

<sup>1</sup>Efficiency.

made in RNase-free water for qPCR analysis. The qPCR reaction volume was 10  $\mu$ l, including 5  $\mu$ l of SYBR Premix Ex Taq, 0.2  $\mu$ l of ROX, 0.2  $\mu$ l of each primer (10  $\mu$ M), 1  $\mu$ l of the template DNA (10 ng/ $\mu$ l), and 3.4  $\mu$ l of RNase-free water. The program was set to 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s for annealing/extension.

The final melting curve was detected at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The final absolute amount of the target group or species was estimated, by relating the cycle threshold (CT) value to the standard curves. The results were then transformed into log<sub>10</sub> copies/ml or g of sample for further analysis.

## Statistical Analysis

Fermentation metabolites and qPCR data were analyzed using the R software version 3.6.3 by the (R Core Team 2020, Vienna, Austria). Data were subjected to a linear mixed model, using the package lme4 version as described by Pinheiro et al. (2013). Including diet, gut regions, sample fraction, and all possible interactions between them, as fixed effects, and block or animal as a random effect. Multiple mean comparisons were tested, using Tukey's adjustment. Differences at  $p \leq 0.05$  were considered significant results. Pearson correlation coefficients were computed to determine the correlations between fermentation metabolites and microbiota concentrations. Correlation coefficient values ( $r$ ) with  $r > 0.44$  for  $p < 0.1$ ,  $r > 0.52$ ,  $p < 0.05$ ,  $r > 0.66$  for  $p < 0.01$ , and  $r > 0.79$  for  $p < 0.001$ . Correlation coefficient values greater than zero indicate a positive correlation while values less than zero indicate a negative correlation between variables.

## RESULTS

### Short-Chain Fatty Acid Metabolites

Amino acid supplementation changed the production of volatile fatty acids in gut regions (Table 3). The highest total VFA production was obtained (+73 and 64%) in the foregut, followed by the hindgut and the small intestine, with a lower (−24%;  $p < 0.05$ ) in the hindgut vs. foregut filtrates. The foregut had a higher acetate molar percentage and acetate to propionate ratio ( $p < 0.05$ ), than the hindgut and small intestine filtrates. Nevertheless, propionate, valerate, and branched-chain VFA (isobutyrate and isovalerate) ( $p < 0.05$ ) followed the reverse trend; being higher in the small intestine > hindgut > foregut.

Except in the small intestine, goats fed Met alone or in combination (ML), had lower acetate, and acetate to propionate ratios, but higher propionate ( $p < 0.05$ ) than those fed control or Lys. Nevertheless, goats fed on the amino acid supplements had greater branched-chain VFA ( $p < 0.05$ ) than those in the control group. Individual fatty acids were modulated by the interaction of the gut with diet: goats fed Met alone or in combination had less acetate, and acetate to propionate ratio while; having more propionate ( $p < 0.05$ ) in the foregut and hindgut than those fed control or Lys. In addition, consistently, greater branched-chain VFA ( $p < 0.01$ ) was apparent; both in the foregut and hindgut of goats fed the amino acid supplements than in the control group. Despite the small intestine having the highest propionate and branched-chain VFA ( $p < 0.05$ ), these values remained unaffected by the diet groups.

### Dissolved Gas Products

Amino acid supplementation influenced ammonia and gaseous production, including  $dH_2$ ,  $dH_2S$ , and  $dCH_4$ , in different gut regions of goats (Table 4 and Figures 2, 3). The ammonia levels (+88 and 85%, respectively) were higher in the foregut and hindgut than in the small intestine, with the hindgut having less ammonia (−21%) than the foregut (Table 4 and Figure 2A). Foregut and hindgut had greater  $dH_2$  (+93 and 87%;  $p < 0.05$ ), respectively, than in the small intestine with an apparently lower (−47.5%;  $p < 0.05$ ) value in the contents of the hindgut vs. foregut (Table 4 and Figure 2B). In addition, the foregut and hindgut had greater  $dCH_4$  (+86 and 79%;  $p < 0.05$ ) than in the small intestine and less (−30;  $p < 0.05$ ) in the hindgut than the foregut (Table 4 and Figure 2C). Consistently, the foregut and hindgut had enhanced  $dH_2S$  (+89 and 86%) levels than the small intestine and lower (−22%)  $dH_2S$  levels in the hindgut than the

TABLE 3 | Fatty acid metabolites in gut regions of goats supplemented with amino acid.

Gut regions	Diet <sup>2</sup>	Items							
		VFA <sup>1</sup>	Acetate	Propionate	Butyrate	Valerate	Isobutyrate	Isovalerate	Ace/prop
Small intestine	Control	18.1	42.1 <sup>c</sup>	31.0 <sup>a</sup>	14.0	4.00	4.20 <sup>a</sup>	4.70 <sup>a</sup>	1.35 <sup>c</sup>
	Met	17.2	42.1 <sup>c</sup>	30.1 <sup>a</sup>	15.9	3.98	3.94 <sup>a</sup>	3.98 <sup>a</sup>	1.39 <sup>c</sup>
	Lys	18.0	41.2 <sup>c</sup>	32.6 <sup>a</sup>	14.1	4.10	3.96 <sup>a</sup>	4.04 <sup>a</sup>	1.26 <sup>c</sup>
	ML	19.3	42.0 <sup>c</sup>	31.3 <sup>a</sup>	13.0	4.70	4.64 <sup>a</sup>	4.36 <sup>a</sup>	1.34 <sup>c</sup>
Foregut	Control	66.7	65.6 <sup>a</sup>	16.0 <sup>c</sup>	14.0	2.12	1.53 <sup>c</sup>	0.75 <sup>c</sup>	4.10 <sup>a</sup>
	Met	67.5	54.9 <sup>b</sup>	22.5 <sup>b</sup>	14.9	2.16	2.67 <sup>b</sup>	2.87 <sup>b</sup>	2.44 <sup>b</sup>
	Lys	68.3	63.6 <sup>a</sup>	15.2 <sup>c</sup>	14.1	1.99	2.62 <sup>b</sup>	2.49 <sup>b</sup>	4.18 <sup>a</sup>
	ML	69.5	53.9 <sup>b</sup>	23.5 <sup>b</sup>	15.0	2.20	2.89 <sup>b</sup>	2.51 <sup>b</sup>	2.29 <sup>b</sup>
Hindgut	Control	49.8	62.5 <sup>a</sup>	19.1 <sup>c</sup>	14.5	2.02	0.98 <sup>c</sup>	0.90 <sup>c</sup>	3.27 <sup>a</sup>
	Met	50.4	51.8 <sup>b</sup>	26.3 <sup>b</sup>	14.8	2.32	2.90 <sup>b</sup>	1.88 <sup>b</sup>	1.97 <sup>b</sup>
	Lys	51.3	61.5 <sup>a</sup>	17.7 <sup>c</sup>	13.9	2.03	2.46 <sup>b</sup>	2.41 <sup>b</sup>	3.47 <sup>a</sup>
	ML	52.1	51.9 <sup>b</sup>	25.7 <sup>b</sup>	15.0	2.71	2.57 <sup>b</sup>	2.12 <sup>b</sup>	2.02 <sup>b</sup>
	SEM	5.01	3.67	2.50	1.46	0.20	0.01	0.02	0.03
<i>P</i> -value	Gut	0.0201	0.0301	0.0401	0.1524	0.0201	0.0400	0.0300	0.0302
	Diet	0.1210	0.0400	0.0200	0.1310	0.1124	0.0201	0.0412	0.0200
	Gut*Diet	0.3212	0.0301	0.0410	0.2435	0.2010	0.0302	0.0402	0.0310

<sup>1</sup>VFA total volatile fatty acids (mM), individual volatile fatty acids (mol/100 mol) acetate to propionate ratio (mol/mol), <sup>2</sup>basic diet without supplementation (Control), control supplemented with methionine (Met), and control supplemented with lysine (Lys), and control supplemented with methionine and lysine (ML). Results beared different letters indicate significant, while results beared same letters indicate not significant variation.

**TABLE 4** | Gas metabolites in gut regions of goats supplemented with amino acid.

Gut regions	Diet <sup>2</sup>	Items				
		pH	Ammonia (mM)	dH <sub>2</sub> <sup>1</sup>	dCH <sub>4</sub>	dH <sub>2</sub> S
Small intestine	Control	7.10	1.20 <sup>e</sup>	1.40 <sup>e</sup>	0.30 <sup>e</sup>	29.9 <sup>e</sup>
	Met	7.00	1.30 <sup>e</sup>	0.77 <sup>e</sup>	0.31 <sup>e</sup>	33.0 <sup>e</sup>
	Lys	6.05	1.31 <sup>e</sup>	1.37 <sup>e</sup>	0.34 <sup>e</sup>	27.0 <sup>e</sup>
	ML	7.09	1.41 <sup>e</sup>	0.80 <sup>e</sup>	0.28 <sup>e</sup>	34.0 <sup>e</sup>
Foregut	Control	6.87	6.37 <sup>d</sup>	24.3 <sup>a</sup>	3.41 <sup>a</sup>	190.4 <sup>c</sup>
	Met	7.20	10.8 <sup>bc</sup>	14.9 <sup>c</sup>	1.70 <sup>cd</sup>	325.3 <sup>a</sup>
	Lys	6.70	11.4 <sup>bc</sup>	21.5 <sup>ab</sup>	2.92 <sup>ab</sup>	187.3 <sup>c</sup>
	ML	6.90	16.4 <sup>a</sup>	13.7 <sup>c</sup>	1.81 <sup>cd</sup>	301.8 <sup>a</sup>
Hindgut	Control	6.85	4.94 <sup>d</sup>	14.2 <sup>c</sup>	2.11 <sup>c</sup>	131.2 <sup>d</sup>
	Met	6.98	8.85 <sup>c</sup>	7.62 <sup>d</sup>	1.21 <sup>d</sup>	249.0 <sup>b</sup>
	Lys	7.06	8.01 <sup>c</sup>	13.1 <sup>c</sup>	2.37 <sup>c</sup>	151.0 <sup>d</sup>
	ML	6.96	13.7 <sup>b</sup>	6.63 <sup>d</sup>	1.42 <sup>d</sup>	245.8 <sup>b</sup>
	SEM	0.24	0.12	0.1	0.02	2.59
<i>P</i> -value	Gut	0.2134	0.0302	0.0434	0.0302	0.0200
	Diet	0.1425	0.0300	0.0329	0.0400	0.0334
	Gut*Diet	0.1201	<0.0001	<0.0001	<0.0001	<0.0001

<sup>1</sup>Dissolved hydrogen ( $\mu\text{M}$ ), dissolved methane (mM), dissolved hydrogen sulfur (mM).

<sup>2</sup>Basic diet without supplementation (Control), control supplemented with methionine (Met), and control supplemented with lysine (Lys), control supplemented with methionine and lysine (ML). Results beard different letters indicate significant, while results beard same letters indicate not significant variation.

foregut (Table 4 and Figure 2D). Goats fed ML had the highest ammonia compared with other treatments, whilst goats fed either Met alone or in combination had greater ammonia ( $p < 0.05$ ) compared, with those in control (Table 4 and Figure 2E). In

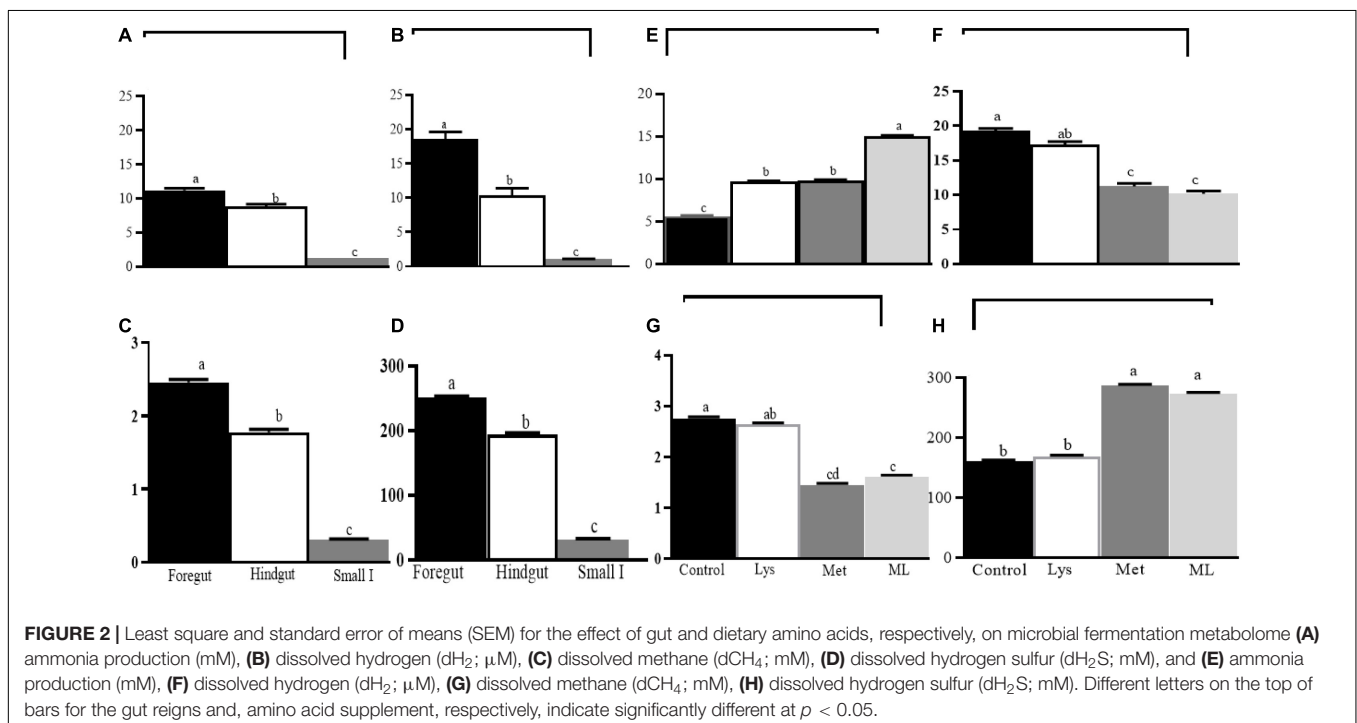
addition, goats fed either Met alone or in combination, reduced dH<sub>2</sub>, dCH<sub>4</sub>, while having greater dH<sub>2</sub>S production, than those in control or Lys (Table 4 and Figures 2F–H). It was consistent with higher ammonia in the contents of the foregut, followed by the hindgut in the amino acid supplements than in the control with ML having the highest ammonia levels (Table 4 and Figure 3A;  $p < 0.01$ ). In addition, goats fed either Met alone or in combination, had reduced dH<sub>2</sub>, dCH<sub>4</sub>, while having increased dH<sub>2</sub>S ( $p < 0.05$ ) than in control or Lys with a notably higher value in the foregut than in the hindgut and small intestine (Table 4 and Figures 3B–D;  $p < 0.01$ ).

## Association of Metabolome and Dissolved Gasses

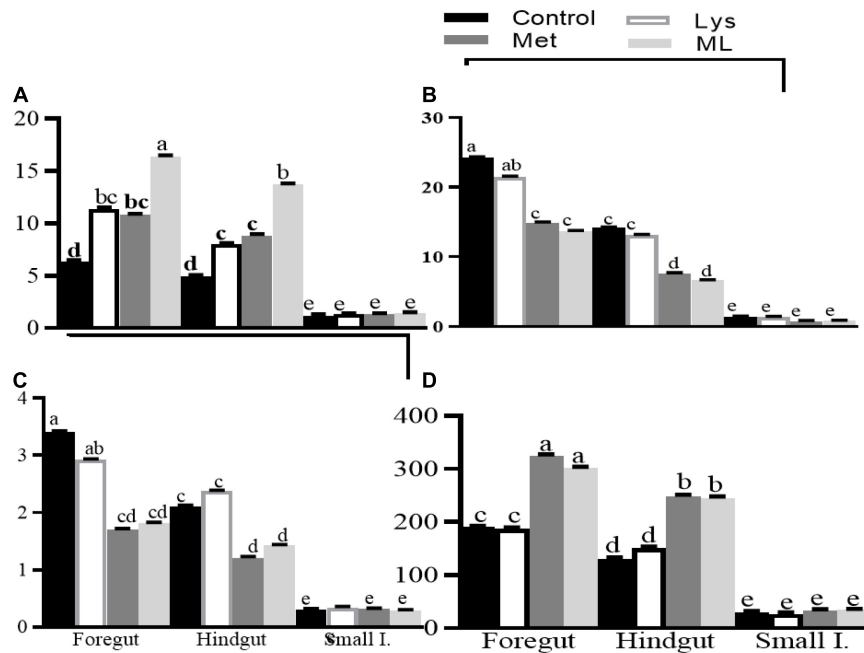
There were positive correlations between dH<sub>2</sub>, dCH<sub>4</sub> with ammonia, total VFA and molar percentages of propionate, isobutyrate, and isovalerate, and negative correlations with acetate, butyrate, and valerate, both in the foregut and hindgut, with no significant correlations in the small intestinal contents (Table 5). Furthermore, there was a strong positive correlation between dH<sub>2</sub> and dCH<sub>4</sub>, while having a negative correlation with dH<sub>2</sub>S in the foregut and hindgut. Nevertheless, there was a negative correlation between dCH<sub>4</sub> and dH<sub>2</sub>S in the foregut and hindgut. In addition, there was no significant correlation between dissolved gasses and fermentation metabolome in the contents of the small intestine.

## Microbiome

Amino acid supplementation had modulated gene copies of gut microbial ecosystems associated with particles and liquid fractions in goats (Table 6). The highest 16S rRNA gene



**FIGURE 2** | Least square and standard error of means (SEM) for the effect of gut and dietary amino acids, respectively, on microbial fermentation metabolome (A) ammonia production (mM), (B) dissolved hydrogen (dH<sub>2</sub>;  $\mu\text{M}$ ), (C) dissolved methane (dCH<sub>4</sub>; mM), (D) dissolved hydrogen sulfur (dH<sub>2</sub>S; mM), and (E) ammonia production (mM), (F) dissolved hydrogen (dH<sub>2</sub>;  $\mu\text{M}$ ), (G) dissolved methane (dCH<sub>4</sub>; mM), (H) dissolved hydrogen sulfur (dH<sub>2</sub>S; mM). Different letters on the top of bars for the gut regions and, amino acid supplement, respectively, indicate significantly different at  $p < 0.05$ .



**FIGURE 3 |** Least square and standard error of means (SEM) on microbial fermentation metabolites (A) ammonia production (mM), (B) dissolved hydrogen (dH<sub>2</sub>; μM), (C) dissolved methane (dCH<sub>4</sub>; mM), and (D) dissolved hydrogen sulfur (dH<sub>2</sub>S; mM) in different gut regions of goats in response to amino acid supplements. Different letters for the dietary treatments within each gut region indicate significant differences at *p* < 0.01.

**TABLE 5 |** Correlation<sup>1</sup> between dissolved hydrogen and fermentation metabolites in the gut regions of goats supplemented with amino acid.

Item	Dissolved H <sub>2</sub>			Dissolved CH <sub>4</sub>		
	Small I	Foregut	Hindgut	Small I	Foregut	Hindgut
Ammonia (mM)	0.10	0.90	0.65	0.09	0.82	0.67
Total volatile fatty acids (mM)	0.02	0.89	0.71	0.01	0.85	0.64
Acetate	0.03	0.82	0.62	0.00	0.92	0.72
Propionate	0.06	0.84	0.64	0.06	0.90	0.69
Butyrate	0.01	0.77	0.69	0.07	0.82	0.74
Valerate	0.04	0.73	0.71	0.11	0.65	0.69
Isobutyrate	0.03	0.82	0.68	0.03	0.78	0.58
Isovalerate	0.05	0.79	0.62	0.02	0.85	0.71
<b>Individual fatty acids (mol/100 mol)</b>						
Acetate	-0.08	-0.95	-0.71	-0.10	-0.84	-0.79
Propionate	0.13	0.82	0.69	0.06	0.79	0.59
Butyrate	-0.11	-0.61	-0.50	-0.12	-0.89	-0.62
Valerate	-0.09	-0.71	-0.64	-0.08	-0.75	-0.72
Isobutyrate	0.13	0.72	0.57	0.07	0.81	0.64
Isovalerate	0.17	0.89	0.74	0.13	0.73	0.66
Acetate to propionate ratio	-0.07	-0.83	-0.81	-0.10	-0.79	-0.70
<b>Dissolved gasses (mM)</b>						
Dissolved methane (dCH <sub>4</sub> )	0.04	0.93	0.81			
Dissolved hydrogen sulfur (dH <sub>2</sub> S)	-0.06	-0.95	-0.83	-0.05	-0.91	-0.74

<sup>1</sup>Correlation coefficient values (*r*) with *r* > 0.44 for *p* < 0.1, *r* > 0.52, *p* < 0.05, *r* > 0.66 for *p* < 0.01, *r* > 0.79 for *p* < 0.001.

copies of methanogens, bacteria, and 18S rRNA gene copies of fungi and protozoa (*p* < 0.01) were observed in the order of foregut > hindgut > small intestine. Additionally, 16S rRNA gene copies of starch (*Salmonella ruminantium*, *Prevotella*

*ruminicola*, and *Ruminobacter amylophilus*) and fiber utilizing bacterial species (*Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes*) were also consistently greater in the foregut and hindgut than the small intestine. Goats fed

amino acid supplements had increased 16S rRNA gene copies of bacteria, methanogens, and 18S rRNA of protozoa, fungi, and bacterial species (starch and fiber utilizing) ( $p < 0.05$ ) than those in control. In addition, the interaction of gut regions with diet had modulated gene copies of microbiota (Table 6). In response to the amino acid supplements total bacteria, methanogens, protozoa, fungi, and functional bacterial species ( $p < 0.01$ ) were increased in the foregut and hindgut compared with control.

Regardless of the amino acid supplemented, greater populations of methanogens, bacteria, fungi, protozoa, and fiber utilizing bacterial species (*R. albus*, *R. flavefaciens*, and *F. succinogenes*), while less *S. ruminantium*, *P. ruminicola* ( $p < 0.05$ ) were observed in the particles, than liquid fractions. In addition, particles associated populations of the methanogens, bacteria, fungi, protozoa, and fiber utilizing bacterial species ( $p < 0.01$ ) increased, both in the foregut and hindgut than liquid fractions. Nevertheless, populations of *S. ruminantium* and *P. ruminicola* were higher in the liquid than particles associated fractions, both in the foregut and hindgut. Consistently, goats in the amino acid supplements had increased populations of total microbiota, fiber utilizing bacterial species associated with particles vs. liquid fractions, both in the foregut and hindgut than in the control treatment with a notably highest value in the foregut. Moreover, the foregut and hindgut in the amino acids had increased total populations of bacteria, methanogens, protozoa, fungi, and fiber utilizing bacteria species ( $p < 0.01$ ) associated with particles rather than liquid fractions in the control group. The highest values of these microbiotas were observed in the foregut than the hindgut, and the lowest in the small intestine (Table 6).

## Association of Gasses and Microbiome

Concentrations of microbial groups were in correlation with almost all fermentation metabolites, both in the foregut and hindgut (Table 7). Concentrations of total VFAs,  $dH_2$ , and ammonia, and molar proportions of acetate, isobutyrate, and isovalerate, were positively correlated with all microbial groups in the foregut and hindgut. On the other hand, the concentration of  $dH_2S$  was positively correlated with the DNA concentrations of bacteria, protozoa, and fungi, and negatively correlated with methanogens. Inversely,  $dCH_4$  was negatively correlated with bacteria, protozoa, and fungi, but positively correlated with methanogens and fiber degrading bacterial species. Furthermore, the molar percentage of propionate was positively correlated with bacteria and fungi, while, negatively correlated with protozoa and methanogens, and fiber degrading bacterial species. On the other hand, molar percentages of butyrate and valerate were negatively correlated with concentrations of all microbial groups considered in the foregut and hindgut (Table 7).

## DISCUSSION

We have proposed that sulfur-containing amino acids could redirect hydrogen toward an alternative sink ( $H_2S$ ) than methanogenesis and modulates metabolites and microbiota associated with particles and liquid fractions in the gut regions

of goats. This was supported by a significant shift of these values both in the foregut and hindgut, with little effect on the small intestinal contents. This is likely because, the small intestine is predominated by enzymatic digestion, rather than microbial fermentation in the foregut and hindgut segments, implying less or no effect on fermentation products in the small intestine, in response to the amino acid supplements.

The influence of Met alone or in combination on the shift of fermentation metabolites such as dissolved gasses, VFAs, and the microbial community was visible, both in the foregut and hindgut, but, had little effect on the small intestine (Figure 3). This shows that the methane mitigating effects of these amino acid supplements are induced, not only by rumen fermentation modifications but also by hindgut fermentation changes. In this study, the decreased acetate to propionate ratio and  $CH_4$ , both in the foregut and hindgut of goats fed, either Met alone or in combination, indicated the use of these supplements in mitigating methanogenesis which was consistent with decreased total gas and  $CH_4$  in methionine supplemented more than in the control group in an *in vitro* trial (Abbasi et al., 2019).

However, an increased propionate in goats fed Met alone or in combination, could be caused by the sulfur contained in the amino acids. This claim is supported by previous studies that highlighted the role of sulfur supplementation at different doses (1 to 2.5%) in increased propionate in the range of (1 to 10.9%) in ruminants (Bal and Ozturk, 2006; Promkot et al., 2007; Supapong and Cherdthong, 2020a,b). This observation was consistent with a previous study that reported goats feeding on high sulfur in corn gluten; (CG) reduced  $CH_4$  production and yield, and this was associated with decreased rumen liquid  $dH_2$  and  $dCH_4$ , and increased  $dH_2S$ , as compared with those fed low sulfur in corn meal (CM; Teklebrhan et al., 2020). In addition, the inclusion of 2% sulfur with 2.5% urea in the fermented total mixed ration (FTMR), improved digestibility, fermentation, microbial crude protein synthesis, and milk quality in dairy cows (Supapong and Cherdthong, 2020a,b), suggesting that sulfur redirects  $H_2$  toward energetically beneficial pathways for the animal against methanogenesis.

The  $dH_2$  plays a central role in regulating fermentation pathways; low  $dH_2$  stimulates the acetate production pathway, while high  $dH_2$  stimulates the propionate production pathway (Janssen, 2010). This was consistent with a positive correlation of  $dH_2$  with propionate proportion; and a negative correlation with acetate proportion in the rumen (Wang et al., 2016b, 2018; Teklebrhan et al., 2020). Similarly, in the current study, we have observed a positive correlation of  $dH_2$  with propionate proportion and a negative correlation with acetate proportion, both in the foregut and hindgut. A shift in fermentation pathways affects  $CH_4$  production because, acetate biosynthesis is associated with net  $H_2$  release while, propionate formation is associated with reduced  $H_2$  formation (Janssen, 2010).

A recent *in vitro* study has reported a reduced  $H_2$  recovery in the methionine supplement than in the control (Hassan et al., 2021). This is in line with the current study, the decreasing  $H_2$  in the foregut and hindgut of goats fed Met alone or in combination might be due to the following main reasons: (1) Sulfur-containing amino acids such, as Met, may over



**TABLE 6** | Microbiota<sup>2</sup> in different gut regions of goats supplemented with amino acid.

Gut regions	Fraction	Diet <sup>1</sup>	Total microbial population				Bacterial species					
			Bacteria	Methanogens	Protozoa	Fungi	<i>P. ruminicola</i>	<i>S. ruminantium</i>	<i>R. amylophilus</i>	<i>R. albus</i>	<i>R. flavefaciens</i>	<i>F. succinogenes</i>
Small intestine	Particle	Control	4.92 <sup>g</sup>	4.01 <sup>f</sup>	4.62 <sup>g</sup>	3.29 <sup>g</sup>	4.97 <sup>f</sup>	4.39 <sup>f</sup>	4.09	4.39 <sup>g</sup>	4.10 <sup>g</sup>	3.96 <sup>g</sup>
		Met	5.15 <sup>g</sup>	4.18 <sup>f</sup>	4.83 <sup>g</sup>	4.04 <sup>g</sup>	4.59 <sup>f</sup>	4.27 <sup>f</sup>	3.99	4.49 <sup>g</sup>	4.19 <sup>g</sup>	4.06 <sup>g</sup>
		Lys	5.01 <sup>g</sup>	4.09 <sup>f</sup>	4.60 <sup>g</sup>	4.17 <sup>g</sup>	4.92 <sup>f</sup>	4.38 <sup>f</sup>	3.96	4.45 <sup>g</sup>	3.99 <sup>g</sup>	3.89 <sup>g</sup>
		ML	5.07 <sup>g</sup>	4.06 <sup>f</sup>	4.69 <sup>g</sup>	4.89 <sup>g</sup>	5.01 <sup>f</sup>	4.52 <sup>f</sup>	4.04	4.53 <sup>g</sup>	4.08 <sup>g</sup>	4.08 <sup>g</sup>
	Liquid	Control	4.82 <sup>g</sup>	3.93 <sup>f</sup>	4.24 <sup>g</sup>	3.02 <sup>g</sup>	5.07 <sup>f</sup>	4.36 <sup>f</sup>	4.02	4.22 <sup>g</sup>	4.03 <sup>g</sup>	4.78 <sup>g</sup>
		Met	5.05 <sup>g</sup>	4.18 <sup>f</sup>	4.40 <sup>g</sup>	4.32 <sup>g</sup>	4.79 <sup>f</sup>	4.42 <sup>f</sup>	3.47	4.39 <sup>g</sup>	4.10 <sup>e</sup>	3.99 <sup>g</sup>
		Lys	5.50 <sup>g</sup>	3.89 <sup>f</sup>	3.97 <sup>g</sup>	4.43 <sup>g</sup>	4.84 <sup>f</sup>	4.51 <sup>f</sup>	3.40	4.28 <sup>e</sup>	3.57 <sup>g</sup>	4.92 <sup>g</sup>
		ML	5.27 <sup>g</sup>	4.16 <sup>f</sup>	4.74 <sup>g</sup>	4.27 <sup>g</sup>	4.62 <sup>f</sup>	4.39 <sup>f</sup>	4.10	4.39 <sup>g</sup>	4.01 <sup>g</sup>	3.97 <sup>g</sup>
Foregut	Particle	Control	10.6 <sup>b</sup>	8.84 <sup>b</sup>	9.06 <sup>b</sup>	8.58 <sup>b</sup>	9.21 <sup>c</sup>	8.01 <sup>d</sup>	9.01	10.9 <sup>b</sup>	10.8 <sup>b</sup>	10.1 <sup>b</sup>
		Met	11.9 <sup>a</sup>	9.97 <sup>a</sup>	10.8 <sup>a</sup>	9.78 <sup>a</sup>	9.10 <sup>c</sup>	9.63 <sup>c</sup>	10.0	10.7 <sup>a</sup>	11.1 <sup>a</sup>	11.7 <sup>a</sup>
		Lys	11.4 <sup>a</sup>	9.67 <sup>a</sup>	10.9 <sup>a</sup>	9.88 <sup>a</sup>	9.01 <sup>c</sup>	9.16 <sup>c</sup>	10.9	11.0 <sup>a</sup>	10.7 <sup>a</sup>	10.8 <sup>a</sup>
		ML	12.2 <sup>a</sup>	9.82 <sup>a</sup>	11.3 <sup>a</sup>	10.2 <sup>a</sup>	10.6 <sup>b</sup>	10.4 <sup>b</sup>	10.2	10.9 <sup>a</sup>	11.0 <sup>a</sup>	10.6 <sup>a</sup>
	Liquid	Control	8.07 <sup>d</sup>	6.14 <sup>d</sup>	6.99 <sup>e</sup>	6.73 <sup>cd</sup>	10.9 <sup>b</sup>	10.0 <sup>b</sup>	9.03	8.03 <sup>d</sup>	8.02 <sup>d</sup>	8.82 <sup>d</sup>
		Met	9.70 <sup>c</sup>	7.95 <sup>c</sup>	8.52 <sup>c</sup>	7.74 <sup>c</sup>	11.0 <sup>a</sup>	11.0 <sup>a</sup>	10.3	9.89 <sup>c</sup>	9.76 <sup>c</sup>	9.78 <sup>c</sup>
		Lys	9.87 <sup>c</sup>	7.91 <sup>c</sup>	8.67 <sup>c</sup>	7.82 <sup>c</sup>	10.8 <sup>a</sup>	10.9 <sup>a</sup>	10.6	9.91 <sup>c</sup>	9.94 <sup>c</sup>	9.86 <sup>c</sup>
		ML	9.99 <sup>c</sup>	8.02 <sup>bc</sup>	9.04 <sup>b</sup>	8.20 <sup>bc</sup>	11.7 <sup>a</sup>	11.2 <sup>a</sup>	10.0	9.86 <sup>c</sup>	9.84 <sup>c</sup>	10.0 <sup>c</sup>
Hindgut	Particle	Control	7.24 <sup>e</sup>	6.19 <sup>d</sup>	6.63 <sup>e</sup>	6.01 <sup>e</sup>	7.61 <sup>e</sup>	7.89 <sup>e</sup>	8.78	7.15 <sup>e</sup>	7.01 <sup>e</sup>	8.05 <sup>e</sup>
		Met	9.59 <sup>c</sup>	7.04 <sup>c</sup>	7.88 <sup>d</sup>	6.98 <sup>cd</sup>	8.01 <sup>d</sup>	8.87 <sup>d</sup>	9.98	8.89 <sup>d</sup>	8.19 <sup>d</sup>	9.79 <sup>d</sup>
		Lys	9.69 <sup>c</sup>	7.14 <sup>c</sup>	7.95 <sup>d</sup>	7.01 <sup>cd</sup>	8.02 <sup>d</sup>	8.82 <sup>d</sup>	9.89	8.97 <sup>d</sup>	8.27 <sup>d</sup>	9.78 <sup>d</sup>
		ML	9.81 <sup>c</sup>	7.10 <sup>c</sup>	7.89 <sup>d</sup>	6.81 <sup>cd</sup>	8.09 <sup>d</sup>	9.45 <sup>c</sup>	9.97	9.09 <sup>d</sup>	8.16 <sup>d</sup>	9.69 <sup>d</sup>
	Liquid	Control	6.04 <sup>f</sup>	4.29 <sup>d</sup>	4.13 <sup>g</sup>	4.97 <sup>g</sup>	8.11 <sup>d</sup>	8.09 <sup>d</sup>	8.89	5.09 <sup>f</sup>	6.91 <sup>f</sup>	6.19 <sup>f</sup>
		Met	6.99 <sup>e</sup>	5.82 <sup>e</sup>	5.88 <sup>f</sup>	5.98 <sup>f</sup>	9.99 <sup>c</sup>	9.01 <sup>c</sup>	9.62	7.23 <sup>e</sup>	7.89 <sup>e</sup>	7.89 <sup>e</sup>
		Lys	7.26 <sup>e</sup>	5.94 <sup>e</sup>	5.84 <sup>f</sup>	5.85 <sup>f</sup>	10.0 <sup>c</sup>	9.32 <sup>c</sup>	9.78	6.99 <sup>e</sup>	7.97 <sup>e</sup>	7.93 <sup>e</sup>
		ML	7.01 <sup>e</sup>	6.10 <sup>d</sup>	5.73 <sup>f</sup>	6.01 <sup>e</sup>	9.97 <sup>c</sup>	10.0 <sup>b</sup>	9.80	7.09 <sup>e</sup>	7.16 <sup>e</sup>	7.95 <sup>e</sup>
<i>P-value</i>	Gut (G)	Diet (D)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
		Fraction	0.0303	0.0300	0.04102	0.0200	0.0223	0.0421	0.0311	0.0321	0.0400	0.0323
		Gut*Diet	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
		Gut*F	<0.0001	<0.0001	<0.0001	<0.0001	0.0234	0.0310	0.0120	<0.0001	<0.0001	<0.0001
		Diet*F	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
		G*D*F	<0.0001	<0.0001	<0.0001	<0.0001	0.2102	0.3101	0.1901	<0.0001	<0.0001	<0.0001

<sup>1</sup>Basic diet without supplementation (control), control supplemented with methionine (Met), and control supplemented with lysine (Lys), and control supplemented with methionine and lysine (ML).

<sup>2</sup>Microbiota log<sub>10</sub> gene copy number per g of particle and liquid fractions of digesta in different gut regions of goats. Results beard different letters indicate significant, while results beard same letters indicate not significant variation.

**TABLE 7** | Correlation<sup>1</sup> between concentrations of microbial groups and fermentation metabolites in the gut regions of goats supplemented with amino acid.

	Bacteria <sup>1</sup>		methanogen		protozoa		Fungi		<i>R. albus</i>		<i>R. flavefaciens</i>		<i>F. succinogenes</i>	
	Foregut	Hindgut	Foregut	Hindgut	Foregut	Hindgut	Foregut	Hindgut	Foregut	Hindgut	Foregut	Hindgut	Foregut	Hindgut
Protozoa	0.92	0.65	0.85	0.80			0.57	0.51	0.64	0.56	0.62	0.54	0.91	0.47
Fungi	0.78	0.73	0.78	0.51	0.54	0.54			0.73	0.57	0.87	0.63	0.82	0.62
Methanogen	0.89	0.70			0.78	0.65	0.86	0.75	0.67	0.51	0.71	0.69	0.73	0.51
<i>R. albus</i>	0.90	0.54	0.90	0.79	0.57	0.59	0.68	0.65			0.89	0.73	0.85	0.66
<i>R. flavefaciens</i>	0.76	0.59	0.83	0.64	0.75	0.66	0.85	0.74	0.85	0.67			0.89	0.57
<i>F. succinogenes</i>	0.86	0.60	0.89	0.74	0.87	0.49	0.79	0.63	0.78	0.53	0.96	0.67		
To VFA <sup>2</sup>	0.86	0.78	0.64	0.75	0.86	0.67	0.89	0.69	0.83	0.53	0.86	0.89	0.86	0.65
dH <sub>2</sub> <sup>3</sup>	0.75	0.63	0.74	0.64	0.59	0.86	0.79	0.73	0.69	0.45	0.58	0.86	0.75	0.68
dH <sub>2</sub> S	0.63	0.53	-0.53	-0.74	0.64	0.57	0.49	0.64	0.59	0.67	0.97	0.86	0.86	0.68
dCH <sub>4</sub>	-0.85	-0.64	0.58	0.47	-0.58	-0.68	-0.85	-0.64	-0.85	-0.75	-0.68	-0.67	-0.86	-0.63
Ammonia <sup>4</sup>	0.85	0.83	0.79	0.74	0.74	0.75	0.71	0.72	0.84	0.78	0.86	0.68	0.76	0.67
Acetate <sup>5</sup>	0.68	0.63	0.75	0.63	0.64	0.75	0.69	0.62	0.73	0.68	0.64	0.84	0.69	0.62
Propionate	0.65	0.68	-0.68	-0.83	-0.75	-0.58	0.74	0.72	-0.78	-0.97	-0.85	-0.69	-0.83	-0.68
Butyrate	-0.63	-0.54	-0.84	-0.64	-0.57	-0.75	-0.68	-0.72	-0.62	-0.61	-0.72	-0.75	-0.83	-0.69
Valerate	-0.27	-0.54	-0.49	-0.53	-0.67	-0.57	-0.49	-0.54	-0.56	-0.75	-0.74	-0.82	-0.75	-0.58
Isobutyrate	0.85	0.74	0.68	0.84	0.57	0.64	0.68	0.62	0.43	0.67	0.72	0.73	0.68	0.72
Isovalerate	0.74	0.63	0.58	0.53	0.50	0.78	0.72	0.52	0.78	0.47	0.68	0.59	0.69	0.58

<sup>1</sup>Microbial concentrations in the gut regions were expressed as log<sub>10</sub> transformed; correlation coefficient values (*r*) with *r* > 0.44 for *p* < 0.1, *r* > 0.52, *p* < 0.05, *r* > 0.66 for *p* < 0.01, *r* > 0.79 for *p* < 0.001. *r*-values < 0.1 is not presented in the table.

<sup>2</sup>Total volatile fatty acids (mM).

<sup>3</sup>Dissolved hydrogen (μM), dissolved methane (mM), dissolved hydrogen sulfur (mM).

<sup>4</sup>Ammonia (mM).

<sup>5</sup>Molar percentages of acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate.

compute methanogens for H<sub>2</sub> because sulfur can be used as a potential H<sub>2</sub> sink (Teklebrhan et al., 2020). (2) Increased free amino acids may increase deamination of the process with increased ammonia accumulation, implying less efficient amino acid utilization for microbial growth, resulting in reduced H<sub>2</sub> release in the gut regions. (3) On the other hand, the increased dH<sub>2</sub> in the foregut and hindgut of goats fed control treatment suggests a thermodynamically less efficient H<sub>2</sub> consumption by methanogens, thus, stimulating the fermentation pathway that releases less H<sub>2</sub> than more H<sub>2</sub>, per unit of glucose fermented in the gut regions. This was associated with increased propionate, over the acetate pathway in the foregut and hindgut of goats fed sulfur-containing amino acids, i.e., Met rather than control or Lys.

Sulfur-containing amino acids shifted H<sub>2</sub> to a different hydrogen sink, increasing dH<sub>2</sub>S production rather than CH<sub>4</sub> production. This occurs under standard gut conditions because sulfidogenic bacteria have a higher affinity for H<sub>2</sub> utilization than methanogens, suggesting thermodynamically methanogenesis is less favored than sulfate reduction (Van Zijderveld et al., 2010; Judy et al., 2019; Lan and Yang, 2019; Teklebrhan et al., 2020). Because sulfur-reducing microbes use sulfur as an electron sinker or acceptor, and the reduced forms of sulfur H<sub>2</sub>S is a metabolic end-product of fermentation from these microorganisms and noticed in the current study. It is related to the reduced forms of sulfur observed in an earlier study (Gould, 2000). This is supported by our findings of increased H<sub>2</sub>S than methanogenesis in the foregut and hindgut; in response to sulfur-containing

amino acid supplementation. Similarly, methanogenesis was reduced in goats fed; a high sulfur-containing diet of CG vs. CM (Teklebrhan et al., 2020), reporting increased H<sub>2</sub>S vis-à-vis CH<sub>4</sub> in the rumen liquid. In addition, an *in vitro* culturing system was found to reduce CH<sub>4</sub> in methionine addition more than in the control group (Abbasi et al., 2019; Hassan et al., 2021). This observation was supported by a negative correlation between concentrations of methanogens, dCH<sub>4</sub>, and dH<sub>2</sub> with dH<sub>2</sub>S, both in the foregut and hindgut contents in the current study.

The gut microbiome is a complex ecosystem of bacteria, methanogens, archaea, fungi, and protozoa, and bacteriophages, which interact with each other and their host (Goodman and Gordon, 2010; Minot et al., 2011). Understanding of their composition, association with their metabolome, and ecological role gives insight into how to improve nutrient utilization efficiencies and health and reduce the carbon footprint of ruminants. The significant associations with microbes and their metabolites suggest that the microbiome plays a significant role in the fermentation of amino acids to ammonia, VFA, CO<sub>2</sub>, CH<sub>4</sub>, and H<sub>2</sub> for microbial protein synthesis in the gut regions of ruminants. In the current study, in response to amino acid supplement, we observed increased copies of 16S rRNA methanogens, bacteria, and 18S rRNA protozoa, fungi, and fiber utilizing bacteria species in the particles associated with liquid fractions in the foregut and hindgut, with a notable greatest value in the foregut. In addition, regardless of the amino acid supplements and sampling fraction, gut regions significantly increased total microbial populations of methanogens, bacteria,

protozoa, fungi, as well as starch and fiber, utilizing bacteria species in the order of foregut > hindgut > small intestine. The decreased copies of these microbiomes could be caused by less digestible matters and a slower fermentation rate in the small intestine than in the foregut and hindgut regions. Similarly, previous studies have reported lower total gene copies of protozoa, methanogens, bacteria, and fiber degrading bacterial species (*F. succinogenes*, *R. albus*, and *R. flavefaciens*) in the cecal and small intestinal contents vs. ruminal contents (Shinkai and Kobayashi, 2007; Frey et al., 2010; Popova et al., 2013). In addition, increasing copies of these genes were documented in the contents of the rumen, rather than in small and large intestines (Zeng et al., 2015, 2017). These lower populations of these microbiomes in the hindgut and small intestine might be, due to the lower rate and activity of fiber degrading enzymes, than in the foregut. This was in agreement with an earlier study by Bauer et al. (2004) that reported less cellulolytic enzyme activities in the contents of the small and large intestines. This might be related to the correlations observed between microbiota and microbial fermentation metabolites, in the foregut and hindgut of goats, which suggests increased gene copies of total microbiota and functional bacterial species in these gut regions in the current study. For example, the increased populations of fiber degrading bacterial species, both in the foregut and hindgut suggests these microbes are useful for fiber degradation, mainly; in the foregut, and fully undegradable fractions in the hindgut. This is supported by significant correlations between the concentration of metabolome and the associated proliferation of fiber degrading bacterial species, both in the foregut and hindgut, as observed in the current study.

Several studies have described that dietary supplements can influence microbial populations and functional bacterial species in ruminal contents (Thoetkiattikul et al., 2013; Pan et al., 2017; Xue et al., 2018; Teklebrhan et al., 2020). Nevertheless, only a few studies have used amino acid supplements for microbial quantification studies and, most of them are *in vitro* highlighting increased microbial copy numbers, using methionine than in the control groups. The authors reported that methionine addition can increase the protozoa and *R. albus* population, both in particles associated and liquid fractions (Abbasi et al., 2019; Hassan et al., 2021). Moreover, Martin et al. (2013) described that methionine supplementation, either alone or in combination, can increase fibrinolytic bacterial species including *R. flavefaciens*, *F. succinogenes*, and *R. albus*. Earlier studies have also reported that ruminal bacteria such as *R. amylophilus* and *Prevotella* spp. are actively engaged in protein degradation (Blackburn and Hobson, 1962; Wallace, 1994). These findings were consistent with higher populations of total bacteria, protozoa, methanogens, fungi, and bacterial species in goats fed on the amino acid supplements, rather than in the control of the current study. The increase of these microbiomes in the foregut and hindgut might be caused by the increase of free amino acid availability to microbes, suggesting that elevating microbial protein synthesis, which is essential for microbial growth and associated with increasing gene copies in the foregut, as well as hindgut. Interestingly, nitrogen losses in the feces and urine were similar to the amino acid supplements vs. control (Zhu et al., 2018), implying that

free amino acid supplements are not always causing nitrogen waste and excretion, if they are with low total dietary protein (12.69% CP), as in the current study. It is consistent with less nitrogen excretion in the feces and urine, when animals are fed on rations containing low protein 12–18% CP and has no adverse effect on the energy balance of ruminants (Broderick et al., 2008; Agle et al., 2010; Erickson and Klopfenstein, 2010).

Most of the microbial quantification studies use rumen liquid samples. This technique may underestimate the number of microbes that remained attached to fiber particles that could have significantly affected the total microbiota inhabiting the ruminant gut. Similarly, earlier studies have suggested that microorganisms attaching to undigested feed particles account for a significant proportion of total ruminal microorganisms (Merry and McAllan, 1983; Craig et al., 1987a,b) stated that particles associated with microbial organic matter (OM), accounted for 70 to 80% of the total microbial mass in the rumen. A previous study has assessed microbes in the rumen liquid or particles associated fractions (Mullins et al., 2013), reporting that total bacteria, fungi, *F. succinogenes*, and *R. albus* populations were higher in the particles associated fractions than in liquid (Mullins et al., 2013). Likewise, in this study, we have investigated the differences in the microbiota of goats fed amino acid in the foregut, small intestine, and hindgut in the particles associated and liquid fractions. The total populations of microbiota and fiber degrading bacterial species of *R. albus*, *F. succinogenes*, and *R. flavefaciens* were increased in the particles associated, with liquid fractions in the current study. This suggests these bacterial species are predominantly engaged in degrading and fermenting plant fibers, which is in accordance with Flint et al. (2008) and Biddle et al. (2013).

## CONCLUSION

The hypothesis of this study is that sulfur amino acids could shift hydrogen toward an alternative sink supported by increased H<sub>2</sub>S instead of methanogenesis and changed fermentation and microbiota, associated with particles and liquid fractions, both in the foregut and hindgut of goats. Goats fed on Met and Lys either alone or in combination had increased 16S rRNA gene copies of total bacteria, methanogens, and 18S rRNA of protozoa, fungi, and fiber; utilizing bacterial species associated with particles than liquid fractions of those in control. In addition, amino acid supplements increased total bacteria, methanogens, protozoa and fungi populations, fiber, and starch utilizing bacterial species both in the foregut and hindgut compared with the control group. This study suggests that sulfur-containing amino acids shift hydrogen to an alternative hydrogen sink, i.e., H<sub>2</sub>S, over methanogenesis and modified gut fermentation metabolites, increasing particle-associated microbiota than liquid both in the foregut and hindgut. This study gives insights into the use of sulfur-containing amino acids, as an alternative dietary mitigation strategy of methanogenesis in ruminants, and it underscores the need for related further research on sulfur amino acids, as a potential sink of hydrogen.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the experimental protocols used in this trial endorsed by the animal care and use committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences and strictly followed the guidelines for animal welfare established by the committee.

## REFERENCES

- Abbasi, I. H. R., Abbasi, F., Liu, L., Bodinga, B. M., Abdel-Latif, M. A., Swelum, A. A., et al. (2019). Rumen-protected methionine a feed supplement to low dietary protein: effects on microbial population, gases production and fermentation characteristics. *AMB Expr.* 9, 1–10. doi: 10.1186/s13568-019-0815-4
- Agle, M., Hristov, A. N., Zaman, S., Schneider, C., Ndegwa, P., and Vaddella, V. K. (2010). Effects of ruminally degraded protein on rumen fermentation and ammonia losses from manure in dairy cows. *J. Dairy Sci.* 93, 1625–1637. doi: 10.3168/jds.2009-2579
- AOAC (1995). *Official Methods Of Analysis*, 16th Edn. Arlington, TX: Association of analytical chemist.
- AOAC International (2006). *Official Methods of Analysis of AOAC International*, 18th Edn. Gaithersburg, MD: AOAC Int.
- Bal, M. A., and Ozturk, D. (2006). Effects of sulfur containing supplements on ruminal fermentation and microbial protein synthesis. *J. Anim. Vet. Sci.* 1, 33–36.
- Barker, H. A. (1961). "Fermentation of nitrogenous organic compounds," in *The Bacteria*, Vol. 2, eds I. C. Gunsalus and R. Y. Stannier (London: Academic Press), 151–207. doi: 10.1016/b978-0-12-395627-9.50011-6
- Bauer, J. H., Goupil, S., Garber, G. B., and Helfand, S. L. (2004). An accelerated assay for the identification of lifespan-extending interventions in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12980–12985. doi: 10.1073/pnas.0403493101
- Biddle, A., Stewart, L., Blanchard, J., and Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by lachnospiraceae and ruminococcaceae in diverse gut communities. *Diversity* 5, 627–640. doi: 10.3390/d5030627
- Blackburn, T., and Hobson, P. (1962). Further studies on the isolation of proteolytic bacteria from the sheep rumen. *J. Gen. Microbiol.* 29, 69–81. doi: 10.1099/00221287-29-1-69
- Broderick, G. A., Stevenson, M. J., Patton, R. A., Lobos, N. E., and Colmenero, J. J. O. (2008). Effect of supplementing rumen-protected methionine on production and nitrogen excretion in lactating dairy cows. *J. Dairy Sci.* 91, 1092–1102. doi: 10.3168/jds.2007-0769
- Craig, W. M., Brown, D. R., Broderick, G. A., and Ricker, D. B. (1987a). Post-prandial compositional changes of fluid- and particle-associated ruminal microorganisms. *J. Anim. Sci.* 65, 1042–1048. doi: 10.2527/jas1987.6541042x
- Craig, W. M., Broderick, G. A., and Ricker, D. B. (1987b). Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. *J. Nutr.* 117, 56–62. doi: 10.1093/jn/117.1.56
- Denman, S. E., and McSweeney, C. S. (2006). Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol. Ecol.* 58, 572–582. doi: 10.1111/j.1574-6941.2006.00190.x
- Erickson, G., and Klopfenstein, T. (2010). Nutritional and management methods to decrease nitrogen losses from beef feedlots. *J. Anim. Sci.* 88, 172–180. doi: 10.2527/jas.2009-2358
- Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R., and White, B. A. (2008). Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat. Rev. Microbiol.* 6, 121–131. doi: 10.1038/nrmicro1817
- Frey, J. C., Pell, A. N., Berthiaume, R., Lapierre, H., Lee, S., Ha, J. K., et al. (2010). Comparative studies of microbial populations in the rumen, duodenum, ileum and faeces of lactating dairy cows. *J. Appl. Microbiol.* 108, 1982–1993. x doi: 10.1111/j.1365-2672.2009.04602
- Goodman, A. L., and Gordon, J. I. (2010). Our unindicted coconspirators: human metabolism from a microbial perspective. *Cell Metab.* 12, 111–116. doi: 10.1016/j.cmet.2010.07.001
- Gould, D. H. (2000). Update on sulfur-related polioencephalomalacia. *Vet. Clin. N. Am. Food. Anim. Pract.* 16, 481–496. doi: 10.1016/s0749-0720(15)30082-7
- Guyader, J., Tavendale, M., Martin, C., and Muetzel, S. (2016). Dose-response effect of nitrate on hydrogen distribution between rumen fermentation end products: an in vitro approach. *Anim. Prod. Sci.* 56, 224–230. doi: 10.1071/AN15526
- Hassan, F., Guo, Y., Li, M., Tang, Z., Peng, L., Liang, X., et al. (2021). Effect of methionine supplementation on rumen microbiota, fermentation, and amino acid metabolism in-vitro culture contain nitrate. *Microorganism* 9, 1–26. doi: 10.3390/microorganisms9081717
- Hook, S. E., Northwood, K. S., Wright, A. D., and McBride, B. W. (2009). Long-term monensin supplementation does not significantly affect the quantity or diversity of methanogens in the rumen of the lactating dairy cow. *Appl. Environ. Microbiol.* 75, 374–380. doi: 10.1128/AEM.01672-08
- Janssen, P. H. (2010). Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed. Sci. Technology.* 160, 1–22. doi: 10.1016/j.anifeedsci.2010.07.002
- Judy, J. V., Bachman, G. C., Brown-Brandl, T. M., Fernando, S. C., Hales, K. E., Miller, P. S., et al. (2019). Reducing methane production with corn oil and calcium sulfate: responses on whole-animal energy and nitrogen balance in dairy cattle. *J. Dairy Sci.* 102, 1–14. doi: 10.3168/jds.2018-14567
- Koike, S., and Kobayashi, Y. (2001). Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol. Lett.* 204, 361–366. doi: 10.1111/j.1574-6968.2001.tb10911.x
- Lan, W., and Yang, C. (2019). Ruminal methane production: associated microorganisms and the potential of applying hydrogen-utilizing bacteria for mitigation. *Sci. Total Environ.* 654, 1270–1283. doi: 10.1016/j.scitotenv.2018.11.180
- Lu, D. X., and Zhang, P. Y. (1996). *Scientific Technology of Feeding Goat*. Beijing: Chinese Academy of Agricultural Sciences.
- Martin, C., Mirande, C., Morgavi, D. P., Forano, E., Devillard, E., and Mosoni, P. (2013). Methionine analogues HMB and HMBi increase the abundance of cellulolytic bacterial representatives in the rumen of cattle with no direct

## AUTHOR CONTRIBUTIONS

TT designed, conducted, and analyzed the experiment and wrote the manuscript. ZT revised and edited the manuscript. Both authors read and approved the manuscript for submission.

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- effects on fiber degradation. *Anim. Feed. Sci. Technol.* 182, 16–24. doi: 10.1016/j.anifeeds.2013.03.008
- Merry, R. J., and McAllan, A. B. (1983). A comparison of the chemical composition of mixed bacteria harvested from the liquid and solid fractions of rumen digesta. *Br. J. Nutr.* 50, 701–709. doi: 10.1079/bjn19830142
- Minot, S., Sinha, R., Chen, J., Li, H., Keilbaugh, S. A., Wu, G. D., et al. (2011). The human gut virome: inter-individual variation and dynamic response to diet. *Genome Res.* 21, 1616–1625. doi: 10.1101/gr.122705.111
- Mullins, C. R., Mamedova, A. J., Carpenter, Y., Ying, M. S., Allen, I., and Yoon, B. J. (2013). Bradford analysis of rumen microbial populations in lactating dairy cattle fed diets varying in carbohydrate profiles and *Saccharomyces cerevisiae* fermentation product. *J. Dairy Sci.* 96, 5872–5881. doi: 10.3168/jds.2013-6775
- Nisman, B. (1954). The Stickland reaction. *Bact. Rev.* 1, 16–42. doi: 10.1128/br.18.1.16-42.1954
- Pan, X., Xue, F., Nan, X., Tang, Z., Wang, K., and Beckers, Y. (2017). Illumina sequencing approach to characterize thiamine metabolism related bacteria and the impacts of thiamine supplementation on ruminal microbiota in dairy cows fed high-grain diets. *Front. Microbiol.* 8:1818. doi: 10.3389/fmicb.2017.01818
- Pinheiro, J., Bornkamp, B., Glimm, E., and Bretz, F. (2013). Model-based dose finding under model uncertainty using general parametric models. *Statist. Med.* 33, 1646–1661. doi: 10.1002/sim.6052
- Popova, M., Morgavi, D. P., and Martin, C. (2013). Methanogens and methanogenesis in the rumens and ceca of lambs fed two different high-grain-content diets. *Appl. Environ. Microbiol.* 79, 1777–1786. doi: 10.1128/AEM.03115-12
- Promkot, C., Wanapat, M., Wachirapakorn, C., and Navanukraw, C. (2007). Influence of sulfur on fresh cassava foliage and cassava hay incubated in rumen fluid of beef cattle. *Asian Australas. J. Anim. Sci.* 20, 1424–1432. doi: 10.5713/ajas.2007.1424
- R Core Team (2020). *A Language and Environment for Statistical Computing*. Vienna: R foundation for statistical computing.
- Shinkai, T., and Kobayashi, Y. (2007). Localization of ruminal cellulolytic bacteria on plant fibrous materials as determined by fluorescence in situ hybridization and real time PCR. *Appl. Environ. Microbiol.* 73, 1–7. doi: 10.1128/AEM.01896-06
- Sinclair, L. A. (2014). Reducing dietary protein in dairy cow diets: implications for nitrogen utilization, milk production, welfare, and fertility. *Animal* 8, 262–274. doi: 10.1017/S1751731113002139
- Stevenson, D. M., and Weimer, P. J. (2007). Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotech.* 75, 165–174. doi: 10.1007/s00253-006-0802-y
- Sun, Z. H., Tan, Z. L., Yao, H., Tang, Z. R., Shan, J. G., Hu, J. P., et al. (2007). Effects of intra-duodenal provision of limiting amino acids on serum concentrations of immunoglobulins and tissue concentrations of DNA and RNA in growing goats fed a maize stover-based diet. *Small Rumin. Res.* 69, 159–166. doi: 10.1016/j.smallrumres.2006.01.006
- Supapong, C., and Cherdthong, A. (2020a). Effect of sulfur and urea fortification of fresh cassava root in fermented total mixed ration on the improvement milk quality of tropical lactating cows. *Vet. Sci.* 7, 1–13. doi: 10.3390/vetsci7030098
- Supapong, C., and Cherdthong, A. (2020b). Effect of sulfur concentrations in fermented total mixed rations containing fresh cassava root on rumen fermentation. *Anim. Prod. Sci.* 16, 1429–1434. doi: 10.1071/AN18779
- Sylvester, R. J., Oosterlinck, W., and van der Meijden, A. P. (2004). A single immediate postoperative instillation of chemotherapy decreases the risk of recurrence in patients with stage Ta T1 bladder cancer: a meta-analysis of published results of randomized clinical trials. *J. Urol.* 171(6 Pt 1), 2186–2190, quiz 2435. doi: 10.1097/01.ju.0000125486.92260.b2
- Teklebrhan, T., Rong, W., Min, W., Jiang, N. W., Li, W., Tan, X., et al. (2020). Effect of dietary corn gluten inclusion on rumen fermentation, microbiota and methane emissions in goats. *Anim. Feed. Sci. Technol.* 259, 1–8. doi: 10.1016/j.anifeeds.2019.114314
- Thoetkiattikul, H., Mhuantong, W., Laothanachareon, T., Tangphatsornruang, S., Pattarajinda, V., Eurwilaichitr, L., et al. (2013). Comparative analysis of microbial profiles in cow rumen fed with different dietary fiber by tagged16S rRNA gene pyrosequencing. *Curr. Microbiol.* 2, 130–137. doi: 10.1007/s00284-013-0336-3
- Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Symposium: carbohydrate methodology, metabolism, and nutritional implications in dairy cattle. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2
- Van Zijderveld, S. M., Van, W. J. J., Gerrits, J. A., Apajalahti, J. R., Newbold, J. D., et al. (2010). Nitrate and sulfate: effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *J. Dairy Sci.* 93, 5856–5866. doi: 10.3168/jds.2010-3281
- Wallace, R. J. (1994). “Amino acid and protein synthesis, turnover, and breakdown by ruminal microorganisms,” in *Principles of Protein Nutrition of Ruminants*, ed. J. M. Asplund (London: CRC Press), 71–111.
- Wang, M., Ungerfeld, E. M., Wang, R., Zhou, C. S., Basang, Z. Z., and Anderson, R. (2016a). Supersaturation of dissolved hydrogen and methane in rumen of tibetan sheep. *Front. Microbiol.* 7:850. doi: 10.3389/fmicb.2016.00850
- Wang, M., Wang, R., Janssen, P. H., Zhang, X. M., Sun, X. Z., Pacheco, D., et al. (2016b). Sampling procedure for the measurement of dissolved hydrogen and volatile fatty acids in the rumen of dairy cows. *J. Anim. Sci.* 94, 1159–1169. doi: 10.2527/jas.2015-9658
- Wang, M., Wang, R., Liu, M., Beauchemin, K. A., Sun, X. Z., Tang, S. X., et al. (2018). Dietary starch and rhubarb supplement increase ruminal dissolved hydrogen without altering rumen fermentation and methane emission in goat. *Animals* 13, 1–8. doi: 10.1017/s1751731118002419
- Weatherburn, M. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* 39, 971–974. doi: 10.1021/ac60252a045
- Xue, F., Nan, X., Sun, F., Pan, X., Guo, Y., Jiang, L., et al. (2018). Metagenome sequencing to analyze the impacts of thiamine supplementation on ruminal fungi in dairy cows fed high concentrate diets. *AMB. Expr.* 8, 1–12. doi: 10.1186/s13568-018-0680-6
- Yu, Z., and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* 36, 808–812. doi: 10.2144/04365ST04
- Zeng, Y., Zeng, D., Ni, X., Zhu, H., Jian, P., Zhou, Y., et al. (2017). Microbial community compositions in the gastrointestinal tract of Chinese Mongolian sheep using Illumina MiSeq sequencing revealed high microbial diversity. *AMB Expr.* 7, 1–10. doi: 10.1186/s13568-017-0378-1
- Zeng, Y., Zeng, D., Zhang, Y., Ni, X. Q., Tang, Y. R., Zhu, H., et al. (2015). Characterization of the cellulolytic bacteria communities along the gastrointestinal tract of Chinese Mongolian sheep by using PCR-DGGE and real-time PCR analysis. *World J. Microb. Biot.* 7, 1103–1113. doi: 10.1007/s11274-015-1860-z
- Zhu, X., Jiao, J., Zhou, C., Tang, S., Wang, M., Kang, J., et al. (2018). Effects of dietary methionine and lysine supplementation on nutrients digestion, serum parameters and mRNA expression of related amino acid sensing and transporting genes in growing goats. *Small Rumin. Res.* 166, 1–6. doi: 10.1016/j.smallrumres.2018.07.002

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