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Rumen-protected lysine supplementation improved amino acid balance, nitrogen utilization and altered hindgut microbiota of dairy cows

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ABSTRACT

This study was conducted to evaluate the effects of dietary crude protein (CP) and rumen-protected lysine (RPL) supplementation on lactation performance, amino acid (AA) balance, nitrogen (N) utilization and hindgut microbiota in dairy cows. Treatments were in a 2 × 2 factorial arrangement, and the main effects were CP concentration (16% vs. 18%) and RPL supplementation (with or without RPL at 40 g/cow per day). Forty cows were randomly allocated to 4 groups: low-CP diet (LP), low-CP diet plus RPL (LPL), high-CP diet (HP), high-CP diet plus RPL (HPL). The experiment was conducted for 8 weeks. Results showed that RPL increased the dry matter intake ($P < 0.01$), milk protein yield ($P = 0.04$) and energy corrected milk ($P = 0.04$), and tended to increase milk fat yield ($P = 0.06$) and fat corrected milk ($P = 0.05$). Cows in the HP group tended to have higher milk urea N ($P = 0.07$). Plasma concentrations of Arg, Ile, Lys, Met, Pro, total essential AA and total nonessential AA were increased by RPL ($P < 0.05$). The total essential AA, total nonessential AA and most AA (except Ile, Phe, Gly and Pro) were increased in the HP group ($P < 0.05$). N excretion was increased in the HP group through an increase in urea N excretion ($P < 0.01$) and an upward trend in plasma urea N ($P = 0.07$). In addition, RPL tended to increase milk protein N secretion ($P = 0.08$), milk N ($P = 0.07$) and microbial protein synthesis ($P = 0.06$), and decreased plasma urea N ($P < 0.001$). In the hindgut, the bacterial community were different between the LP and LPL groups ($P < 0.01$). The probiotic abundances of *Christensenellaceae_R-7_group* and *Acinetobacter* were increased by RPL ($P = 0.03$ and 0.03, respectively). The pathogenic abundances of *Clostridium_sensu_stricto_1* ($P < 0.001$) and *Turicibacter* ($P < 0.01$) were decreased by RPL. In conclusion, supplementing RPL with low dietary CP could balance AA supply and increase milk protein yield, resulting in an improvement in N utilization efficiency, and altered the composition of the hindgut microbiota to favor the lactation performance of dairy cows.

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1. Introduction

Milk protein is a type of high-quality protein easily absorbed by the human body, which is an important indicator for evaluating the quality of milk. Supplementing cows with a higher crude protein (CP) concentration diet has been shown to be an effective measure to improve milk protein (Lee et al., 2012a). With the continuous expansion of dairy farming, the demand for protein feed is increasing. However, the import and export of feed materials have been hindered, and the price of protein feed has been rising, resulting in higher production costs. Making better use of protein

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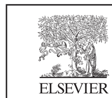

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feed is a priority for dairy operators. Feeding a high CP diet based on soybean meal can maintain the nutritional requirements of high-yield dairy cows, however while in a state of negative amino acid (AA) balance, utilization efficiency of protein is low (Titgemeyer et al., 1989). Feeding a low CP diet is one of the ways to improve the utilization efficiency of protein feeds and reduce nitrogen (N) emissions in the environment; however it would reduce production to a certain extent, which is not in line with economic interests (Apelo et al., 2014). Balancing AA is considered an effective way to maintain milk protein synthesis in dairy cows, which allows for higher protein utilization efficiency (Schwab and Broderick, 2017).

Lysine, as the main limiting AA in ruminants (NRC, 2001), is an important precursor for milk protein synthesis in dairy cows (Lin et al., 2018). Rumen-protected lysine (RPL) supplementation could increase duodenal Lys flow and improve the extraction efficiency of other essential AA (EAA) by mammary glands (Guinard and Rulquin, 1994). Previous studies on the supplementation of RPL at different dietary CP concentrations showed mixed results. Some studies reported that supplementing RPL with different CP diets, ranging from 14.8% to 16.5% CP, could increase milk protein synthesis (Li et al., 2014; Giallongo et al., 2016), while synthesis was not changed in others (Wang et al., 2010; Chen et al., 2018; Girma et al., 2019). One of the reasons for the inconsistent production response would be the balance of dietary metabolizable protein (MP). A corn and soybean meal-based diet is widely used to improve yield, in which Lys is limited even if MP is balanced (Titgemeyer et al., 1989), so a 3:1 ratio of Lys to Met in MP is recommended (Wang et al., 2010).

Research on diets supplemented with AA often focuses on rumen metabolism in ruminants, ignoring the importance of microbial fermentation and the microbiome of the hindgut. Compared with digestion in the rumen, the rumen undegradable protein and ruminal microbial protein (MCP) are digested in the small intestine, which also plays an important role in digestion and absorption of nutrients. Previous studies have demonstrated that rumen protected AA alters hindgut microbiota, which could play important roles in regulating performance and metabolism (Ren et al., 2020; Teklebrhan and Tan, 2022). Different CP concentration and supplementation levels of rumen protected AA might provide a different amount of substrate for microbial fermentation, and whether CP concentration and RPL could affect the microbiota of the hindgut and how they work have not been reported before.

We hypothesized that (1) RPL supplementation could maintain milk protein synthesis of dairy cows fed a low CP diet, (2) RPL could improve N utilization and reduce N excretion to the environment, and (3) RPL supplementation and different CP diets could alter the hindgut microbiota. Therefore, the objectives of this study were not only to investigate the effects of different dietary CP concentrations (16% vs. 18%; DM basis) supplemented with or without RPL on AA supply, N utilization efficiency and lactation performance of dairy cows, but to profile the changes in fermentation characteristics and microbiota of the hindgut.

2. Materials and methods

2.1. Animal ethics statement

The experiment was approved by the Institutional Animal Care and Use Committee of Zhejiang A&F University of China (Hangzhou), and the experiment procedures were in accordance with the guidelines for animal research. The experiment was performed using Chinese Holstein cows at a commercial dairy farm in Shanghai, China. All animal experiments complied with the ARRIVE guidelines.

2.2. Animals and experimental design

Forty Chinese Holstein cows (BW = 711 ± 55 kg, mean ± SD) were selected and balanced for their milk yield (32.6 ± 3.7 kg, mean ± SD), parity (2.1 ± 0.94), and days in milk (DIM; 163 ± 19 d). The cows were randomly allocated into 1 of 4 treatment groups (n = 10 in each group) in a 2 × 2 factorial arrangement. The CP levels used in this study were 16% and 18% (dry matter basis), and the concentrations of RPL used were 0 and 40 g/d per cow (68% Lys and 44% bioavailability, LysiGEM, Kemin Industries Inc., USA). The 4 treatment groups were low CP diet (LP), low CP diet supplemented with RPL (LPL), high CP diet (HP), and high CP diet supplemented with RPL (HPL). Before feeding the basal total mixed ration (TMR), the RPL mixed with a small amount of fresh TMR was supplemented directly in front of each cow for individual consumption. The experiment was conducted in a tie-stall barn dairy farm and lasted for 8 weeks. Cows had free access to feed and water.

2.3. Sampling and laboratory analyses

During the entire experiment, dry matter intake (DMI) was measured and recorded for 2 consecutive days per week. The TMR samples were collected monthly and frozen at −20 °C. Composited samples were dried for 48 h at 65 °C and ground through a 1-mm sieve before analysis. The DM was determined by further drying at 105 °C for 3 h, and chemical analyses were based on the final absolute DM. The Macro-Kjeldahl N test was used to analyse total N in feed samples, and the CP (N × 6.25) content of feed samples was calculated using standard methods (AOAC, 1990). Acid detergent fiber was analyzed using acid detergent (20 g/L cetyl trimethyl ammonium bromide solution) and 1 M H_2SO_4 (AOAC, 1990), and neutral detergent fiber was analyzed using neutral detergent (30 g/L sodium dodecyl sulfate solution), sodium sulfite and heat-stable α-amylase (Soest et al., 1991). Net energy concentration, dietary MP and AA balance of the diet was estimated using CPM CNCPS v3.0.8.1 (Cornell University, Ithaca, NY). The DMI of each cow was calculated by measuring TMR offered and residual feed. The ingredients and chemical compositions of the diets are listed in Table 1.

Milk yield was measured weekly. Aliquots of milk samples from each cow (50 mL) were collected at 3 consecutive milkings (approximately 03:30, 10:30 and 16:30), and mixed proportionally (4:3:3). Milk preservative (potassium dichromate) was added for subsequent analysis of milk fat, protein, lactose, total solids, milk somatic cell count (SCC) and milk urea nitrogen (MUN) using infrared spectroscopy MilkoScan (Foss Electric, Hillerød, Denmark).

At the end of experiment, blood samples were collected from the coccygeal vein by venipuncture into heparinized tubes approximately 2 h after feeding. Plasma was separated immediately by centrifugation at 3,500 × g for 10 min, collected and analysed for plasma urea N (PUN; Urea Assay Kit; Jiancheng Bioengineering Institute, Nanjing, China). Aliquots of the plasma samples were analysed for plasma AA using a Hitachi L-8900 amino acid analyser (Hitachi, Tokyo, Japan).

The ruminal contents were collected approximately 3 h after feeding using an oral rumen fluid sampler composed of a stainless-steel shell body and latex tube. A 200-mL syringe was connected at one end of the tube for extracting rumen fluid samples. The first 100 mL of ruminal content was discarded to avoid saliva contamination, and then 100 mL of ruminal content was collected for the analysis of ruminal microbial protein (MCP) (Zinn and Owens, 1986). Free bases were obtained by hydrolysis of nucleotides using perchloric acid followed by precipitation of free purines with silver nitrate to obtain a silver nitrate purine complex. The silver nitrate purine complex was reacted with hydrochloric acid to form

Table 1
Ingredient and chemical composition of experimental diets.

Item	Diet[1]			
	LP	LPL	HP	HPL
Ingredient, % of DM				
Corn grain, ground	25.73	25.73	23.83	23.83
Soybean meal	11.28	11.28	17.84	17.84
Extruded soybean	2.61	2.61	2.42	2.42
Whole cottonseed	5.94	5.94	5.50	5.50
Molasses cane	2.89	2.89	2.68	2.68
Alfalfa hay	7.91	7.91	7.33	7.33
Alfalfa silage	5.93	5.93	5.50	5.50
Oats hay	4.00	4.00	3.71	3.71
Corn silage	24.62	24.62	22.80	22.80
Wheat bran	3.90	3.90	3.61	3.61
Premix[2]	2.88	2.88	2.67	2.67
Bergafat F-100[3]	1.67	1.67	1.55	1.55
Optigen[4]	0.25	0.25	0.23	0.23
MgO	0.08	0.08	0.08	0.08
Sodium diacetate	0.13	0.13	0.12	0.12
KemTRACE chromium[5]	0.02	0.02	0.02	0.02
Liyourul[6]	0.10	0.10	0.10	0.10
Rumen-protected lysine, g/d	0	40	0	40
Chemical composition, % of DM				
DM	56.1	56.1	57.7	57.7
CP	16.1	16.1	18.3	18.3
NDF	27.5	27.5	26.6	26.6
ADF	19.5	19.5	18.8	18.8
NE_L[7], Mcal/kg of DM	1.75	1.75	1.78	1.78

DM = dry matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; NE_L = net energy for lactation.
[1] LP = low-protein (16% CP) diet; LPL = LP plus rumen-protected lysine; HP = high-protein (18% CP) diet; HPL = HP plus rumen-protected lysine.
[2] Premix contains (per kilogram): 180 kIU of vitamin A; 45 kIU of vitamin D3; 1400 IU of vitamin E; 170 mg of Cu; 360 mg of Fe; 680 mg of Zn; 910 mg of Mn; 4 mg of Co; 20 mg of I; 6 mg of Se; 7 mg of Se yeast; 50 to 100 g of Ca; 110 to 180 g of NaCl; 40 g of Mg; 10 g of P; 150 mg of copper methionine; 700 mg of zinc methionine and 10 g of methionine hydroxy-analogue.
[3] Bergafat F-100 (Berg and Schmidt Nutrition Sdn. Bhd., Malaysia): bypass fats from palm oil processing of raw materials for ruminants.
[4] Optigen (Alltech Inc., Nicholasville, KY, USA): urea as nonprotein nitrogen source for ruminants.
[5] KemTRACE chromium (Kemin Technologies Co., Ltd. China): organic chromium source.
[6] Liyourul (Jiangsu Sinitic Biological Technology Co., Ltd., Jiangsu, China): exogenous enzyme source, cellulase at 150,000 U/kg and xylanase at 200,000 U/kg.
[7] Net energy concentration of the diets was estimated using CPM CNCPS v3.0.8.1 (Cornell University, Ithaca, NY).

acid resolubilized purines, which were then quantitated spectrophotometrically at 260 nm. MCP was determined by the ratio of purines to N of isolated bacteria.

Spot urine samples were collected on week 8 by massaging the vulva. Aliquots of urine samples were immediately acidified with 0.036 M H_2SO_4 (1:4, vol/vol) and then analysed for urine total N. The urinary urea N and creatinine were measured using commercial kits (Urea Assay Kit and Creatinine Assay Kit; Jiancheng Bioengineering Institute, Nanjing, China). Daily urine volume was calculated by urinary creatinine concentration, and the creatinine excretion rate was assumed to be 29 mg/kg of BW (Lee et al., 2012a).

Fecal samples were collected 8 times (about 100 g each time) for 3 d in the last experimental week (03:00, 12:00, and 21:00 on d 1; 06:00, 15:00, and 24:00 on d 2; and 09:00 and 18:00 on d 3) from the rectum. Before analysis, all fecal samples were pooled, mixed, and homogenized using a sterile slap homogenizer. Part of the mixture was acidified immediately using 10% H_2SO_4 (about 20 mL/100 g), dried at 65 °C for 48 h and ground through a 1-mm sieve to analyse total N. About 4 g sample was mixed in 4 mL of distilled water for volatile fatty acid extraction and analyses. The volatile fatty acid was analysed using gas chromatography (Agilent 7890B GC, CA, USA). In addition, around a 3-g fecal sample from each cow was packed into a 5-mL sterile frozen storage tube and stored at −80 °C for the analysis of microbiota.

2.4. Fecal microbiota 16S rDNA sequencing and analysis

2.4.1. Fecal microorganism DNA extraction and PCR amplification

The E.Z.N.A. soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) was used to extract the total DNA of fecal samples ($n = 10$ in each group) according to the instructions of the manufacturer. The purity and concentration of extracted DNA were checked using a NanoDrop 2000UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA), and the usability was detected using 1% agarose gel electrophoresis. Targeting the hypervariable regions V3–V4, forward primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify V3 and V4 using a thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR procedure included 3 min of denaturation at 95 °C, 28 cycles of 30 s at 95 °C, 30 s for annealing at 55 °C, and 45 s for elongation at 72 °C, and a final extension at 72 °C for 10 min. PCR reactions were performed in triplicate, with 20 μL of mixture containing 4 μL of 5× FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The resultant PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor-ST (Promega, USA) according to the manufacturer's guidelines. The amplicons were sequenced on Illumina MiSeq platform (Illumina, San Diego, USA).

2.4.2. Analysis of sequencing data

Raw fastq files were quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (1) The reads were truncated at any site receiving an average quality score below 20 over a 50 bp sliding window. (2) Sequences whose overlap was longer than 10 bp were merged according to their overlap with mismatch no more than 2 bp. (3) Sequences of each sample were separated according to barcodes (exact matches) and Primers (allowing 2 nucleotide mismatches), and reads containing ambiguous bases were removed. The divisive amplicon denoising algorithm (DADA2) was used to perform amplicon sequence variant (ASV) sequence clustering. The taxonomy of each 16S rRNA gene sequence was analysed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the database using a confidence threshold of 70%.

2.4.3. Correlation analysis

The correlation analysis was conducted using an online platform called Majorbio Cloud Platform (www.majorbio.com). The correlation between fecal bacteria and lactation performance was calculated by Spearman's correlation coefficient. A heat map diagram was constructed to enable visualization of data, in which red represented negative correlation and blue represented positive correlation. Correlation significance was indicated by asterisks.

2.5. Data analysis

Data analysis was performed using the MIXED procedure in SPSS Statistics (version 24, IBM, New York, USA). The model included cow, week (wk), dietary CP concentration, RPL supplementation, dietary CP concentration × RPL supplementation, dietary CP concentration × wk, RPL supplementation × wk, dietary CP concentration × RPL supplementation × wk, and residual error. The repeated procedure was used for variables repeatedly measured over time, such as milk yield, milk composition and DMI. The data on BW, plasma concentrations of AA, N utilization, fecal

fermentation and microbiota were analysed without repeated measures. In addition, one-way ANOVA was also conducted to compare the differences among the 4 treatment groups. The least squares mean was compared using LSD, and statistical differences were declared significant at $P \leq 0.05$. Tendencies were considered at $0.05 < P \leq 0.10$.

3. Results

3.1. Dietary MP and AA characteristics

Estimated dietary MP and AA balance in cows are presented in Table 2. The MP in the LPL group was imbalanced, but adequate in the HP group. The predicted proportions of Lys to MP were 6.76% and 6.78% in the LP and HP group, respectively, lower than optimum content of Lys in MP recommended by NRC (2001). The supply of RPL resulted in 7.11% Lys of MP in both the LPL and HPL groups. The ratio of Lys to Met reached 3:1 in both the LPL and HPL groups.

Table 2
Estimated dietary metabolizable protein and amino acid balance in dairy cows fed different dietary crude protein concentrations and rumen-protected lysine supplementation.

Item	Diet[1]			
	LP	LPL	HP	HPL
Protein supply and balance[2], g/d				
MP supply	2,267	2,322	2,350	2,447
MP requirements	2,238	2,398	2,342	2,401
MP balance	29	−76	8	46
MP-allowed milk, kg/d	34.2	33.4	34.6	36.2
Digestible Lys balance[2], g/d				
Requirements	132	141	137	141
Supply from the diet	153	155	159	164
Supply from rumen-protected lysine	0	10	0	10
Balance	21	24	22	33
Ratio of Lys to Met[2]				
Lys, of % MP	6.76	7.11	6.78	7.11
Met, of % MP	2.40	2.37	2.32	2.30
Lys:Met ratio	2.82	3.00	2.93	3.09

MP = metabolizable protein; Lys:Met ratio = the ratio of lysine to methionine.
[1] LP = low-protein (16% CP) diet; LPL = LP plus rumen-protected lysine; HP = high-protein (18% CP) diet; HPL = HP plus rumen-protected lysine.
[2] Values were estimated using CPM CNCPS v3.0.8.1 (Cornell University, Ithaca, NY). The requirements, supply, and balance (supplied − required) were based on actual mean DMI, milk yield, and milk composition, respectively. The rumen-protected lysine contains 68% Lys with 44% bioavailability.

3.2. Milk production and dry matter intake

Milk production and composition are presented in Table 3. Dietary CP concentration, RPL, and their interaction did not affect milk yield, milk fat content, or SCC in this study ($P > 0.05$). Milk protein yield ($P = 0.04$) and energy corrected milk (ECM, $P = 0.04$) were significantly increased by RPL. In addition, there was a tendency for RPL to increase milk fat yield ($P = 0.06$), milk total solids ($P = 0.07$) and fat corrected milk (FCM, $P = 0.05$). MUN concentration was affected by dietary CP concentration ($P < 0.01$). In the HP group, MUN concentration of cows was greater in HPL compared with those in HP, while no difference was observed between cows in the LP and LPL groups. A wk × CP interaction for MUN concentration was detected (Fig. 1A, $P < 0.01$). The wk × Lys interactions for lactose content (Fig. 1B, $P = 0.05$) and MUN concentration (Fig. 1C, $P = 0.01$), and a tendency for wk × Lys interaction for milk protein content were observed (Fig. 1D, $P = 0.10$). The wk × CP × Lys interactions for milk protein percentage (Fig. 2A, $P < 0.001$), total solids percentage (Fig. 2B, $P = 0.04$) and MUN concentration (Fig. 2C, $P < 0.001$), and a tendency for wk × CP × Lys interaction for milk protein yield (Fig. 2D, $P = 0.07$) were detected. The DMI was not affected by dietary CP concentration, while RPL significantly increased DMI by 2.82% ($P < 0.01$).

3.3. Plasma free AA

Concentrations of free AA in plasma are presented in Table 4. Total EAA, total nonessential AA (NEAA) and most AA (except Ile, Phe, Gly and Pro) were increased by high CP ($P < 0.05$). Plasma concentrations of Arg, Ile, Lys, Met, Pro, total EAA and total NEAA were increased by RPL ($P < 0.05$). The interaction of CP × RPL was found in plasma concentrations of Met, Pro, total EAA minus Lys (EAA-Lys) and total NEAA ($P = 0.01$, 0.05, 0.01 and 0.03,

Table 3
Effects of dietary crude protein concentration and rumen-protected lysine supplementation on DMI, BW and milk production.

Item[1]	Treatment[2]				SEM	*P*-value[3]					
	LP	LPL	HP	HPL		CP	RPL	CP × RPL	wk × CP	wk × RPL	wk × CP × RPL
DMI, kg/d	21.2[b]	21.6[ab]	21.2[b]	21.9[a]	0.10	0.46	<0.01	0.42	<0.01	<0.01	<0.01
BW change, kg	21.7	12.7	19.1	17.6	13.07	0.97	0.85	0.89	–	–	–
Milk yield											
Milk, kg/d	33.5	35.0	34.4	35.2	0.39	0.49	0.16	0.68	0.99	0.99	0.86
FCM[4], kg/d	30.6[b]	34.9[a]	34.1[a]	34.8[a]	0.60	0.17	0.05	0.14	0.76	0.38	0.29
ECM[5], kg/d	33.0[b]	37.3[a]	36.5[a]	37.3[a]	0.60	0.15	0.04	0.16	0.71	0.40	0.28
Milk composition											
Fat, %	3.42	4.01	3.96	4.09	0.115	0.19	0.13	0.31	0.84	0.63	0.15
Fat, kg/d	1.15[b]	1.39[a]	1.36[a]	1.40[a]	0.037	0.15	0.06	0.18	0.84	0.27	0.22
Protein, %	3.13	3.29	3.25	3.38	0.046	0.25	0.12	0.89	0.82	0.10	<0.01
Protein, kg/d	1.05[b]	1.15[a]	1.11[ab]	1.16[a]	0.016	0.23	0.04	0.43	0.80	0.74	0.07
Lactose, %	5.10	5.16	5.13	5.15	0.022	0.84	0.39	0.71	0.08	0.05	0.16
Total solids, %	11.8	12.6	12.5	12.8	0.14	0.17	0.07	0.37	0.91	0.30	0.04
MUN, mg/dL	13.1[c]	13.2[c]	15.7[b]	17.6[a]	0.26	<0.01	0.10	0.07	<0.01	0.01	<0.01
SCC, × 1000/mL	191	249	179	70	39.8	0.24	0.75	0.30	0.22	0.49	0.53
Milk/DMI	1.59	1.62	1.63	1.61	0.020	0.67	0.80	0.49	0.22	<0.01	0.83

DMI = dry matter intake; BW = body weight; RPL = rumen-protected lysine; wk = week; MUN = milk urea nitrogen; SCC = somatic cell count.
[a − c] Means within a row with different superscripts differ and are marked according to ANOVA results ($P < 0.05$).
[1] Data were collected once a week.
[2] LP = low-protein (16% CP) diet; LPL = LP plus RPL; HP = high-protein (18% CP) diet; HPL = HP plus RPL.
[3] Wk, effect of week; CP, effect of dietary CP concentration; RPL, effect of RPL supplementation; CP × RPL, interaction of CP and RPL; wk × CP, interaction of wk and CP; wk × RPL, interaction of wk and RPL; wk × CP × RPL, interaction of wk and CP and RPL.
[4] Fat corrected milk (FCM) = 0.4 × milk fat yield (kg) + 15 × milk yield (kg).
[5] Energy corrected milk (ECM) = 0.323 × milk fat yield (kg) + 7.13 × milk protein yield (kg) + 12.82 × milk yield (kg).

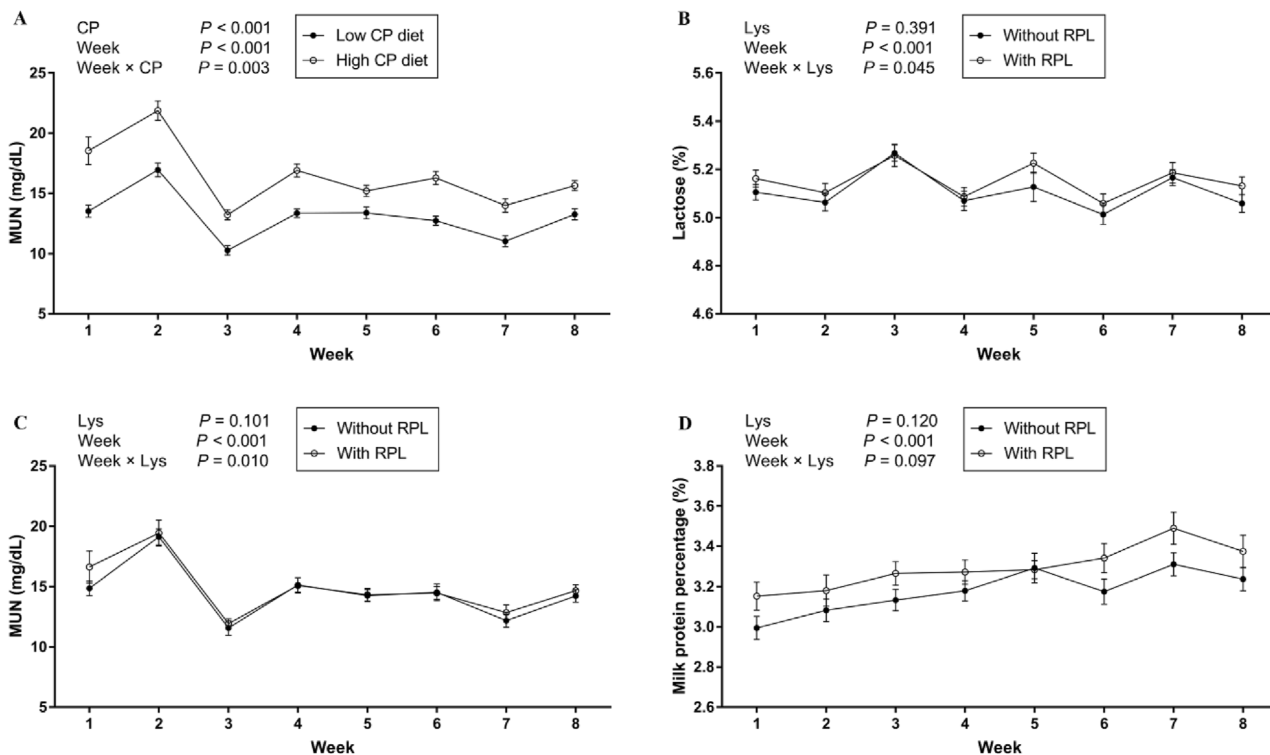

Fig. 1. Effects of dietary crude protein concentration and rumen-protected lysine (RPL) supplementation on milk urea nitrogen, lactose percentage and protein percentage in dairy cows. Diets were Low CP (CP = 16%) with RPL (40 g/d per cow) or without RPL, or High CP (CP = 18%) with or without RPL. Values are means; error bars represent standard error. MUN = milk urea nitrogen.

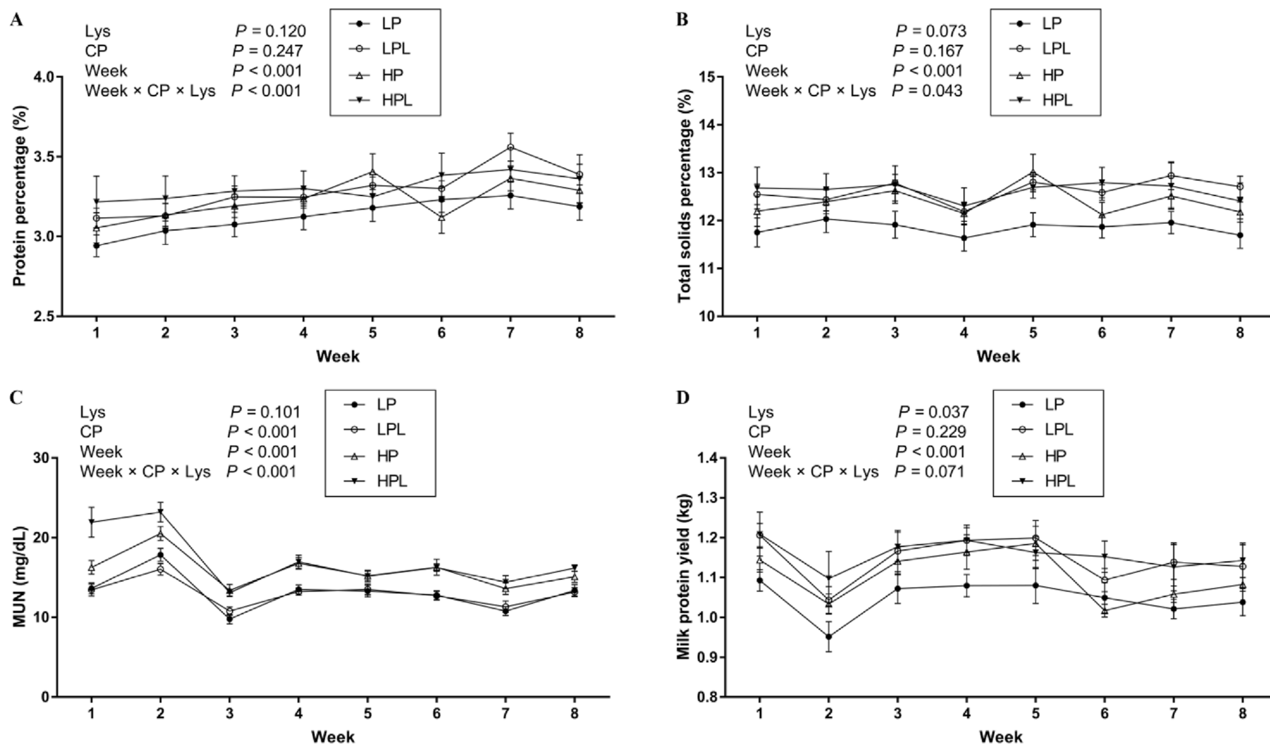

Fig. 2. Effects of dietary crude protein concentration and rumen-protected lysine (RPL) supplementation on milk protein percentage, total solids percentage, urea nitrogen and milk protein yield in dairy cows. LP = low-protein (16% CP) diet; LPL = LP plus RPL; HP = high-protein (18% CP) diet; HPL = HP plus RPL. Values are means; error bars represent standard error. MUN = milk urea nitrogen.

Table 4
Effects of dietary crude protein concentration and rumen-protected lysine supplementation on concentrations of free AA in plasma.

Item	Treatment[1]				SEM	P-value[2]		
	LP	LPL	HP	HPL		CP	RPL	CP × RPL
EAA, μM								
Arg	58.0[b]	59.8[b]	62.2[a]	63.5[a]	0.49	<0.001	0.04	0.79
His	38.7	38.6	41.1	40.6	0.48	0.02	0.74	0.79
Ile	28.7	30.3	29.7	31.4	0.40	0.17	0.03	0.95
Leu	98.1	96.7	95.0	95.3	0.48	0.02	0.58	0.35
Lys	317[c]	386[b]	341[c]	480[a]	11.5	<0.001	<0.001	<0.01
Met	184[b]	200[a]	202[a]	203[a]	1.7	<0.001	<0.01	0.01
Phe	103	107	106	105	0.8	0.88	0.45	0.18
Thr	722[b]	734[b]	761[a]	760[a]	3.1	<0.001	0.08	0.07
Val	64.3[b]	65.8[ab]	67.1[a]	67.1[a]	0.38	<0.01	0.32	0.33
Total EAA	1,614[c]	1,717[b]	1,704[b]	1,846[a]	14.5	<0.001	<0.001	0.13
Total EAA-Lys	1,298[c]	1,331[b]	1,363[a]	1,366[a]	5.2	<0.001	<0.01	0.01
NEAA, μM								
Ala	281[b]	283[b]	299[a]	300[a]	1.8	<0.001	0.46	0.77
Asp	12.6	13.4	14.0	14.2	0.23	0.02	0.24	0.53
Cys	1.50[b]	1.53[b]	1.80[ab]	2.01[a]	0.057	<0.001	0.22	0.36
Glu	76.2[b]	77.0[ab]	79.8[a]	78.3[ab]	0.45	<0.01	0.67	0.16
Gly	61.1	62.5	63.4	64.3	0.53	0.06	0.28	0.75
Pro	56.4[b]	66.6[a]	58.1[b]	60.1[b]	1.14	0.22	<0.01	0.05
Ser	6.85[b]	6.86[b]	7.14[ab]	7.25[a]	0.045	<0.001	0.42	0.50
Tyr	177[b]	179[b]	190[a]	190[a]	1.4	<0.001	0.55	0.64
Total NEAA	673[c]	691[b]	713[a]	716[a]	3.2	<0.001	<0.01	0.03

CP = crude protein; RPL = rumen-protected lysine; EAA = essential amino acids; EAA-Lys = essential amino acids except lysine; NEAA = nonessential amino acids.
[a – c] Means within a row with different superscripts differ and are marked according to ANOVA results ($P < 0.05$).
[1] LP = low-protein (16% CP) diet; LPL = LP plus RPL; HP = high-protein (18% CP) diet; HPL = HP plus RPL.
[2] CP, effect of dietary CP concentration; RPL, effect of RPL supplementation; CP × RPL, interaction of CP and RPL.

respectively). On the low-CP diet, plasma concentrations of Met, Pro, EAA-Lys and total NEAA were greater in cows supplied with RPL compared with those supplied without RPL, but no differences were observed between cows in the HP and HPL groups. Plasma concentration of total EAA-Lys was increased by both dietary CP concentration ($P < 0.001$) and RPL ($P < 0.01$).

3.4. Nitrogen utilization and ruminal MCP synthesis

The results of N utilization and ruminal MCP synthesis are presented in Table 5. N intake was lower in cows fed lower CP compared with those fed higher CP ($P < 0.01$). Cows fed higher CP raised urinary N excretion ($P < 0.01$) through a notable increase in urea excretion ($P < 0.01$). In addition, higher CP significantly decreased milk N as proportion of N intake ($P < 0.01$) and tended to increase urine N as proportion of N intake ($P = 0.07$). Supplementing RPL tended to increase milk protein N secretion ($P = 0.08$) and milk N presented as proportion ($P = 0.07$). Neither dietary supplementation of RPL nor CP concentration affected fecal N excretion ($P > 0.05$). Besides, RPL decreased plasma concentration of PUN ($P < 0.01$). Higher CP tended to increase plasma concentration of PUN ($P = 0.07$), and RPL tended to increase ruminal MCP synthesis ($P = 0.06$).

3.5. Fecal fermentation characteristics

Most of the fecal fermentation variables measured in this experiment were not affected by dietary CP concentration, RPL, or

Table 5
Effects of dietary crude protein concentration and rumen-protected lysine supplementation on nitrogen utilization and microbial protein synthesis in dairy cows.

Item	Treatment[1]				SEM	P-value[2]		
	LP	LPL	HP	HPL		CP	RPL	CP × RPL
N intake, g/d	547[b]	535[b]	621[a]	629[a]	7.6	<0.001	0.78	0.19
N secretion and excretion, g/d								
Milk protein N	166	181	173	183	3.2	0.22	0.08	0.46
Total urinary N	132[b]	137[b]	185[a]	174[a]	6.8	<0.01	0.82	0.53
UUN	75.3[b]	81.4[b]	117[a]	118[a]	5.6	<0.001	0.71	0.80
UUN/total urinary N, %	56.8	60.6	65.1	69.8	2.70	0.03	0.72	0.55
Fecal N	197	189	203	209	5.1	0.22	0.91	0.50
Total N in excreta and milk	495[b]	506[b]	561[a]	566[a]	8.1	<0.001	0.54	0.83
As proportion of N intake, %								
Milk N	30.4[ab]	33.7[a]	28.0[b]	29.2[b]	0.68	<0.01	0.07	0.37
Urine N	24.2	25.5	29.8	27.9	1.09	0.07	0.86	0.46
Fecal N	35.9	35.3	32.6	33.2	0.84	0.12	0.99	0.70
Total N in excreta and milk	90.5	94.5	90.4	90.2	1.23	0.39	0.45	0.41
PUN, mM	5.64[a]	4.36[b]	6.35[a]	4.76[b]	0.190	0.07	<0.001	0.61
Ruminal MCP-N, mg/mL	7.76	11.0	9.47	10.9	0.61	0.51	0.06	0.45

CP = crude protein; RPL = rumen-protected lysine; N = nitrogen; UUN = urinary urea nitrogen; PUN = plasma urea nitrogen; Ruminal MCP-N = nitrogen in ruminal microbial protein.
[a, b] Means within a row with different superscripts differ and are marked according to ANOVA results ($P < 0.05$).
[1] LP = low-protein (16% CP) diet; LPL = LP plus RPL; HP = high-protein (18% CP) diet; HPL = HP plus RPL.
[2] CP, effect of dietary CP concentration; RPL, effect of RPL supplementation; CP × RPL, interaction of CP and RPL.

their interactions (Table 6). RPL had a tendency to reduce the fecal ratio of acetic acid to propionic acid ($P = 0.09$) and the proportion of acetic acid ($P = 0.08$), and increase the proportions of propionic acid ($P = 0.08$) and valeric acid ($P = 0.06$). Higher CP decreased the fecal ratio of acetic acid to propionic acid ($P < 0.01$) and the proportion of acetic acid ($P < 0.01$), and increased the proportion of propionic acid ($P < 0.01$).

3.6. Fecal bacterial richness, diversity and composition

A total of 2,228,374 reads from 4476 ASVs were observed in 40 fecal samples with an average of 55,709 reads per sample. Rarefaction curves based on Sobs index showed that sequences were adequate to reflect the alpha diversity of microbial communities in all samples and could accurately reflect the diversity of the microbial community (Supplementary Fig. S1). We evaluated the richness and diversity of the microbiota by the alpha diversity indices (Table 7). Results showed that a CP × Lys interaction existed in the values of Chao, Sobs and Shannon indices ($P < 0.05$). The values in the LPL group were significantly greater than those in the LP group, but no differences were observed between cows in the HP and HPL groups. The beta diversity analysis was performed to study the similarity or difference of microbial community composition (Fig. 3). PCoA pictures of the microbial compositions in 4 groups were achieved based on Bray–Curtis distance. The points represent fecal microorganisms, and the closer the points are, the greater the similarity is. Results showed that the fecal bacterial community in the LPL group was clearly separated from that in the LP group (Fig. 3A) and differences between the LP and HP groups were found (Fig. 3C). However, no significant difference was observed between the HP and HPL groups, or between the LPL and HPL groups (Fig. 3B and D). A Venn diagram performed at the ASV level showed that the shared ASV numbers (948 ASVs) in 4 groups accounted for 21.2% of the total ASVs (Supplementary Fig. S2).

Based on the SILVA 138 bacteria database and RDP classifier Bayesian algorithm, 16 phyla and 305 genera were identified. At phylum level, the relative community sequence abundances of Firmicutes, Bacteroidota, Actinobacteriota, Patescibacteria, Proteobacteria and Spirochaetota were greater than 0.5% (Table 8). As the dominant bacterium, the average abundance of Firmicutes was 75.42% ± 0.91%. Other bacteria represented 8.11% ± 0.52%, 6.13% ± 0.82%, 4.48% ± 0.51%, 3.81% ± 0.64% and 1.34% ± 0.13% of the total sequences, respectively. RPL increased the relative abundance of phylum Proteobacteria ($P = 0.03$), and decreased the relative abundance of phylum Firmicutes ($P = 0.02$).

At genus level, the top twenty microorganisms (relative abundances > 1%) were analyzed (Table 9), of which *Romboutsia* was the most dominant genus, representing 13.4% ± 0.52% of the total sequences. The second-most dominant genus was *Paeniclostridium*, representing 9.49% ± 0.59% of the total sequences. RPL significantly increased the abundance of *Acinetobacter* ($P = 0.03$) and *Christensenellaceae_R-7_group* ($P = 0.03$), and decreased the abundance of *Clostridium_sensu_stricto_1* ($P < 0.001$), *Turicibacter* ($P < 0.01$) and *unclassified_f__Peptostreptococcaceae* ($P < 0.01$). Compared with cows fed lower CP, higher CP increased the abundance of *Paeniclostridium* ($P = 0.04$), and decreased the abundance of *Lachnospiraceae_NK3A20_group* ($P = 0.01$). In addition, CP × RPL interactions were observed for *Oscillospiraceae_UCG-005* ($P < 0.01$) and *Christensenellaceae_R-7_group* ($P = 0.02$). The bacterial abundances of *Oscillospiraceae_UCG-005* and *Christensenellaceae_R-7_group* were increased in the LPL group compared to the LP group. Besides, a tendency of CP × RPL interaction was observed for *Clostridium_sensu_stricto_1* ($P = 0.06$). RPL only decreased the bacterial abundance in cows fed lower CP, but not in those fed higher CP. The visual circus figure shows the proportion of dominant genera in each group and reflects the distribution of dominant genera in different groups (Supplementary Fig. S3).

3.7. Correlation analysis among different fecal bacteria, production performance and fecal fermentation

The correlations between the relative abundance of fecal bacteria at genus level (abundance >1%) and lactation performance are

Table 6
Effects of dietary crude protein concentration and rumen-protected lysine (RPL) supplementation on fecal volatile fatty acids in dairy cows.

Item	Treatment[1]				SEM	*P*-value[2]		
	LP	LPL	HP	HPL		CP	RPL	CP × RPL
VFA, mM								
Acetic acid	71.0	73.6	75.4	67.4	4.54	0.92	0.75	0.54
Propionic acid	9.0	10.0	10.5	10.3	0.68	0.53	0.81	0.67
Isobutyric acid	0.37	0.55	0.51	0.35	0.074	0.86	0.96	0.28
Butyric acid	5.86	6.55	6.95	7.49	0.576	0.41	0.61	0.95
Isovaleric acid	0.22	0.47	0.38	0.36	0.057	0.84	0.35	0.24
Valeric acid	0.25	0.55	0.51	0.54	0.063	0.31	0.21	0.28
Total VFA	86.7	91.7	94.3	86.4	5.32	0.92	0.90	0.57
VFA proportions, %								
Acetic acid	81.8^{a}	80.9^{a}	80.1^{ab}	78.6^{b}	0.39	<0.01	0.08	0.64
Propionic acid	10.4^{b}	10.7^{b}	11.0^{ab}	11.9^{a}	0.18	<0.01	0.08	0.33
Isobutyric acid	0.44	0.54	0.49	0.37	0.064	0.66	0.95	0.40
Butyric acid	6.79	6.93	7.42	8.21	0.246	0.05	0.33	0.50
Isovaleric acid	0.26	0.45	0.39	0.39	0.050	0.74	0.37	0.38
Valeric acid	0.30	0.55	0.52	0.57	0.041	0.11	0.06	0.20
A:P ratio	7.89^{a}	7.66^{a}	7.30^{ab}	6.64^{b}	0.152	<0.01	0.09	0.41

VFA = volatile fatty acids; A:P ratio = the ratio of acetic acid to propionic acid.
[a, b] Means within a row with different superscripts differ and are marked according to ANOVA results ($P < 0.05$).
[1] LP = low-protein (16% CP) diet; LPL = LP plus RPL; HP = high-protein (18% CP) diet; HPL = HP plus RPL.
[2] CP, effect of dietary CP concentration; RPL, effect of RPL supplementation; CP × RPL, interaction of CP and RPL.

Table 7
Effects of dietary crude protein concentration and rumen-protected lysine (RPL) supplementation on fecal bacterial abundance and diversity in dairy cows.

Item	Treatment[1]				SEM	*P*-value[2]		
	LP	LPL	HP	HPL		CP	RPL	CP × RPL
Chao	562^{c}	672^{a}	647^{ab}	575^{bc}	15.2	0.83	0.50	<0.01
Shannon index	4.59^{b}	4.99^{a}	4.69^{ab}	4.53^{b}	0.070	0.17	0.37	0.04
Sobs	555^{b}	665^{a}	636^{ab}	566^{b}	14.9	0.74	0.45	<0.01
Coverage, %	99.86^{a}	99.85^{a}	99.79^{b}	99.83^{ab}	0.009	<0.01	0.34	0.11

[a – c] Means within a row with different superscripts differ and are marked according to ANOVA results ($P < 0.05$).
[1] LP = low-protein (16% CP) diet; LPL = LP plus RPL; HP = high-protein (18% CP) diet; HPL = HP plus RPL.
[2] CP, effect of dietary CP concentration; RPL, effect of RPL supplementation; CP × RPL, interaction of CP and RPL.

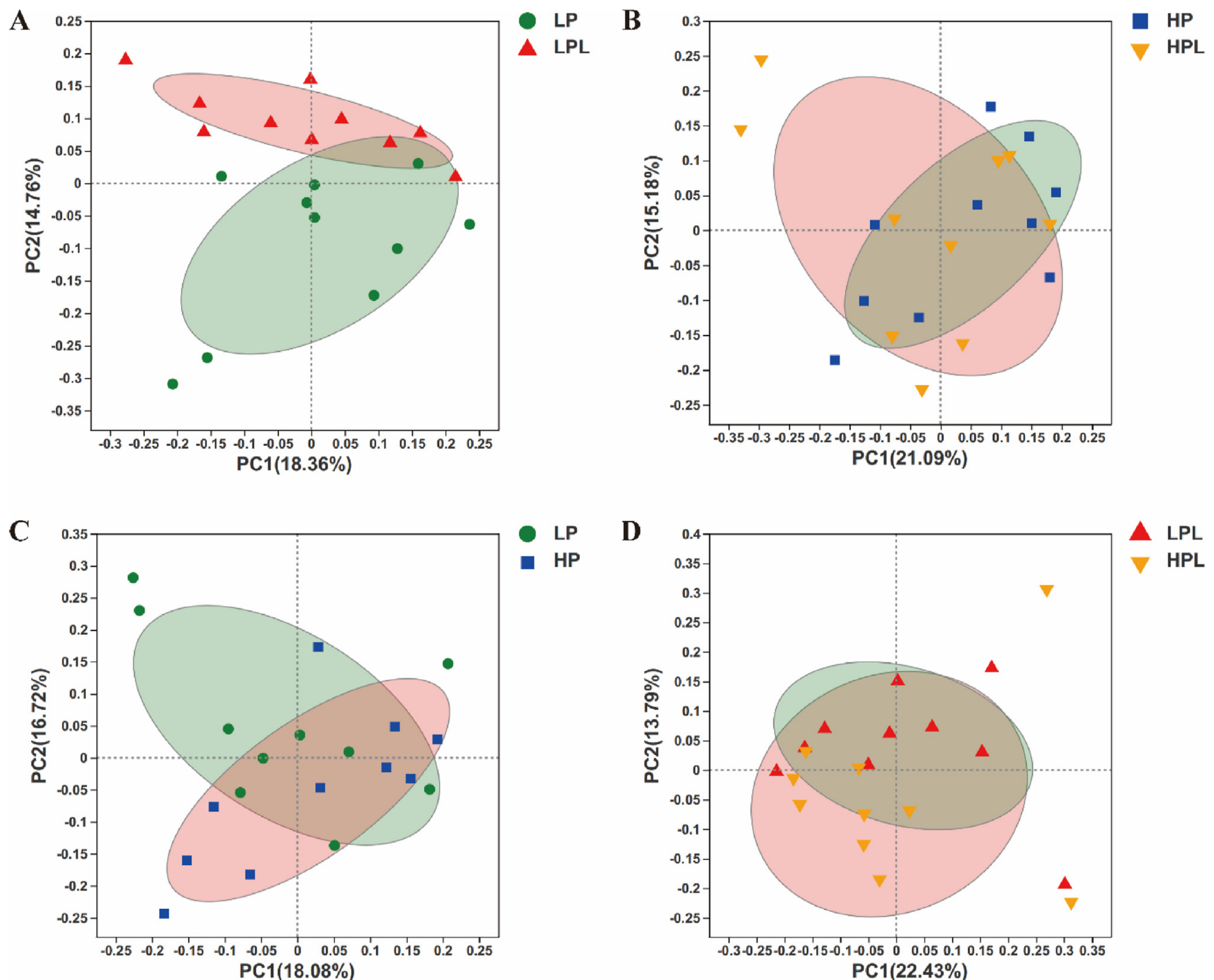

Fig. 3. Principal coordinate analysis (PCoA) with Bray–Curtis dissimilarity of the fecal microbial community between the 4 groups. LP = low-protein (16% CP) diet; LPL = LP plus rumen-protected lysine; HP = high-protein (18% CP) diet; HPL = HP plus rumen-protected lysine. (A) LP vs. LPL, significance: $R = 0.19$, $P < 0.01$. (B) HP vs. HPL, significance: $R = 0.03$, $P = 0.22$. (C) LP vs. HP, significance: $R = 0.12$, $P = 0.04$. (D) LPL vs. HPL, significance: $R = 0.07$, $P = 0.08$.

presented in Fig. 4. The relative abundance of *Clostridium_sensu_stricto_1* was negatively correlated with FCM ($R = -0.312$, $P < 0.05$), milk fat yield ($R = -0.379$, $P < 0.05$) and milk fat content ($R = -0.316$, $P < 0.05$). The relative abundance of *Oscillospiraceae_UCG-005* was positively correlated with FCM ($R = 0.333$, $P < 0.05$) and milk fat content ($R = 0.339$, $P < 0.05$). The relative abundance of *norank_f__Muribaculaceae* was positively correlated with milk protein yield ($R = 0.339$, $P < 0.05$). The relative abundance of *Christensenellaceae_R-7_group* was positively correlated with milk fat content ($R = 0.315$, $P < 0.05$) and milk protein content ($R = 0.315$, $P < 0.05$). The relative abundance of *unclassified_f__Peptostreptococcaceae* was negatively correlated with milk fat yield ($R = -0.346$, $P < 0.05$). The relative abundance of *g__norank_f__Ruminococcaceae* was positively correlated with milk fat content ($R = 0.333$, $P < 0.05$).

Table 8
Effects of dietary crude protein concentration and rumen-protected lysine (RPL) supplementation on fecal bacterial community (%) at phylum level (0.5% < relative abundance) in dairy cows.

Item	Treatment[1]				SEM	*P*-value[2]		
	LP	LPL	HP	HPL		CP	RPL	CP × RPL
Firmicutes	77.9[a]	75.4[ab]	77.2[a]	71.2[b]	0.91	0.16	0.02	0.32
Bacteroidota	6.86	8.84	9.02	7.71	0.522	0.63	0.75	0.12
Actinobacteriota	5.87	6.07	4.57	7.99	0.820	0.85	0.28	0.34
Patescibacteria	5.16	3.84	3.94	4.97	0.509	0.97	0.89	0.27
Proteobacteria	1.94[b]	4.21[ab]	2.90[ab]	6.20[a]	0.644	0.24	0.03	0.68
Spirochaetota	1.45	0.97	1.64	1.31	0.126	0.29	0.11	0.76
Other (<0.5%)	0.82	0.91	0.83	0.71	0.113	0.69	0.93	0.66

[a, b] Means within a row with different superscripts differ and are marked according to ANOVA results ($P < 0.05$).
[1] LP = low-protein (16% CP) diet; LPL = LP plus RPL; HP = high-protein (18% CP) diet; HPL = HP plus RPL.
[2] CP, effect of dietary CP concentration; RPL, effect of RPL supplementation; CP × RPL, interaction of CP and RPL.

4. Discussion

One of the objectives of this study was to evaluate the effect of different dietary CP concentrations supplemented with or without RPL on production performance in lactating dairy cows. The DMI of cows can be affected by many complex factors, from the cellular level up to environmental conditions (Allen, 2000). Increasing dietary CP concentration using rumen undegradable protein improved DMI and protein and energy metabolism of dairy cows from calving to 21 d postpartum (Amanlou et al., 2017). However,

Table 9
Effects of dietary crude protein concentration and rumen-protected lysine (RPL) supplementation on fecal bacterial community (%) at genus level (1% < relative abundance) in dairy cows.

Phylum	Genus	Treatment[1]				SEM	P-value[2]		
		LP	LPL	HP	HPL		CP	RPL	CP × RPL
Firmicutes	*Romboutsia*	13.9	12.0	14.7	13.1	0.52	0.34	0.10	0.87
	Paeniclostridium	8.30	8.24	10.8	10.6	0.59	0.04	0.92	0.96
	Clostridium_sensu_stricto_1	9.66[a]	5.23[c]	7.98[ab]	6.48[bc]	0.454	0.78	<0.001	0.06
	Oscillospiraceae_UCG-005	4.19[c]	6.93[a]	6.58[ab]	4.74[bc]	0.385	0.89	0.53	<0.01
	Lachnospiraceae_NK3A20_group	5.08[ab]	6.48[a]	3.90[b]	4.00[b]	0.363	0.01	0.27	0.34
	Turicibacter	6.05[a]	3.38[c]	5.15[ab]	4.17[bc]	0.304	0.92	<0.01	0.12
	unclassified_f__Lachnospiraceae	2.91	3.90	3.13	3.18	0.221	0.57	0.25	0.30
	norank_f__Eubacterium_coprostanoligenes_group	4.25	2.79	2.15	2.49	0.372	0.11	0.44	0.22
	g__norank_f__norank_o__Clostridia_UCG-014	2.81	3.01	2.06	2.31	0.222	0.11	0.62	0.95
	Christensenellaceae_R-7_group	1.98[b]	3.64[a]	2.33[b]	2.26[b]	0.199	0.15	0.03	0.02
	unclassified_f__Peptostreptococcaceae	2.38[a]	1.79[b]	2.12[ab]	1.83[b]	0.080	0.45	<0.01	0.31
	g__norank_f__norank_o__RF39	1.79	1.83	1.32	1.69	0.154	0.33	0.52	0.60
	g__norank_f__Ruminococcaceae	0.55	1.06	1.69	1.78	0.337	0.18	0.67	0.76
	g__norank_f__UCG-010	1.00	1.52	1.23	1.27	0.081	0.94	0.08	0.14
Bacteroidota	*norank_f__Muribaculaceae*	2.56	3.62	3.13	2.90	0.222	0.86	0.36	0.16
	Rikenellaceae RC9 gut group	1.65	2.17	2.07	1.68	0.146	0.91	0.83	0.13
Actinobacteriota	*Bifidobacterium*	4.17	4.71	3.32	6.99	0.833	0.67	0.22	0.36
Patescibacteria	*Candidatus Saccharimonas*	5.15	3.18	3.93	4.96	0.508	0.98	0.88	0.26
Proteobacteria	*Acinetobacter*	1.07[b]	3.26[ab]	1.62[ab]	4.63[a]	0.591	0.40	0.03	0.72
Spirochaetota	*Treponema*	1.44	0.96	1.64	1.31	0.126	0.28	0.11	0.76

[a – c] Means within a row with different superscripts differ and are marked according to ANOVA results ($P < 0.05$).
[1] LP = low-protein (16% CP) diet; LPL = LP plus RPL; HP = high-protein (18% CP) diet; HPL = HP plus RPL.
[2] CP, effect of dietary CP concentration; RPL, effect of RPL supplementation; CP × RPL, interaction of CP and RPL.

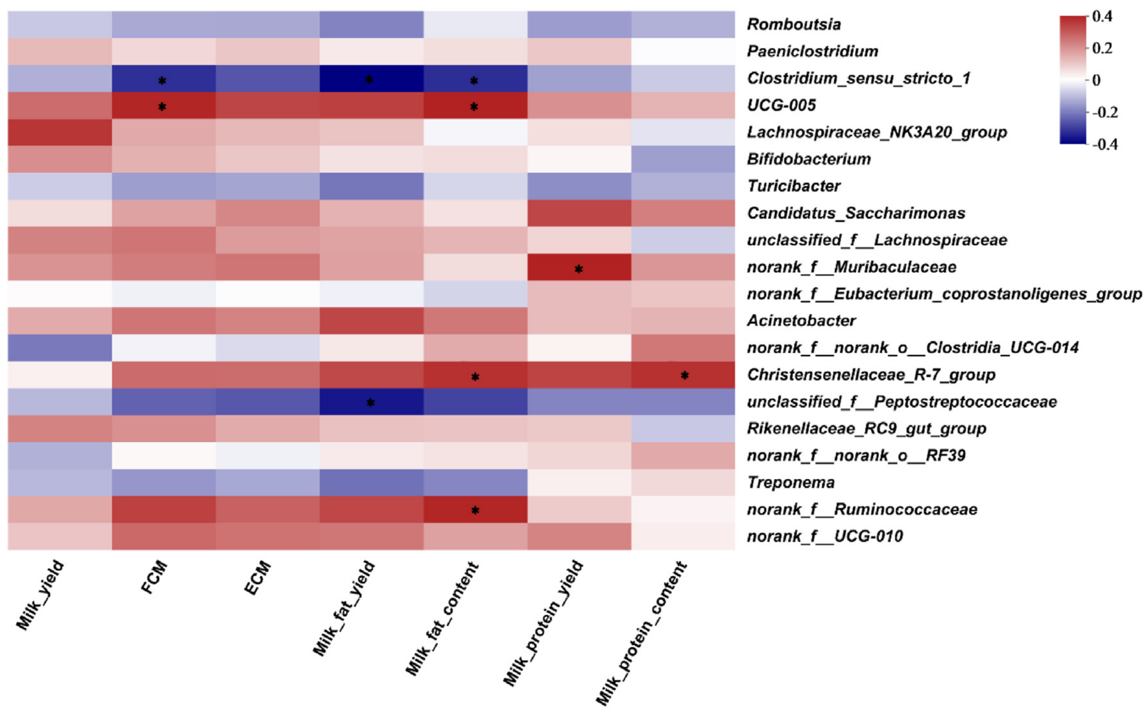

Fig. 4. Heatmap diagram of correlations between fecal bacterial and production performance and milk compositions at genus level. Red was positively correlated and blue was negatively correlated. Correlation significance P-value is indicated by asterisks. * $0.01 < P \le 0.05$.

the increasing dietary CP concentration had no effect on DMI in our study. We found that the RPL increased the DMI, which was similar to the findings of Girma et al. (2019) and Swanepoel et al. (2010). In addition, the RPL increased the DMI when the cows were fed high CP, but not at low CP. In the study of Lee et al. (2012b), the supplementation of RPL elevated the DMI under the MP-deficient diet, while the MP of low CP and high CP diets were sufficient in this study. As suggested by Girma et al. (2019), the increased DMI would be due to greater MP and Lys intake and more endogenous synthesis of carnitine, which is a methylated form of Lys (Shug et al., 1982), and the DMI tended to increase as carnitine dose increased from 1 to 3 g/d (Carlson et al., 2006). Further study about the mechanism of Lys on DMI is needed.

In this study, results showed that milk protein yield was significantly increased by RPL. Amino acids are important precursors for milk protein synthesis in dairy cows (Schwab and Broderick, 2017). Hence, the concentrations of plasma AA were measured to estimate the utilization of AA in current study. Similar

increases in plasma Lys concentration in the LPL and HPL groups confirmed the absorption of RPL. RPL supplementation could meet the needs of limiting AA for dairy cows, producing more intestinally absorbable Lys for milk protein synthesis (Fleming et al., 2019). In addition, we found that RPL had no effect on milk protein yield in dairy cows fed higher CP, while RPL could contribute to synthesis of milk protein in cows fed lower CP. This difference might be related to the balance of AA supply for mammary glands (Zhou et al., 2021). A well-balanced AA supply is critical for milk protein synthesis (Patton et al., 2014). In this study, plasma concentrations of Lys, total EAA-Lys, total NEAA were increased in the LPL group compared with LP group. When the energy supply for cows is insufficient, AA is be used to generate energy through oxidative decomposition instead of milk protein synthesis (Lee et al., 2012a). In this study, the net energy for lactation in the diets fed to the LP and LPL groups exceeded the cows' requirements, suggesting that the significantly lower plasma concentration of total EAA-Lys in the LP group compared with the LPL group was not caused by energy deficiency. In addition, the catabolism and transamination of Lys can produce branched chain AA and various NEAA, of which the rate would increase when AA supply exceeds the demands of dairy cows (Lee et al., 2019). Compared with the LP group, the plasma concentration of Pro, de novo synthesized using Lys as precursor, was higher in the LPL group, suggesting that Lys could be degraded to produce other AA (Lapierre et al., 2006). Besides, plasma concentrations of EAA, NEAA and total EAA-Lys in the HP and HPL groups were similar. In the current study, dietary MP in the LP and LPL groups was deficient, whereas MP reached balance in the HP and HPL groups. The MP requirement represented the total supply of EAA and NEAA needed by a cow to support production (Patton et al., 2014). Hence, different CP concentrations supplemented with RPL resulted in different milk protein synthesis, which might be due to the sufficiency of dietary MP. Furthermore, the tendency for increased ruminal MCP synthesis in the LPL and HPL groups was observed. RPL would be partially degraded in rumen for the nutritional needs of ruminal microbes, promoting the growth of the rumen microbe and increasing the efficiency of ruminal MCP synthesis (Liu et al., 2021). As an important source of intestinally absorbable AA, ruminal MCP could benefit production performance and milk quality (Schwab and Broderick, 2017).

The amount and composition of AA were critical to the synthesis of milk protein. The ratio of Lys to Met was 2.82:1 and 3:1 in the LP and LPL groups, respectively. Wang et al. (2010) reported that a proper ratio (3:1) of Lys to Met in MP could improve milk protein synthesis most effectively. The optimal ratio of Lys to Met could reduce the negative impact of MP deficiency (Liu et al., 2013). Therefore, the positive effect of RPL on milk protein yield in the LPL group compared with the LP group could possibly be the result of the balance of Lys and Met in MP, even though MP was deficient. Both Met and Lys are important limiting AA in dairy cows (NRC, 2001). Compared with the LP group, the higher plasma concentration of Met in LPL cows provided precursors to promote milk protein synthesis, and the absorption of Met in the mammary glands would reach the upper limit. Appuhamy et al. (2011) also highlighted the combined effect of Met and Lys on milk protein synthesis. In addition, Arg and Ile could act as regulators of the mTOR signalling pathway to promote milk protein synthesis (Ban et al., 2004). Hence, we suggested that the higher milk protein yield in this study would be partly associated with the differences in plasma concentrations of Arg and Ile.

Based on the differences in milk protein yield, we further investigated the effect of different dietary CP concentrations and RPL supplementation on N metabolism. In this study, in addition to the synthesis of milk protein, most of the N consumed by dairy cows was excreted through feces and urine. Compared with cows fed lower CP, the cows fed higher CP had greater total N excretion through increasing total urinary N excretion rather than fecal N excretion, as the total urinary N was mainly determined by urea N. This result was similar to the findings of Mutsvangwa et al. (2016). The milk protein N was not affected by dietary CP concentration. In this study, soybean meal was used as the main protein supplement to elevate the dietary CP concentration, which might limit the supply of Met. The decreased proportion of Met to MP might cause lower Met content in intestinal protein and synthesis of milk protein. In addition, a previous study found that increasing the dietary CP concentration had a positive effect on milk protein synthesis during early to mid-lactation of dairy cows, while the response to dietary protein supplementation decreased as lactation progressed (Wu and Satter, 2000).

Sinclair et al. (2014) indicated that reducing dietary CP concentrations from 18.4% to 15.1% of DM did not affect milk protein yield. Besides, cows fed lower CP had higher milk N presented as proportion of N intake compared with those fed higher CP. Reducing dietary CP concentration could improve N utilization efficiency (milk N presented as proportion of N intake) and reduce total N excretion into the environment (Colmenero and Broderick, 2006). The highest ratio of milk N to N intake was found in the LPL group, suggesting that RPL supplementation could improve N utilization efficiency to a greater extent when the dietary CP concentration is reduced. PUN was a useful indicator of protein metabolism in dairy cows (Puppel and Kuczynska, 2016). The lower PUN in cows supplemented with RPL suggested that RPL could balance AA and reduce deamination of other AA to ammonia, so that more N would flow to milk protein and increase N utilization efficiency (Wang et al., 2010).

Previous studies on dietary CP concentration and RPL mainly concentrated on the rumen, while studies on hindgut fermentation are relatively few. Dietary CP concentration usually alters fecal fermentation; however the dominant microbiota at phylum level was not changed, which might be related to the parity of the cows as the shift in fermentation pattern is more likely to occur in the fecal microbiota of older cows (Zhang et al., 2019). At genus level, dietary CP concentration significantly increased the relative abundance of *Paeniclostridium*, and decreased the abundance of *Lachnospiraceae_NK3A20_group*. *Paeniclostridium* has been described as a pathogenic bacterium in feces (Kim et al., 2017), and found enriched in the gut of mastitis cows (Wang et al., 2022). *Lachnospiraceae_NK3A20_group* has been described as a probiotic that produces butyrate and attenuates inflammation by inhibiting production of proinflammatory cytokines by neutrophils in the gut (Li et al., 2021). Hence, we suggested that more caution should be exercised in the selection of dietary CP concentration in future studies.

The proportions of acetic acid, propionic acid and valeric acid tended to be affected by RPL in this study, and the ratio of acetic acid to propionic acid tended to decrease. The microbiome plays a significant role in the fermentation of AA to ammonia, VFA etc., for MCP synthesis, and changes in fermentation conditions might alter the richness and diversity of microorganisms (Petri et al., 2019). Hence, we further investigated the effect on hindgut microbiota. The supplementation and restriction of Lys have been proven to change gut microflora in monogastric animals (Yin et al., 2017; Zhou et al., 2018), which was mainly associated with the amount of substrate for bacterial proliferation. In addition, a previous study demonstrated that rumen-protected leucine improved starch utilization by regulating fermentation profiles and amylolytic microbes of the hindgut in dairy calves (Ren et al., 2020). Supplementation with sulfur AA modulated the fermentation metabolome and gut microbiota, thus altering the methanogenesis in ruminants (Teklebrhan and Tan, 2022). In this study, the relative

abundance of Firmicutes and Proteobacteria was changed by RPL, showing that RPL could affect the microbial community of the hindgut of dairy cows. The changes might be caused by the increase of free AA available to microbes, suggesting that improved MCP synthesis is essential for microbial growth and associated with increased gene copies. Although the MCP synthesis in the hindgut was not evaluated, the increased plasma AA concentrations and tendency to elevate ruminal MCP supported our speculation. The fecal bacterial abundance and diversity were increased in the LPL group compared with the LP group, and no difference was observed between the HP and HPL groups, suggesting that the effect of RPL on hindgut microbiota would be in a dietary CP concentration-dependent manner.

The RPL supplementation mainly affected the relative abundances of several probiotics and pathogenic bacteria at genus level in this study. The major genus of Firmicutes in feces was *Romboutsia*, which is often described as a biological indicator of intestinal mucosal health (Mangifesta et al., 2018). No difference in the abundance of *Romboutsia* was found among all groups, indicating that CP or RPL had no effect on intestinal mucosal health. RPL supplementation decreased the abundance of bacteria *Turicibacter*, which is positively associated with colitis (Rettedal et al., 2009). The proliferative inhibition of RPL on pathogen *Turicibacter* might suggest an indirect effect of RPL on hindgut health. Another pathogen decreased by RPL was *Clostridium_sensu_stricto_1*. Spearman correlation analysis showed that genus *Clostridium_sensu_stricto_1* was negatively correlated with production performance, including FCM, milk fat yield and milk fat content. Zhang et al. (2017) reported that Arg supplementation could alleviate intestinal mucosal injury by inhibiting *Clostridium_sensu_stricto_1* in broilers. RPL supplementation led to an increase in plasma Arg concentration in this study, supporting the decreased abundance of *Clostridium_sensu_stricto_1*. Furthermore, the genus *Christensenellaceae_R-7_group* was positively correlated with milk protein content and milk fat content. RPL supplementation significantly increased the abundance of *Christensenellaceae_R-7_group*, a probiotic that is widely found in the intestinal tract and mucosa, which is considered to be involved in AA and lipid metabolism (Waters and Ley, 2019). Notably, the effect of RPL on abundances of probiotics and pathogenic bacteria was only observed in cows fed lower CP, consistent with the production performance. Moreover, RPL might enhance the efficacy of probiotics by inhibiting pathogen growth in the hindgut.

The abundance of genus *UCG-005* belonging to family Oscillospiraceae was increased by RPL when fed lower CP. Studies on *UCG-005* function are few, however Oscillospiraceae are considered a protein degrader involved in the breakdown of intestinal mucins, which might provide free AA as C and N precursors to promote microbial proliferation (Raimondi et al., 2021; Amaretti et al., 2019). Besides, RPL decreased the relative abundance of an unclassified genus, belonging to family Peptostreptococcaceae, a microorganism that is related to intestinal inflammation and was inversely associated with milk fat yield in this study. Lys restriction could cause an enhanced inflammatory response in the organs of piglets (Yin et al., 2017), while the effect of Lys on hindgut inflammation in dairy cows is unknown. In addition, the increase in the abundance of *Acinetobacter* by RPL might be related to fermentation parameters of the hindgut. Shen et al. (2019) reported that an increase in ruminal acetate decreased the relative abundance of genus *Acinetobacter*, and the fecal proportion of acetate was found to be decreased by RPL supplementation in this study. In addition, the *Acinetobacter* was positively correlated with average milk protein yield in lactating buffalo (Zou et al., 2019), while no correlation was found in this study.

5. Conclusion

In this study, RPL supplementation increased milk protein yield and N utilization efficiency by balancing AA supply in cows fed lower CP compared with those fed higher CP, and cows had lower N excretion to the environment when fed lower CP, which was mainly due to urea-N excretion. In addition, RPL tended to increase bacterial abundance and change the composition of hindgut microorganisms in cows fed lower CP. The composition of probiotics *Christensenellaceae_R-7_group* tended to elevate, and the composition of pathogenic bacteria *Clostridium_sensu_stricto_1* and *Turicibacter* tended to reduce, indicating better hindgut health, thus improving the lactation performance of dairy cows. Our results provide the first evidence of RPL altering hindgut microbiota. More research is warranted to look at the metabolic effects of RPL on the rumen and hindgut.

Author contributions

Xiaoshi Wei: Writing - Review & Editing and Data Curation. **Hao Wu**: Writing - Original Draft and Investigation. **Zixiang Wang**: Formal analysis. **Jinpeng Zhu**: Investigation and Data Curation. **Weijie Wang**: Validation and Visualization. **Junhong Wang**: Investigation. **Yanming Wang**: Project administration. **Chong Wang**: Visualization and Funding acquisition.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.08.001.

References

Allen MS. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. J Dairy Sci 2000;83:1598–624.
Amanlou H, Farahani TA, Farsuni NE. Effects of rumen undegradable protein supplementation on productive performance and indicators of protein and energy metabolism in Holstein fresh cows. J Dairy Sci 2017;100(5):3628–40.
Amaretti A, Gozzoli C, Simone M, Raimondi S, Righini L, Perez-Brocal V, Garcia-Lopez R, Moya A, Rossi M. Profiling of protein degraders in cultures of human gut microbiota. Front Microbiol 2019;10:2614. -2614.
AOAC. Official methods of analysis. 15th ed. Association of Official Analytical Chemists; 1990.
Apelo SIA, Bell AL, Estes K, Ropelewski J, de Veth MJ, Hanigan MD. Effects of reduced dietary protein and supplemental rumen-protected essential amino acids on the nitrogen efficiency of dairy cows. J Dairy Sci 2014;97(9):5688–99.

Appuhamy J, Knapp JR, Becvar O, Escobar J, Hanigan MD. Effects of jugular-infused lysine, methionine, and branched-chain amino acids on milk protein synthesis in high-producing dairy cows. J Dairy Sci 2011;94(4):1952–60.
Ban H, Shigemitsu K, Yamatsuji T, Haisa M, Naomoto Y, et al. Arginine and Leucine regulate p70 S6 kinase and 4E-BP1 in intestinal epithelial cells. Int J Mol Med 2004;13(4):537–43.
Carlson DB, Litherland NB, Dann HM, Woodworth JC, Drackley JK. Metabolic effects of abomasal l-carnitine infusion and feed restriction in lactating Holstein cows. J Dairy Sci 2006;89:4819–34.
Chen Y, Harrison JH, Bunting LD. Effects of replacement of alfalfa silage with corn silage and supplementation of methionine analog and lysine-HCl on milk production and nitrogen feed efficiency in early lactating cows. Anim Feed Sci Technol 2018;242:120–6.
Colmenero JJ, Broderick GA. Effect of dietary crude protein concentration on milk production and nitrogen utilization in lactating dairy cows. J Dairy Sci 2006;89(5):1704–12.
Fleming AJ, Lapierre H, White RR, Tran H, Kononoff PJ, Martineau R, Weiss WP, Hanigan MD. Predictions of ruminal outflow of essential amino acids in dairy cattle. J Dairy Sci 2019;102(12):10947–63.
Giallongo F, Harper MT, Oh J, Lopes JC, Lapierre H, Patton RA, Parys C, Shinzato I, Hristov AN. Effects of rumen-protected methionine, lysine, and histidine on lactation performance of dairy cows. J Dairy Sci 2016;99(6):4437–52.
Girma DD, Ma L, Wang F, Jiang QR, Callaway TR, Drackley JK, Bu DP. Effects of close-up dietary energy level and supplementing rumen-protected lysine on energy metabolites and milk production in transition cows. J Dairy Sci 2019;102(8): 7059–72.
Guinard J, Rulquin H. Effects of graded amounts of duodenal infusions of lysine on the mammary uptake of major milk precursors in dairy cows. J Dairy Sci 1994;77(12):3565–76.
Kim JY, Kim YB, Song HS, Chung WH, Lee C, Ahn SW, Lee SH, Jung MY, Kim TW, Nam YD, Roh SW. Genomic analysis of a pathogenic bacterium, Paeniclostridium sordellii CBA7122 containing the highest number of rRNA operons, isolated from a human stool sample. Front Pharmacol 2017;8:840.
Lapierre H, Pacheco D, Berthiaume R, Ouellet DR, Lobley GE. What is the true supply of amino acids for a dairy cow? J Dairy Sci 2006;89(E. Suppl):E1–14.
Lee C, Hristov AN, Heyler KS, Cassidy TW, Lapierre H, Varga GA, Parys C. Effects of metabolizable protein supply and amino acid supplementation on nitrogen utilization, milk production, and ammonia emissions from manure in dairy cows. J Dairy Sci 2012a;95(9):5253–68.
Lee C, Hristov AN, Cassidy TW, Heyler KS, Lapierre H, Varga GA, de Veth MJ, Patton RA, Parys C. Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. J Dairy Sci 2012b;95(10):6042–56.
Lee C, Lobos NE, Weiss WP. Effects of supplementing rumen-protected lysine and methionine during prepartum and postpartum periods on performance of dairy cows. J Dairy Sci 2019;102(12):11026–39.
Li W, Chang Q, Liu G, Shan A, Li F. Effect of distillers dried grains with solubles diet supplemented with rumen-protected lysine and methionine on milk production and milk amino acids in the lactating cow. Int J Dairy Technol 2014;67(1): 47–54.
Li G, Lin J, Zhang C, Gao H, Lu H, Gao X, Zhu R, Li Z, Li M, Liu Z. Microbiota metabolite butyrate constrains neutrophil functions and ameliorates mucosal inflammation in inflammatory bowel disease. Gut Microb 2021;13(1):1968257.
Lin X, Li S, Zou Y, Zhao F, Liu J, Liu H. Lysine stimulates protein synthesis by promoting the expression of ATB0,+ and activating the mTOR pathway in bovine mammary epithelial cells. J Nutr 2018;148(9):1426–33.
Liu Y, Peng H, Schwab CG. Enhancing the productivity of dairy cows using amino acids. Anim Prod Sci 2013;53(11):1156–9.
Liu H, Yang G, Degen A, Ji K, Jiao D, Liang Y, et al. Effect of feed level and supplementary rumen protected lysine and methionine on growth performance, rumen fermentation, blood metabolites and nitrogen balance in growing Tan lambs fed low protein diets. Anim Feed Sci Technol 2021;279:115024.
Mangifesta M, Mancabelli L, Milani C, Gaiani F, de'Angelis N, de'Angelis GL, Sinderen DV, Ventura M, Turroni F. Mucosal microbiota of intestinal polyps reveals putative biomarkers of colorectal cancer. Sci Rep-UK 2018;8(1): 13974.
Mutsvangwa T, Davies KL, McKinnon JJ, Christensen DA. Effects of dietary crude protein and rumen-degradable protein concentrations on urea recycling, nitrogen balance, omasal nutrient flow, and milk production in dairy cows. J Dairy Sci 2016;99(8):6298–310.
NRC. Nutrient requirements of dairy cattle. 7 th ed. Washing: Nat. Acad. Press; 2001.
Patton RA, Hristov AN, Lapierre H. Protein feeding and balancing for amino acids in lactating dairy cattle. Vet Clin N Am-Food A 2014;30(3):599–621.
Petri RM, Münnich M, Zebeli Q, Klevenhusen F. Graded replacement of corn grain with molassed sugar beet pulp modulates the fecal microbial community and hindgut fermentation profile in lactating dairy cows. J Dairy Sci 2019;102(6): 5019–30.
Puppel K, Kuczynska B. Metabolic profiles of cow's blood; a review. J Sci Food Agric 2016;96(13):4321–8.
Raimondi S, Musmeci E, Candeliere F, Amaretti A, Rossi M. Identification of mucin degraders of the human gut microbiota. Sci Rep-UK 2021;11(1):11094.
Ren H, Bai H, Su X, Pang J, Li X, Wu S, Cao Y, Cai C, Yao J. Decreased amylolytic microbes of the hindgut and increased blood glucose implied improved starch utilization in the small intestine by feeding rumen-protected leucine in dairy calves. J Dairy Sci 2020;103(5):4218–35.
Rettedal E, Vilain S, Lindblom S, Lehnert K, Scofield C, George S, Clay S, Kaushik RS, Rosa AJM, Francis D, Brozel VS. Alteration of the ileal microbiota of weanling piglets by the growth-promoting antibiotic chlortetracycline. Appl Environ Microbiol 2009;75(17):5489–95.
Schwab CG, Broderick GAA. 100-Year Review: protein and amino acid nutrition in dairy cows. J Dairy Sci 2017;100(12):10094–112.
Shen H, Xu ZH, Shen ZM, Lu ZY. The Regulation of ruminal short-chain fatty acids on the functions of rumen barriers. Front Physiol 2019;10:1305. -1305.
Shug AL, Schmidt MJ, Golden GT, Fariello RG. The distribution and role of carnitine in the mammalian brain. Life Sci 1982;31:2869–74.
Sinclair KD, Garnsworthy PC, Mann GE, Sinclair LA. Reducing dietary protein in dairy cow diets: implications for nitrogen utilization, milk production, welfare and fertility. Animal 2014;8(2):262–74.
Soest PJV, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 1991;74(10):3583–97.
Swanepoel N, Robinson P, Erasmus L. Amino acid needs of lactating dairy cows: impact of feeding lysine in a ruminally protected form on productivity of lactating dairy cows. Anim Feed Sci Technol 2010;157:79–94.
Teklebrhan T, Tan Z. Diet supplementation with sulfur amino acids modulated fermentation metabolome and gut microbiome in goats. Front Microbiol 2022;24(13):870385.
Titgemeyer EC, Merchen NR, Berger LL. Evaluation of soybean meal, corn gluten meal, blood meal and fish meal as sources of nitrogen and amino acids disappearing from the small intestine of steers. J Anim Sci 1989;67(1):262–75.
Wang C, Liu H, Wang Y, Yang Z, Liu J, Wu Y, Yan T, Ye H. Effects of dietary supplementation of methionine and lysine on milk production and nitrogen utilization in dairy cows. J Dairy Sci 2010;93(8):3661–70.
Wang Y, Nan X, Zhao Y, Jiang L, Wang H, Zhang F, Hua D, Liu J, Yang L, Yao J, Xiong B. Discrepancies among healthy, subclinical mastitic, and clinical mastitic cows in fecal microbiome and metabolome and serum metabolome. J Dairy Sci 2022;105(9):7668–88.
Waters JL, Ley RE. The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. BMC Biol 2019;17(1):83.
Wu Z, Satter LD. Milk production during the complete lactation of dairy cows fed diets containing different amounts of protein. J Dairy Sci 2000;83(5):1042–51.
Yin J, Han H, Li Y, Liu Z, Zhao Y, Fang R, Huang X, Zheng J, Ren W, Wu F, Liu G, Wu X, Wang K, Sun L, Li C, Li T, Yin Y. Lysine restriction affects feed intake and amino acid metabolism via gut microbiome in piglets. Cell Physiol Biochem 2017;44(5): 1749–61.
Zhang B, Lv Z, Li H, Guo S, Liu D, Guo Y. Dietary l-arginine inhibits intestinal Clostridium perfringens colonisation and attenuates intestinal mucosal injury in broiler chickens. Br J Nutr 2017;118(5):321–32.
Zhang G, Wang Y, Luo H, Qiu W, Zhang H, Hu L, Wang Y, Dong G, Guo G. The association between inflammaging and age-related changes in the ruminal and fecal microbiota among lactating Holstein cows. Front Microbiol 2019;10:1803.
Zhou H, Chen D, Mao X, He J, Yu J, Zheng P, Luo J, Gao J, Htoo J, Yu B. Effects of dietary lysine levels on jejunal expression of amino acids transporters and hindgut microflora in weaned pigs. J Anim Feed Sci 2018;27(3).
Zhou M, Xu L, Zhao F, Liu H. Regulation of milk protein synthesis by free and peptide-bound amino acids in dairy cows. Biol Behav 2021;10(10):1044.
Zinn RA, Owens FN. A rapid procedure for purine measurement and its use for estimating NE. Can J Anim Sci 1986;66(1):157–66.
Zou C, Gu Q, Zhou X, Xia Z, Muhammad WI, Tang Q, Liang M, Lin B, Qin G. Ruminal microbiota composition associated with ruminal fermentation parameters and milk yield in lactating buffalo in Guangxi, China-A preliminary study. J Anim Physiol Anim Nutr 2019;103(5):1374–9.