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Original Research Article

Impact of dietary supplementation with β-alanine on the rumen microbial crude protein supply, nutrient digestibility and nitrogen retention in beef steers elucidated through sequencing the rumen bacterial community

Jinming Hu [1], Shuo Zhang [1], Mengmeng Li, Guangyong Zhao*

State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, 100193 Beijing, China

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ABSTRACT

This study investigated the effects of β-alanine (β-Ala) on rumen fermentation, nutrient digestibility, nitrogen (N) metabolism, plasma biochemical parameters, and rumen bacterial communities in beef steers. Six steers with initial liveweight of 252.8 ± 5.2 kg and 3 treatments of supplementing with 0, 30, or 60 g β-Ala per day to basal diet were allocated in a replicated 3 × 3 Latin square design. Each experimental period was 20 d, of which the first 15 d were for adaptation and the subsequent 5 d were for sampling. The results showed that β-Ala linearly increased the ruminal concentration of microbial crude protein (MCP) ($P = 0.005$), but it did not affect the ruminal concentrations of ammonia N and total volatile fatty acids ($P > 0.10$). β-Ala also linearly increased the dry matter (DM) ($P = 0.009$), organic matter (OM) ($P = 0.017$) and crude protein (CP) ($P = 0.043$) digestibility, tended to decrease the acid detergent fiber digestibility ($P = 0.077$), but it did not affect the neutral detergent fiber digestibility ($P = 0.641$). β-Ala quadratically increased the relative abundance of ruminal Bacteroidota ($P = 0.021$) at the phylum level, and increased *Prevotella* ($P = 0.028$) and *Prevotellaceae_UCG-003* ($P = 0.014$), and decreased the relative abundance of *NK4A214_group* ($P = 0.009$) at the genus level. Feeding steers with β-Ala linearly increased the urinary N ($P = 0.006$), urea excretions ($P = 0.002$) and the N retention ($P = 0.004$), but it did not affect the N utilization efficiency ($P = 0.120$). β-Ala quadratically increased the plasma concentration of the total antioxidant capacity ($P = 0.011$) and linearly increased the plasma concentration of insulin-like growth factor-1 ($P < 0.001$). In summary, dietary supplementation with β-Ala improved the rumen MCP supply and increased the digestibility of DM, OM, CP and the N retention. Further research is necessary to verify the ruminal degradability of β-Ala and to investigate the mechanism of the impact of absorbed β-Ala on the anti-oxidative ability in steers.

1. Introduction

β-Alanine (β-Ala) is the only naturally occurring β-amino acid (AA) that exists in many feedstuffs such as whole corn silage (Bai et al., 2021) and *Medicago truncatula* (Broeckling et al., 2005). β-Ala differs from α-Ala (L-Ala) in that its amino group is located at the beta position of the carboxyl carbon and lacks a chiral center. Although β-Ala and L-Ala differ in the position of only one amino group, the roles they play exhibit significant disparities (Wu, 2009). L-Ala can be directly used for protein synthesis whereas β-Ala serves as a non-proteinogenic AA (Griffith, 1986). In the animal body, however, β-Ala can be used for the synthesis of carnosine,

* Corresponding author.
E-mail address: zhaogy@cau.edu.cn (G. Zhao).
[1] These authors contributed equally.
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which can improve the activity of glycine-N-methyltransferase in the kidney of mice, and consequently regulate the ratio of S-adenosylmethionine to S-adenosylhomocysteine in the liver (Liu et al., 2020). S-adenosylmethionine acts as a methyl donor for AA and protein synthesis in animals such as mice and dairy cows (Abbasi et al., 2018; Chu et al., 2013). Based on the results of previous studies, it was hypothesized that β-Ala would impact the nitrogen (N) metabolism of animals. To date, however, no reports are available in this aspect.

A previous study showed that supplementing with β-Ala decreased the taurine levels of gastrocnemius muscle and brain in rats (Shaffer and Kocsis, 1981), whereas another study indicated that dietary addition of taurine at 0.2% increased the protein conversion rate, N retention, and N utilization efficiency (NUE) in rats (Liu et al., 1996). The results of the two experiments suggested that supplementing with β-Ala would possibly decrease the N retention and the NUE in rats. In broilers, however, adding β-Ala at 0.5% in diets reduced the feed intake whereas it did not affect the average daily gain (ADG), resulting in an improved feed conversion ratio (Lackner et al., 2021). The results suggested that β-Ala improved the nutrient digestibility and the NUE in broilers. Apparently the results of these experiments on the effects of β-Ala on the nutrient utilization, especially on the N metabolism of animals, are inconsistent. As β-Ala exists in many feeds such as whole corn silage (Bai et al., 2021) and soybeans (Islam et al., 2023) which are the main ingredients of cattle diets, it is necessary to investigate the effects of β-Ala on the nutrient digestibility, NUE, and growth performance in steers and clarify the inconsistency of the results of the previous studies.

The experiment aimed to study the effects of dietary supplementation with β-Ala on the rumen fermentation, nutrient digestibility, and N metabolism in beef steers and verify the mechanisms through sequencing the rumen bacterial community.

2. Materials and methods

2.1. Animal ethics

The procedures of the experiment were approved by China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Inspection Committee (AW82803202-2-1).

2.2. Animals and dietary treatments

Six Simmental steers with an initial liveweight of 252.8 ± 5.2 kg were used as experimental animals. Three levels of β-Ala (Shanghai Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China), i.e. 0, 30 and 60 g/d (equivalent to 0%, 0.73% and 1.45% DM, respectively), were supplemented in a basal diet (Table 1), as experimental treatments. Since no references on the suitable supplementation level of β-Ala in steers are available, the supplemental levels of β-Ala in the present experiment referred to that in broilers (0.5%; Lackner et al., 2021) as the basis. Considering β-Ala could possibly be hydrolyzable in the rumen of steers, the actual supplementation levels were higher than that for broilers. The steers and experimental treatments were assigned in a replicated 3 × 3 Latin square design. Each experimental period included a 15-d adaptation phase and a subsequent 5-d sampling phase.

The animals were tethered and housed in individual pens. Each animal was fed with a total mixed ration (TMR), consisting of 2.35 kg whole corn silage and 1.74 kg of concentrated mixture on dry matter (DM) basis daily. The diet could supply about 90% of the ad libitum DM intake of the animals measured in a pre-trial. No feed residuals were left during the whole experiment. The daily amounts of TMR and β-Ala to each steer were equally divided into two portions and supplied to each steer at 07:00 and 17:00, respectively. β-Ala was mixed well with the TMR before feeding. The animals had free access to fresh drinking water. The body weights of the animals were recorded before morning feeding on d 1 and d 20 of each experimental period.

Table 1
Ingredients and nutritional composition of the basal diet (% DM).

Item	Contents
Ingredients	
Corn silage	57.40
Corn	16.21
Soybean meal	10.57
Wheat bran	13.70
Sodium chloride	1.06
Sodium bicarbonate	1.06
Nutritional composition	
OM	92.90
CP	14.66
NDF	42.45
ADF	20.27
GE, MJ/kg	19.56
NEmf, MJ/kg	6.19

DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; GE = gross energy; NEmf = net energy for maintenance and fattening.
NEmf was calculated based on the dietary GE, OM and NDF contents according to the Nutrient Requirements and Feeding Standards of Beef Cattle (Feng, 2000).

2.3. Sampling

During each sampling period, the total feces from each steer were collected and weighed daily. An amount of 200 g feces was sampled from each steer daily after the feces were homogenized. An aliquot of 10 mL H_2SO_4 (10%, vol/vol) was added to each fecal sample to avoid the N loss. The total urine from each steer was also collected daily using a rubber funnel connected through polyethylene tubing to a plastic bucket in a polystyrene container, surrounded by ice packs. The volume of urine from each steer was recorded daily and well mixed, then 1% of the urine was taken as the sample and added with 10 mL of H_2SO_4 (10%, vol/vol). Feed samples were collected during each sampling period. All samples were kept in a freezer at −20 °C.

On d 3 of each sampling period, the rumen fluid was collected from each animal using an esophagus tube prior to morning feeding. The first tube of rumen fluid was discarded. Then the second tube of rumen fluid of about 100 mL was taken as the sample. The pH of rumen fluid was immediately measured using a pH meter (PHS-3C; Shanghai Yueping Scientific Instrument Co., Ltd., Shanghai, China). Then the rumen fluid was filtered through four layers of surgical gauze and dispensed into three 15-mL centrifuge tubes and two 5-mL cryopreservation tubes. The rumen fluid samples in 15-mL centrifuge tubes were kept in a freezer at −20 °C for analyzing rumen fermentation parameters, and the rumen fluid samples in 5-mL cryopreservation tubes were stored in liquid N for analyzing the rumen bacterial community.

On d 4 of each sampling period before feeding in the morning, 20 mL blood was taken through the jugular vein of each steer using an anticoagulant tube containing sodium heparin (Greiner Bio-one, Germany). The plasma samples were obtained after the blood samples were centrifuged at 3000 × *g* at 4 °C for 15 min. The plasma samples were dispensed into 2-mL cryopreservation tubes and kept in liquid N.

2.4. Chemical analyses

The corn silage and feces samples were freeze-dried for 72 h in a freeze-dryer (LGJ-12; Beijing Songyuan Huaxing Technology Development Co., Ltd., Beijing, China). The dried fecal samples from each steer during each sampling period were compounded to obtain a fecal sample based on the daily output of feces in proportion. The daily urine samples of each steer were directly mixed to obtain a compound sample. The freeze-dried feces samples were crushed with a pestle and a mortar, and the feed samples were ground to pass through a sieve with a pore size of 1 mm.

The DM and crude ash of feeds and feces were analyzed according to the procedures of AOAC (2005) using methods No. 930.15 and 942.05, respectively. The organic matter (OM) content of the feeds and feces was calculated by DM subtracting crude ash content. The total N of feeds, urine, and feces was analyzed using the Kjeldahl method according to AOAC (2005) using method No. 984.13. The neutral detergent fiber (NDF) of feeds and feces were analyzed using the procedures described by Van Soest et al. (1991) with heat-stable α-amylase as well as sodium sulfite and without correction for residual ash as suggested by Mertens et al. (2002). Acid detergent fiber (ADF) was analyzed according to AOAC (2005) using method No. 973.18 on an Ankom A200i fiber analyzer (Ankom Technology, Macedon, NY, USA). The gross energy (GE) of feeds was analyzed on a Parr 6400 automatic oxygen bomb calorimeter (Parr Instrument Company, Moline, IL, USA).

The ammonia nitrogen (NH_3–N) concentration of rumen fluid was analyzed using the colorimetric method of Broderick and Kang (1980). The microbial crude protein (MCP) concentration of rumen fluid was analyzed using the colorimetric method of Makkar (1982) on a spectrophotometer (UV-1801; Beijing Rayleigh Analytical Instruments Co., Ltd., Beijing, China). The concentrations of volatile fatty acids (VFA) of rumen fluid were determined on a gas chromatograph (GC-8600; Beijing Beifen Tianpu Instrument Technology Co., Ltd., Beijing, China) using the procedures described by Yang et al. (2017).

The allantoin and uric acid of urine samples were analyzed based on the methods of Chen and Gomes (1992). The urinary hippuric acid, urea, and creatinine of urine samples were analyzed according to China National Hygiene Standard (WS/T 52-1996), the diacetyl monoxime method (Friedman, 1953), and Jaffe's method (Yatzidis, 1974), respectively, on a spectrophotometer (UV-1801; Beijing Rayleigh Analytical Instruments Co., Ltd.).

The total protein, albumin, triglyceride, urea, total antioxidant capacity (T-AOC), growth hormone (GH), and insulin-like growth factor-1 (IGF-1) of plasma samples were analyzed using commercial kits (Beijing Sinouk Institute of Biological Technology, Beijing, China) on an automatic biochemical analyzer (HY-60021; Beijing Sino-UK Institute of Biological Technology, Beijing, China). The globulin of plasma samples was calculated by total protein minus albumin.

2.5. β-Ala analysis

The β-Ala of urine and rumen fluid were analyzed using the method of Zhang et al. (2008) with HPLC (LC98-1; Beijing Wenfen Analytical Instrument Development Co., Ltd., Beijing, China) using a HyperClone BDS C_{18} column (250 mm × 4.6 mm, 5 μm) with the mobile phase of acetonitrile-0.05 mol/L NaH_2PO_4 solution (20:80, vol/vol; pH 6.5) at a flow rate of 1.0 mL/min. The wave length was 360 nm and the column temperature was 30 °C for detection. The samples of urine and rumen fluid were pretreated as follows. A 10-mL brown volumetric flask was used to mix 1 mL urine or rumen fluid sample, 0.5 mL of NaH_2PO_4 (0.05 M; pH 8.0) and 1 mL 2,4-dinitrofluorobenzene (DNFB) acetonitrile (0.5%, vol/vol). Then, the mixed solution was derivatized for 30 min at 60 °C in a water bath. After the mixture cooled down, ultrapure water was added to the flask to the scale line and mixed well. Then the mixture was centrifuged at 6000 × *g* for 5 min at 4 °C. The supernatant was collected and passed through a filter membrane with a pore size of 0.45 μm for further analysis.

2.6. DNA extraction, PCR amplification, and rumen bacteria sequencing

The Fast DNA SPIN Kit for Soil (MP Biomedicals, Solon, USA) was used to extract the DNA of rumen bacteria. The quality of extracted DNA was assessed using 1% agarose gel. The NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, Wilmington, USA) was applied to determine the concentration and the purity of the extracted DNA.

The hypervariable region V3 to V4 of the bacterial 16S rRNA gene was amplified using the primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR amplification was performed using the ABI GeneAmp 9700 PCR thermocycler (ABI, CA, USA) with TransStart FastPfu DNA Polymerase (AP221-02; TransGen Biotech, Beijing, China), a 20 μL reaction system, 0.8 μmol/L forward and reverse primers, and approximately 10 ng template DNA. The PCR amplification procedures included an initial denaturation at 95 °C for 3 min (1 cycle), then 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. Based on the instructions of Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China), the purified amplicons were pooled equimolarly and subjected to paired-end sequencing on a NovaSeq PE250 platform (Illumina, San Diego, USA).

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered using fastp software (version 0.20.0; Chen et al., 2018), and then merged using FLASH software (version 1.2.7; Magoč and Salzberg, 2011) with the following criterias: (1) Reads with an average quality score less than 20 over a 50-bp sliding window were truncated. The reads shorter than 50 bp after truncation or reads containing ambiguous characters were discarded; (2) Overlapping sequences longer than 10 bp were assembled based on their overlaps, with a maximum mismatch ratio of 0.2 in the overlap region. Reads that failed to assemble were discarded; (3) Samples were identified based on their barcodes and primers, and the sequence direction was adjusted accordingly, allowing for exact barcode matching and a 2 nucleotide mismatch in primer matching.

UPARSE version 7.1 (Edgar, 2013; Stackebrandt and Goebel, 1994) was used to generate the operational taxonomic units (OTU) with a 97% similarity cutoff and the chimeric sequences were removed. The taxonomy of each representative sequence of OTU was determined using RDP Classifier version 2.2 (Wang et al., 2007) against the 16S rRNA database with a confidence threshold of 0.7.

2.7. Calculations and statistical analysis

$$\text{N retention (g/d)} = \text{N intake} - \text{Fecal N} - \text{Urinary N},$$

$$\text{NUE (\%)} = (\text{N retention/N intake}) \times 100.$$

The rumen microbial N (MN) supply was estimated using the daily urinary excretions of total urinary purine derivatives (PD) according to the methods of Chen and Gomes (1992).

$$\text{Urinary PD } (\text{mmol/d}) = 0.85X + (0.385 \times \text{BW}^{0.75}),$$

where X (mmol/d) represents the excretion of absorbed PD; $\text{BW}^{0.75}$ (kg) represents the metabolizable live weight of the steer; 0.85 represents the recovery of absorbed purine as PD in urine; 0.385 (mmol/kg $\text{BW}^{0.75}$ per day) represents the endogenous excretion of PD.

$$\text{Rumen MN supply } (\text{g/d}) = \frac{(X \times 70)}{(0.116 \times 0.83 \times 1000)} = 0.727X,$$

where X (mmol/d) represents the PD absorption; 70 (mg N/mmol) represents the purine N content; 0.116 represents the ratio of purine N to total mixed rumen microbial N; 0.83 represents the rumen microbial purine digestibility.

The data were statistically analyzed using the mixed linear model in SAS 9.4 (SAS Inst. Inc., Cary, NC, USA) as follows:

$$Y_{ijk} = \mu + T_i + P_j + S_k + e_{ijk},$$

where Y_{ijk} is the dependent variable; μ is the overall mean; T_i is the fixed treatment effect; P_j is the period effect; S_k is the steer effect; e_{ijk} is random error.

The linear and quadratic effects were evaluated using the CONTRAST procedure in SAS 9.4. The Duncan method was used for multiple comparisons of the mean values among treatments. $P \leq 0.05$ was declared as significant difference and $0.05 < P \leq 0.10$ as a tendency.

The correlations between the distance-corrected dissimilarity of the domain genera and the nutrient digestibility were evaluated using the Mantel test using the linkET package in R (version 3.5.1).

3. Results

3.1. Rumen fermentation parameters

Table 2 indicates that supplementing with β-Ala increased the ruminal concentrations of MCP ($P = 0.005$) and β-Ala ($P = 0.003$). No significant differences were found in the ruminal pH, the concentrations of total VFA and individual VFA, the ratio of acetate to propionate, and the NH_3–N concentration ($P > 0.10$).

Table 2
Effects of β-Ala on rumen fermentation in beef steers.

Items	β-Ala supplemented, g/d			SEM	*P*-value		
	0	30	60		T	L	Q
pH	6.48	6.46	6.46	0.034	0.907	0.713	0.822
MCP, mg/mL	0.19[b]	0.25[a]	0.26[a]	0.015	0.005	0.002	0.121
VFA, mmol/L							
Total VFA	58.08	53.08	56.58	1.981	0.278	0.629	0.133
Acetate	39.82	36.80	38.31	1.292	0.358	0.468	0.219
Propionate	14.26	12.59	14.48	0.661	0.067	0.777	0.025
Iso-butyrate	1.33	1.22	1.24	0.045	0.271	0.192	0.336
Butyrate	1.52	1.46	1.47	0.191	0.977	0.861	0.905
Iso-valerate	0.53	0.50	0.47	0.024	0.286	0.125	0.806
Valerate	0.60	0.59	0.55	0.045	0.686	0.409	0.843
A/P	2.79	2.79	2.75	0.113	0.381	0.767	0.187
NH_3–N, mg/dL	7.83	8.88	8.57	0.404	0.143	0.164	0.139
β-Ala, mg/L	1.03[b]	1.77[a]	2.28[a]	0.187	0.003	0.001	0.553

β-Ala = β-alanine; SEM = standard error of the mean; T = treatment; L = linear; Q = quadratic; MCP = microbial crude protein; VFA = volatile fatty acids; NH_3–N = ammonia nitrogen; A/P = ratio of acetate to propionate.
[a,b]Within a row, means without a common superscript differ ($P \leq 0.05$).

Table 3
Effects of β-Ala on apparent nutrient digestibility in beef steers.

Items, %	β-Ala supplemented, g/d			SEM	*P*-value		
	0	30	60		T	L	Q
DM	67.51[b]	68.41[ab]	69.71[a]	0.788	0.025	0.009	0.720
OM	68.82[b]	69.64[ab]	70.84[a]	0.827	0.047	0.017	0.749
CP	69.16	71.76	72.82	1.318	0.102	0.043	0.564
NDF	59.97	60.26	60.92	1.352	0.885	0.641	0.918
ADF	46.94	44.93	44.27	1.039	0.169	0.077	0.568

β-Ala = β-alanine; SEM = standard error of the mean; T = treatment; L = linear; Q = quadratic; DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber.
[a,b]Within a row, means without a common superscript differ ($P \leq 0.05$).

3.2. Apparent nutrient digestibility

Table 3 shows that supplementing with β-Ala linearly increased the DM ($P = 0.009$), OM ($P = 0.017$), and CP ($P = 0.043$) digestibility, tended to decrease the ADF digestibility ($P = 0.077$), but it did not affect the NDF digestibility ($P = 0.641$).

3.3. N balance, urine nitrogenous compounds and ADG

Table 4 indicates that supplementing with β-Ala linearly increased the urinary urea excretion ($P = 0.006$), the ratios of urinary urea N to N intake ($P = 0.015$) and urinary urea N to total N excretion ($P = 0.002$). β-Ala did not affect the urinary excretions of uric acid ($P = 0.656$), allantoin ($P = 0.453$), and hippuric acid ($P = 0.839$), but linearly decreased the ratios of uric acid N ($P = 0.016$), allantoin N ($P = 0.049$), and hippuric acid N ($P = 0.007$) to total urinary N and tended to decrease the ratio of creatinine N to urinary N ($P = 0.077$). β-Ala also linearly increased the urinary excretion of the PD ($P = 0.001$) and consequently linearly increased the estimated rumen MN supply ($P = 0.048$). β-Ala was undetectable in the urine samples.

Table 5 shows that supplementing with β-Ala linearly decreased the fecal N excretion ($P = 0.019$) and increased the urinary N excretion ($P = 0.006$) and the total N excretion ($P = 0.019$). It also decreased the ratio of fecal N to urinary N ($P = 0.007$) and increased the N retention ($P = 0.004$) in a linear manner. However, β-Ala did not affect the NUE ($P = 0.277$) and the ADG ($P = 0.430$).

3.4. Plasma biochemical parameters

Supplementing with β-Ala did not affect the total protein ($P = 0.446$), globulin ($P = 0.567$), and triglyceride ($P = 0.408$) concentrations in plasma, but linearly increased the urea ($P = 0.018$) and IGF-1 ($P < 0.001$) concentrations and linearly tended to increase the GH ($P = 0.084$) and quadratically tended to increase the albumin ($P = 0.058$) and T-AOC concentrations ($P = 0.011$) in plasma (Table 6).

3.5. Richness and diversity of the rumen bacterial community

A total of 1,315,676 clean reads were detected with over 73,093 for each sample. The Chao1 and Shannon indices were used to generate the rarefaction curves to assess the adequacy of the sampling depth. The rarefaction curves indicated a diminishing rate of clean reads identification as the number of reads per sample increased. With all rarefaction curves of clean reads tending to plateau at 45,000 tags, the sequencing depth and the amount were saturated (Figs. S1A and B). Similarly, the Good's coverage of all samples exceeded 98%, indicating the high accuracy and

Table 4
Effects of β-Ala on the urine composition in beef steers.

Items	β-Ala supplemented, g/d			SEM	*P*-value		
	0	30	60		T	L	Q
Urine output, L/d	8.19	8.01	9.13	0.689	0.179	0.142	0.232
Urinary N concentration, g/L	4.10	4.66	4.85	0.364	0.206	0.096	0.606
Urinary urea, mmol/d	820.55^{b}	900.97^{b}	1087.50^{a}	56.063	0.006	0.002	0.328
Urea-N/urinary N, %	72.83	70.24	72.14	2.107	0.489	0.755	0.262
Urea-N/N intake, %	23.93^{b}	25.08^{ab}	28.90^{a}	1.536	0.035	0.015	0.363
Urea-N/total N excretion, %	36.66^{b}	39.93^{ab}	44.48^{a}	1.857	0.006	0.002	0.668
Allantoin, mmol/d	71.95	70.34	79.02	4.603	0.453	0.343	0.420
Allantoin-N/urinary N, %	13.15	11.10	10.65	0.987	0.102	0.049	0.405
Uric acid, mmol/d	8.52	7.92	8.41	0.858	0.656	0.876	0.383
Uric acid-N/urinary N, %	1.50^{a}	1.22^{ab}	1.13^{b}	0.127	0.038	0.016	0.375
Creatinine, mmol/d	119.71	120.81	131.65	10.015	0.588	0.351	0.655
Creatinine-N/urinary N, %	16.01	13.93	13.86	1.271	0.130	0.077	0.301
Hippuric acid, mmol/d	60.77	57.61	55.83	5.430	0.839	0.567	0.925
Hippuric acid-N/urinary N, %	2.75^{a}	2.28^{ab}	1.76^{b}	0.194	0.024	0.007	0.936
Urinary PD, mmol/d	68.20^{b}	79.87^{ab}	90.85^{a}	2.976	0.004	0.001	0.932
Estimated MN supply, g/d	27.99	33.49	47.80	5.530	0.120	0.048	0.601
β-Ala	ND	ND	ND	–	–	–	–

β-Ala = β-alanine; SEM = standard error of the mean; T = treatment; L = linear; Q = quadratic; N = nitrogen; PD = purine derivatives; MN = microbial N; ND = undetectable.
[a,b]Within a row, means without a common superscript differ ($P \leq 0.05$).

Table 5
Effect of β-Ala on the N balance and the ADG in beef steers.

Items	β-Ala supplemented, g/d			SEM	*P*-value		
	0	30	60		T	L	Q
DMI, kg/d	4.09	4.09	4.09	–	–	–	–
N intake, g/d	95.96	100.68	105.40	–	–	–	–
Fecal N, g/d	34.03	32.35	28.89	1.414	0.055	0.019	0.576
Urinary N, g/d	32.93^{b}	35.99^{ab}	42.26^{a}	2.026	0.016	0.006	0.472
Total N excretion, g/d	66.87	68.69	70.90	0.960	0.055	0.019	0.879
Fecal N/urinary N	1.06^{a}	0.93^{ab}	0.69^{b}	0.083	0.018	0.007	0.531
Urinary N/N intake, %	34.31	35.76	40.11	1.994	0.101	0.044	0.508
N retention, g/d	29.09^{b}	31.99^{ab}	34.50^{a}	0.960	0.012	0.004	0.879
NUE, %	30.32	31.76	32.74	0.949	0.277	0.120	0.852
ADG, kg/d	0.89	0.84	0.76	0.057	0.430	0.204	0.838

β-Ala = β-alanine; N = nitrogen; ADG = average daily gain; SEM = standard error of the mean; T = treatment; L = linear; Q = quadratic; DMI = dry matter intake; NUE = nitrogen utiliation efficiency.
[a,b]Within a row, means without a common superscript differ ($P \leq 0.05$).

reproducibility of the sequencing. The indices of Chao1 and Shannon were used to present the richness and the diversity of bacteria (Table S1). The nonmetric multidimensional scaling analysis (NMDS) plots were performed based on the Bray Curtis distance. The clearly clustered bacterial communities indicated distinct structures among treatments (Fig. S2).

3.6. Composition of the rumen bacterial community

The taxonomic analysis of the reads revealed that there were more than 9 bacterial phyla in the rumen fluid. The dominant phyla were Bacteroidota and Firmicutes, accounting for 55.03% and 38.75% of total reads, respectively (Fig. 1A). Among these phyla, supplementing with β-Ala quadratically increased the relative abundance of Bacteroidota ($P = 0.021$) (Table 7). At the genus level, 9 genera with high relative abundance were identified in the rumen fluid. The top genera were *Prevotella* (18.85%), *Rikenellaceae_RC9_gut_group* (15.16%), *norank_f__F082* (5.66%), and *Ruminococcus* (5.29%) (Fig. 1B). Table 7 shows that β-Ala exerted a pronounced impact on the dominant bacteria at the genus level, leading to a quadratic change in the relative abundance of *Prevotella* ($P = 0.028$), *NK4A214_group* ($P = 0.009$), and *Prevotellaceae_UCG-003* ($P = 0.014$).

3.7. Biomarker taxa of the rumen bacterial community

Linear discriminant analysis (LDA) effect size (LEfSe) was used to integrate the rank sum tests with taxonomic data, allowing for the identification of biomarker taxa (with a logarithmic LDA score ≥ 3.0) that significantly influenced the structure of the rumen bacterial community. The LDA histogram (Fig. 2) indicates the presence of a total of 20 biomarkers from the phylum level to

Table 6
Effect of β-Ala on the plasma biochemical indexes in beef steers.

Items	β-Ala supplemented, g/d			SEM	*P*-value		
	0	30	60		T	L	Q
Total protein, g/L	39.33	45.09	39.15	3.061	0.446	0.970	0.214
Globulin, g/L	22.38	25.07	22.42	1.754	0.567	0.990	0.300
Albumin, g/L	16.50	20.93	16.84	1.551	0.151	0.884	0.058
Triglyceride, mmol/L	0.15	0.16	0.13	0.019	0.408	0.478	0.263
Urea, mmol/L	1.94^{b}	2.23^{ab}	2.43^{a}	0.213	0.046	0.018	0.762
GH, ng/mL	4.55	5.18	5.52	0.324	0.203	0.084	0.751
IGF-1, ng/mL	174.70^{b}	181.20^{b}	183.90^{a}	2.094	<0.001	<0.001	0.138
T-AOC, U/mL	6.71^{b}	7.93^{a}	7.02^{ab}	0.255	0.029	0.407	0.011

β-Ala = β-alanine; SEM = standard error of the mean; T = treatment; L = linear; Q = quadratic; GH = growth hormone; IGF-1 = insulin-like growth factor-1; T-AOC = total antioxidant capacity.
[a,b]Within a row, means without a common superscript differ ($P \leq 0.05$).

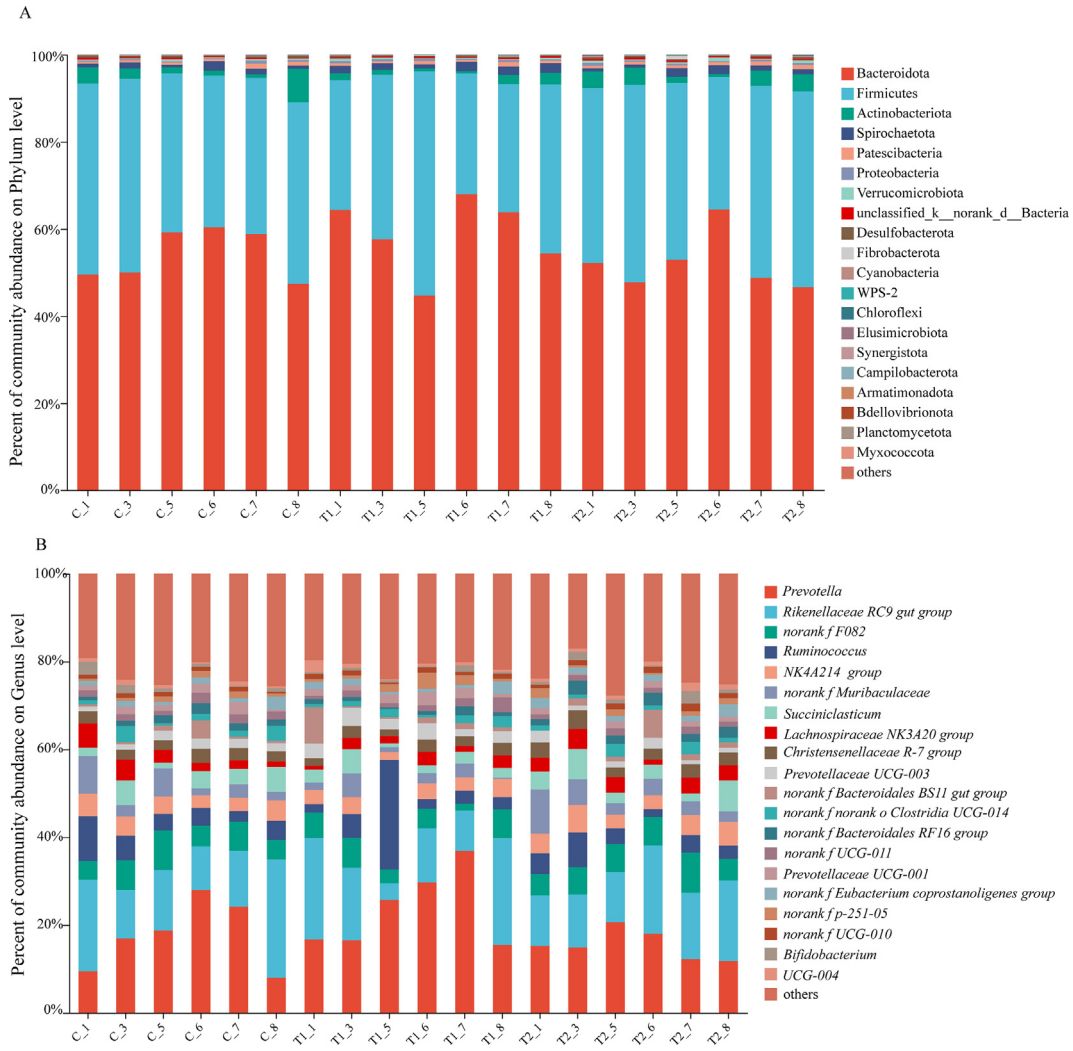

Fig. 1. The ruminal bacterial community composition as affected by β-alanine at (A) the phylum level and (B) the genus level. Different colors of bars represent different species, while the length of the bars represents the relative abundance of each species. Groups C, T1, and T2 represent supplementing with 0, 30, and 60 g β-alanine/d, respectively.

Table 7
Effects of β-Ala on the relative abundances of the rumen bacterial community in beef steers.

Items	β-Ala supplemented, g/d			SEM	*P*-value		
	0	30	60		T	L	Q
Phylum							
Bacteroidota	53.63	59.30	52.16	2.612	0.056	0.578	0.021
Firmicutes	40.06	35.43	40.75	2.414	0.126	0.781	0.051
Actinobacteriota	2.89	1.37	2.92	0.231	0.220	0.970	0.090
Spirochaetota	1.17	1.75	1.30	0.249	0.311	0.729	0.145
Patescibacteria	0.66	0.64	0.71	0.089	0.622	0.550	0.455
Proteobacteria	0.36	0.43	0.40	0.071	0.740	0.664	0.534
Verrucomicrobiota	0.31	0.31	0.47	0.076	0.182	0.294	0.242
Desulfobacterota	0.35	0.26	0.32	0.039	0.268	0.508	0.143
Fibrobacterota	0.04	0.04	0.14	0.066	0.066	0.276	0.684
Genus							
Prevotella	17.92	23.14	15.49	2.756	0.064	0.403	0.028
Rikenellaceae_RC9_gut_group	15.04	15.19	15.27	2.391	0.995	0.925	0.990
Norank_f__F082	6.03	4.60	6.35	0.860	0.408	0.815	0.197
Ruminococcus	4.84	6.78	4.26	2.465	0.791	0.882	0.518
NK4A214_group	4.01^{ab}	3.26^{b}	4.52^{a}	0.389	0.018	0.167	0.009
Succiniclasticum	3.75	2.72	4.07	0.790	0.147	0.625	0.063
Lachnospiraceae_NK3A20_group	3.04	2.01	3.31	0.462	0.162	0.682	0.068
Christensenellaceae_R-7_group	2.61	2.27	3.04	0.214	0.080	0.179	0.057
Prevotellaceae_UCG-003	1.79^{ab}	3.00^{a}	1.62^{b}	0.316	0.038	0.713	0.014

β-Ala = β-alanine; SEM = standard error of the mean; T = treatment; L = linear; Q = quadratic.
^{a,b}Within a row, means without a common superscript differ ($P \leq 0.05$).

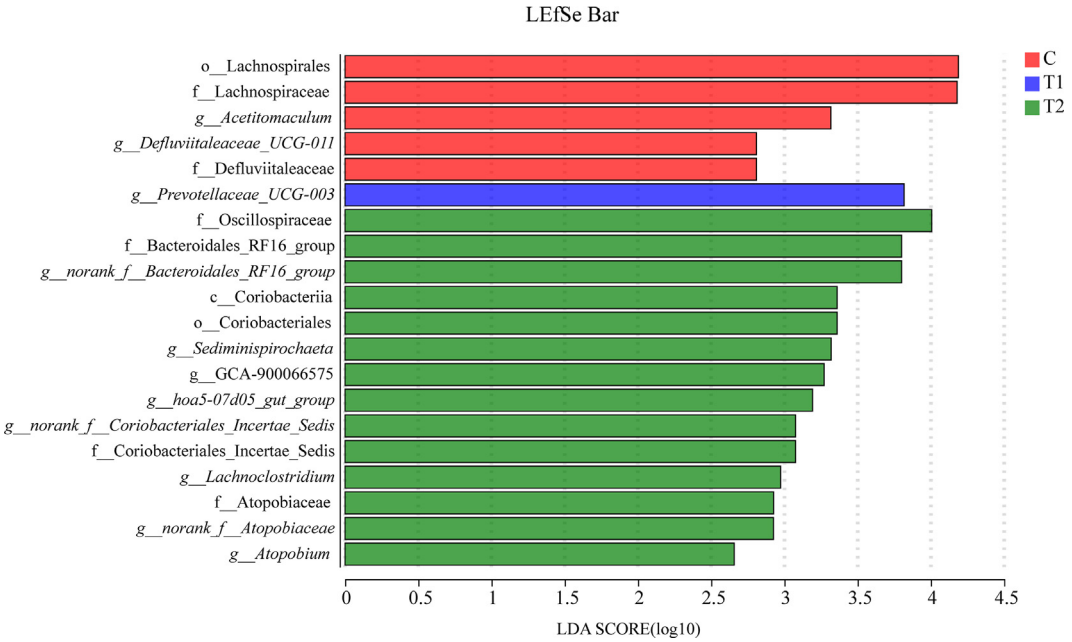

Fig. 2. The linear discriminant analysis effect size (LEfSe) analysis of differential ruminal bacteria as affected by β-alanine. Linear discriminant analysis (LDA) bars represent the effect of the relative abundance of each species on the differentiation among different groups. Groups C, T1, and T2 represent supplementing with 0, 30, and 60 g β-alanine/d, respectively.

the genus level, of which 5, 1 and 14 biomarkers were detected for the groups supplementing with 0, 30 and 60 g β-Ala/d, respectively, as follows: o__Lachnospirales, f__Lachnospiraceae, *g__Acetitomaculum*, *g__Defluviitaleaceae_UCG-011*, f__Defluviitaleaceae; *g__Prevotellaceae_UCG-003*; f__Oscillospiraceae, f__Bacteroidales_RF16_group, *g__norank_f__Bacteroidales_RF16_group*, c__Coriobacteriia, o__Coriobacteriales, *g__Sediminispirochaeta*, *g__GCA-900066575*, *g__hoa5-07d05_gut_group*, *g__norank_f__Coriobacteriales_Incertae_Sedis*, f__Coriobacteriales_Incertae_Sedis, *g__Lachnoclostridium*, f__Atopobiaceae, *g__norank_f__Atopobiaceae*, *g__Atopobium*, where prefix c represents the Class, o represents the Order, f represents the Family, and *g* represents the Genus.

3.8. Correlations between the bacterial genera and the nutrient digestibility

The Mantel test shows that positive correlations were found between the relative abundance of *Prevotellaceae UCG-003* and the DM digestibility ($P < 0.05$, $0.1 < r < 0.3$), the OM digestibility ($P < 0.05$, $0.1 < r < 0.3$), and the CP digestibility ($P < 0.05$, $r > 0.3$). A positive correlation was also found between the relative abundance of *Prevotella* and the CP digestibility ($P < 0.05$, $r > 0.3$) (Fig. 3).

4. Discussion

In ruminants, dietary CP can be extensively degraded into peptides, AA, and NH_3 by ruminal microorganisms. Ruminal NH_3–N can be used for MCP synthesis, absorbed into blood and moved into the lower digestive tract with digesta. As a free AA, L-Ala is highly hydrolyzable in rumen fermentation (Sok et al., 2017). Since β-Ala and L-Ala are only slightly different in molecular structure, it could be speculated that β-Ala could be highly hydrolyzed by ruminal microorganisms to NH_3 and CO_2. However, the results of the present experiment showed that supplementing with β-Ala did not affect the ruminal concentration of NH_3–N. One reason for the results could be that the rumen fluid was taken before morning feeding while another reason could be that part of the NH_3–N from β-Ala was utilized for ruminal MCP synthesis. The results of the present experiment also showed that supplementing with β-Ala increased the ruminal MCP concentration and MN supply predicted based on the urinary excretion of PD. Since β-Ala is a non-proteinogenic AA, the increased ruminal MCP should be synthesized through utilizing the NH_3–N released from β-Ala.

In ruminants, the absorbed NH_3 from the rumen into the blood can be used for urea synthesis in the liver (Reynolds and Kristensen, 2008). Part of the urea in the blood can go back into the rumen through saliva secretion and across the rumen epithelium, while part of the urea can be excreted into urine (Zhao, 2019). Urinary urea excretion is positively correlated with plasma urea concentration (Calsamiglia et al., 2010) and urinary urea accounts for a major part of the urinary nitrogenous compounds in cattle (Gao et al., 2021). The results of the present experiment showed that supplementing with β-Ala increased the plasma urea concentration and urinary excretion of urea, suggesting that part of the NH_3 degraded from β-Ala was absorbed into the blood and utilized for urea synthesis.

The results of the present experiment also showed that dietary supplementation with β-Ala linearly decreased the fecal N excretion and increased the urinary N excretion. The decreased fecal N excretion could be resulted from the increased rumen MCP synthesis because rumen MCP has a more balanced AA composition and can be more easily digestible and utilizable than feed CP (Storm et al., 1983b; Storm and Ørskov, 1983a) while the increased urinary N excretion could be due to the increased urinary excretion of urea.

Qi et al. (2018) reported that dietary supplementation with 1000 mg/kg β-Ala for broilers increased the ADG and elevated the β-Ala content in breast muscle. Wang et al. (2022) reported that dietary addition of β-Ala at 600 mg/kg increased the lean meat percentage and decreased the backfat thickness in finishing pigs. Although the results of the two studies suggested that β-Ala had beneficial effects on the growth performance of broilers and pigs, no direct evidence was available on the impacts of β-Ala on the N retention of broilers and pigs. The results of the present experiment

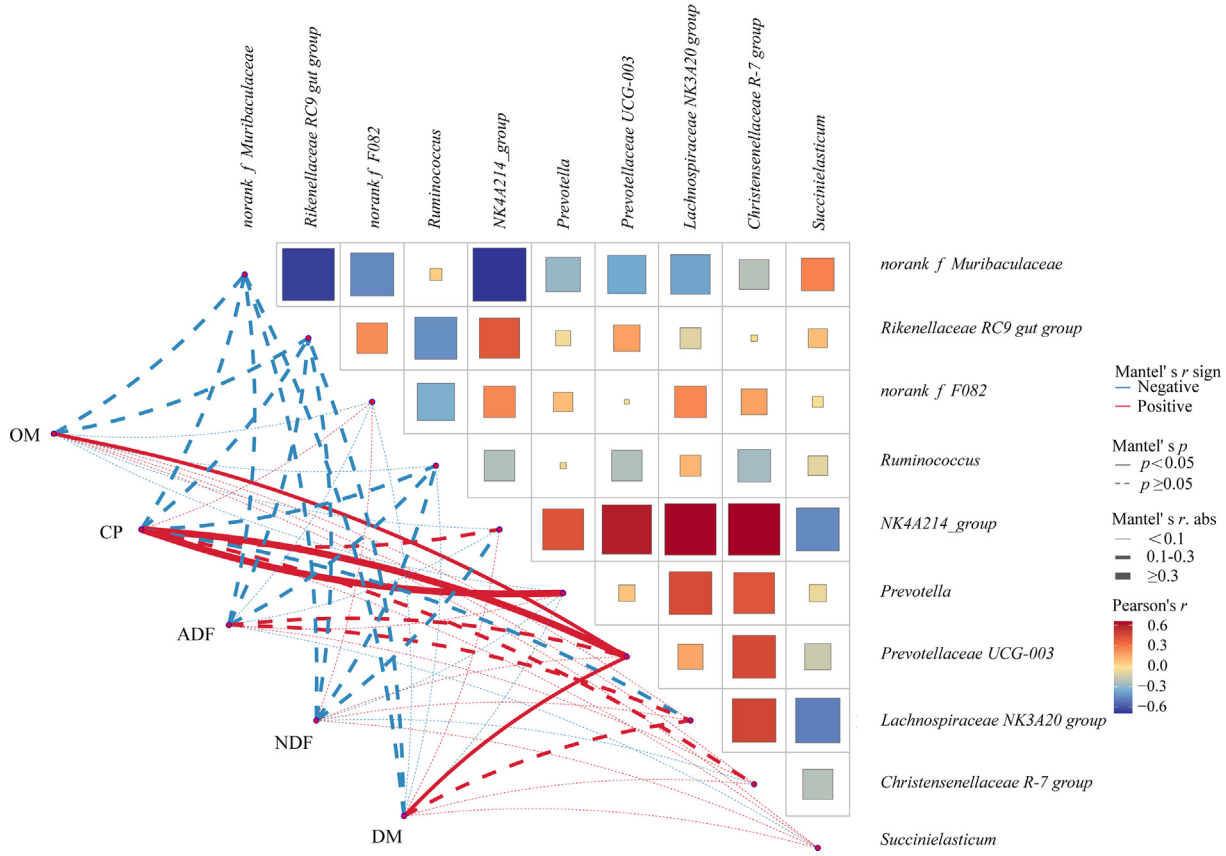

Fig. 3. The correlations between the relative abundances of rumen bacterial genera and the apparent nutrient digestibility. OM = organic matter; CP = crude protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; DM = dry matter.

showed that adding β-Ala at 30 and 60 g/d increased the N intakes of the two treatments by 4.92% and 9.84%, respectively, whereas it increased the N retention by 9.97% and 19.94%, respectively. Apparently, the increased N retention rates were higher than the increased N intake rates. The results suggested that β-Ala not only could be used as an N source but also as a potential promoter for N utilization. It should be noted, however, that supplementing with β-Ala did not affect the NUE in steers. The reason for the results could be that a considerable part of β-Ala was hydrolyzed in rumen fermentation. Feeding experiments with higher level of β-Ala or rumen-protected β-Ala need to be conducted to verify the effects.

The present experiment showed that supplementing with β-Ala did not affect the richness and the diversity (Chao1 and Shannon index) of the rumen bacterial community in steers. At the phylum level, the dominant ruminal bacteria were found to be Bacteroidota and Firmicutes. These results were consistent with Zeineldin et al. (2018) who indicated that the predominant phyla present in the rumen are Firmicutes and Bacteroidetes. Supplementing with β-Ala quadratically increased the relative abundance of rumen Bacteroidota and linearly increased the CP digestibility. Since the phylum Bacteroidota were able to improve feed protein degradation by producing aminopeptidases in the rumen (Hernandez et al., 2022), the linearly increased nutrient digestibility of DM, OM, and CP could be partly attributed to the increased relative abundance of Bacteroidota.

At the genus level, *Prevotella* are the most abundant ruminal bacteria in the Bacteroidetes group. The results of the present experiment showed that the main ruminal bacteria were *Prevotella* and *Rikenellaceae_RC9_gut_group* for all groups and supplementing with β-Ala quadratically increased the relative abundances of *Prevotella* and *Prevotellaceae_UCG-003* and reduced the relative abundance of *NK4A214_group*. Since *Prevotella* are able to improve the feed protein degradation in rumen fermentation (Liu et al., 2019), the increased CP digestibility should be partly due to the increased relative abundance of *Prevotella* and *Prevotellaceae_UCG-003* by the impact of β-Ala. The speculation was confirmed by the Mantel test analysis which showed that the digestibility of DM, OM or CP was positively correlated with the relative abundance of *Prevotella* and *Prevotellaceae_UCG-003* in the rumen.

In pigs, it was reported that *Ruminococcaceae_NK4A214_group* are the predominant genera of gut microbiota to improve the apparent total tract digestibility of ADF (Niu et al., 2022). The results of the present experiment showed that supplementing with β-Ala decreased the relative abundance of ruminal *NK4A214_group* and tended to decrease the ADF digestibility. The impact of β-Ala on decreasing the relative abundance of *NK4A214_group* could be one of the reasons for the tendency to decrease the ADF digestibility.

In the present experiment, supplementing with β-Ala increased the plasma concentration of IGF-1. The results are consistent with Pence et al. (2016) who reported that feeding 17-month-old mice with 3.43 mg/kg β-Ala for 41 d increased the plasma concentrations of IGF-1. However, the mechanism by which β-Ala stimulates the production of IGF-1 remains unclear.

β-Ala can be used for carnosine synthesis in many species of animals such as poultry, pigs and rats (Boldyrev et al., 2013). One of the important functions of carnosine is the antioxidative effect (Wu, 2020). It was reported that supplementing with β-Ala at 600 mg/kg to weaned piglets increased the superoxide dismutase (SOD) activity in the jejunum (Chen et al., 2023), and feeding 1200 mg/kg of β-Ala to 1-d-old pullets for 42 d increased the T-AOC content in

the breast muscle (Qi et al., 2018). The present experiment showed that supplementing steers with β-Ala quadratically increased the plasma concentration of T-AOC, suggesting that part of the absorbed β-Ala was used for carnosine synthesis, resulting in the increased plasma T-AOC concentration.

5. Conclusion

Dietary supplementation with β-Ala linearly increased the DM, OM and CP digestibility but tended to decrease the ADF digestibility. Supplementing with β-Ala also linearly increased MN supply and improved the N retention but did not affect the NUE in beef steers. β-Ala could be highly hydrolyzable in rumen fermentation, but further research is needed to verify this speculation.

Author contributions

Jinming Hu: Methodology, Investigation, Formal analysis, Visualization, Writing-Original. **Shuo Zhang:** Methodology, Investigation, Data curation, Visualization, Writing-Original. **Mengmeng Li:** Writing – Review & Editing. **Guangyong Zhao:** Conceptualization, Methodology, Supervision, Writing – Review & Editing, Project administration.

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.2024.02.006.

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