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Protease or *Clostridium butyricum* addition to a low-protein diet improves broiler growth performance

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Abstract

Low-protein (LP) feeds are used in the poultry industry to combat the increasing consumption of protein resources and reduce environmental pollution caused by excessive nitrogen excretion. Dietary supplementation of protease or *Clostridium butyricum* increases the growth performance of broilers; however, it is unclear whether they counteract the negative effects of LP diets. The effects of protease and *C. butyricum* on growth performance, intestinal morphology, anti-oxidant capacity, anti-inflammatory response, and microbial community of broilers have not been studied extensively. Here, 450 healthy 1-day-old Cobb500 broilers were allocated to five groups, according to different diets: basal diet (Control); LP diet (LP; 2% less crude protein than the control); LP diet + 200 g/t HuPro protease (LPH); LP diet + 1.0×10^9 CFU/t *C. butyricum* (LPC); and basal diet + 200 g/t oxytetracycline (Antibiotic). Supplementing both *C. butyricum* and protease improved the growth performance of broilers. The supplementation of HuPro protease under low-protein conditions could achieve a breeding effect similar to that of the positive control (Antibiotic). Supplementing *C. butyricum* could maintain intestinal barrier function, alleviate the inflammatory response, and increase ileal and cecal short-chain fatty acid concentrations. Both *C. butyricum* and protease altered the bacterial diversity in the cecum, increased *Bacteroidetes* abundance, and resulted in higher abundance of *Rikenellaceae RC9 gut* spp. and lower abundance of *Alistipes* spp. in broilers. This study demonstrates the positive effects of proteases and *C. butyricum* on broilers and serves as a reference for the selection of appropriate supplementation for broilers in the poultry industry.

Key points

- *Low-protein diet had a negative effect on growth performance of broilers.*
- *Protease significantly reduced feed conversion rate.*
- *Clostridium butyricum had positive effects on broilers.*

Keywords Low-protein feed · Broiler · *Clostridium butyricum* · Protease · Growth performance · Intestinal health

Introduction

According to the OECD-FAO Agricultural Outlook 2020–2029, global livestock production is expected to increase by 14% from 2020 to 2029, during which the demand for poultry will grow rapidly (Bearak et al. 2019). This anticipated expansion will necessitate the supply of more raw ingredients (cereal grains, animal proteins, vitamins, and minerals) in poultry feed (Lee et al. 2020). However, limited arable land and slow production of feed materials imply that the protein needs of livestock can no longer be met. Therefore, lowering dietary crude protein (CP) levels in broiler chicken feed has become a research hotspot in the field of animal nutrition. This low-protein (LP) diet can reduce environmental pollution caused by the

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rapid development of animal husbandry because it reduces the excretion of nitrogen and phosphorus in feces and urine (Ma et al. 2021; Wang et al. 2018). However, previous studies have shown that nutrient deficiencies may retard growth performance and lead to metabolic disorders in broilers (Boontiam et al. 2019); therefore, it is necessary to identify feed additives that can improve nutrient availability in feed to overcome this negative effect. Antibiotics have long been used as feed additives to promote growth and prevent common diseases, but the abuse of antibiotics is getting worse. Numerous studies have confirmed that the abuse of antibiotics in animal husbandry exacerbates the problem of bacterial resistance, endangering the health of animals and humans and seriously damaging the ecological environment (Blaser 2016; Zhou et al. 2021). Several countries, such as those in the European Union and China, have issued bans on adding antibiotics to animal feed as growth promoters (Zhou et al. 2021). To avoid the negative consequences of such bans on antibiotics and eliminate the negative effects of LP feed, it is critical to develop new feed additives other than antibiotics that may prevent infections and stimulate the rapid growth of animals with no residues or toxic adverse effects.

Moreover, probiotics are potential feed supplements to inhibit pathogenic microorganisms, regulate the gastrointestinal microbiome of the host, and stimulate growth (Wang et al. 2019; Zhang et al. 2016; Cao et al. 2019; Gaggia et al. 2010). *Clostridium butyricum* is a butyric acid-producing, spore-forming, gram-positive anaerobe found in the soil, and animal droppings (Zhao et al. 2013). It also has biological properties that make it a suitable feed additive, such as high-temperature resistance, acid resistance, and antibiotic sensitivity (Zhang et al. 2011, 2016). In addition, *C. butyricum* can produce short-chain fatty acids (SCFAs), which have been used in the feed and pharmaceutical sectors to prevent or cure intestinal disorders in animals (Wang et al. 2020; Li et al. 2018). Previous studies have demonstrated that feed supplemented with *C. butyricum* can boost growth performance, balance intestinal flora, and promote immune responses in broilers (Cassir et al. 2016; Huang et al. 2019; Han et al. 2018). Butyric acid is the most important metabolite of *C. butyricum* and endows *C. butyricum* with antioxidant effects on the colonic mucosa (Hamer et al. 2009; Ohsawa et al. 2007; Huang et al. 2010). However, there are only few reports on the effects of *C. butyricum* on anti-oxidant activity in broilers.

Similarly, enzymes can be used as feed additives to counteract the negative effects of LP. There is a high demand for nutrients in chicks because they have incomplete enzyme systems and insufficient enzyme activities, and exogenous proteases can improve the digestibility of proteins and promote growth. In addition, protease supplementation can increase endogenous peptidase production and cleave protein-based antinutritional factors (Isaksen et al. 2010). In recent years, studies have been published on the effects of enzyme mixtures, including proteases, on the growth performance of broilers (Adeola and Cowieson 2011; Diarra and Anand 2020); however, only a few studies have been conducted on the use of mono-component proteases in poultry diets. As mono-component proteases directly affect the usage of compound enzymes, it is necessary to investigate the mechanisms and effects of monomer enzymes more clearly.

It is well known that the gut microbiome has a profound effect on livestock production. The microbiome of the cecum is diverse and has recently attracted the attention of researchers (Gong et al. 2002). A substantial body of evidence indicate diet has a substantial effect on the gut microbiome of broilers (Waite and Taylor 2014; Li et al. 2021). Therefore, it is reasonable to hypothesize that the addition of protease or *C. butyricum* can reduce CP levels without negatively affecting the growth performance of broilers and can also affect the intestinal flora of broilers. However, there are no comprehensive studies on the effects of *C. butyricum* or protease on broilers fed LP diets. Therefore, for this study, we designated a group with a normal protein diet supplemented with antibiotics as the positive control (i.e., the normal diet before the antibiotic ban), whereas the experimental group was supplemented with protease or *C. butyricum* under the conditions of an LP diet, saving breeding costs. By using these additives, we hope to achieve the same effect as the positive control group. Specifically, we aimed to compare functional differences in growth performance; serum biochemical parameters; intestinal barrier function; immune response; gut microbiota; SCFAs in the ileum and cecum; anti-oxidant activity; and histomorphology of the jejunum, ileum, and cecum of broilers. This study may provide insights and a basis for selecting LP feed additives in the future.

Materials and methods

Ethics statement

All experiments were approved by the Experimental Animal Center of the Huazhong Agricultural University (HZAUCH-2019–008) and conducted according to its ethical standards. All efforts were made to minimize animal suffering.

Animals and experimental design

The feeding experiment was conducted at Huanghu Breeding Base of Xinhua Yang Biological Co., Ltd. (Huanggang, China), from October 2019 to January 2020. A total of 450 healthy 1-day-old Cobb500 broilers purchased from Xiangda Agriculture and Animal Husbandry Co., Ltd. (Yicheng,

China) were used in a feeding trial of 6 weeks. All broilers were randomly allocated to five treatment groups, with six replicates per treatment and 15 birds per replicate.

A basal diet was formulated based on corn and soybean meal to meet or exceed the nutritional requirements recommended by the National Research Council (1994), and details about the ingredients and nutritional content of the basal diet are shown in Supplementary Table S1. The dietary treatments (Table 1) were (1) basal diet (Control); (2) LP diet (the experimental group with 2% less CP than the control [LP]); (3) LP diet supplemented with 200 g/t HuPro protease (LPH); (4) LP diet supplemented with 1.0×10^9 colony forming units (CFU)/t *C. butyricum* [CGMCC1.5205] (LPC); and (5) basal diet with 200 g/t oxytetracycline (Antibiotic). The control diets had a 22% and 20% CP content in the starter (days 1 to 21) and grower (days 22 to 42) phases, respectively. HuPro protease and *C. butyricum* supplement were provided by Xinhua Yang Biological Co., Ltd. (Wuhan, China). *C. butyricum* powder was obtained after liquid fermentation and spray drying, and the final number of live bacteria in the powder was 1.0×10^9 CFU/t. The protease is the exclusive patented product HuPro20 (CN103497943B), of Xinhua Yang Biological Co., Ltd.; it has a neutral protease with an effective enzyme activity of 20,000 IU/g.

The broilers were raised on the ground in an enclosed, ventilated house at 35 °C in the first week, after which, the temperature was gradually decreased and maintained at 25 °C. Before the experiment, the equipment and coop were thoroughly cleaned and disinfected by fumigation with potassium permanganate and formaldehyde. Immunization, disinfection, deworming procedures, and feeding schedule were similar to those in the commercial settings of Huanghu Breeding Base. All birds had unrestricted access to water and mash feed throughout the experimental period.

Table 1 Groups and diets

Groups	Diets
Control	Basal diet
LP	Basal diet − 2% protein
LPH	Basic diet − 2% protein + HuPro protease (200 g/t)
LPC	Basic diet − 2% protein + *C. butyricum* (1.0×10^9 CFU/t)
Antibiotic	Basal diet + oxytetracycline (200 g/t)

Sample collection and preparation

Twelve broilers (two from each replicate) were selected randomly from each treatment group for sampling on the morning of days 1, 22, and 43 of the experiment (after being fasted for 12 h and without water for 2 h). After weighing, blood samples were collected via venipuncture, and then hemolysis-free serum was obtained via centrifugation at $3000\times g$ for 15 min and stored at 4 °C until analyses of immune function and anti-oxidants. Finally, the jejunum, ileum, and cecum were separated from the abdominal cavity after the birds were sacrificed. Samples of the jejunum, ileum, and cecum (approximately 1–2 cm from the midpoint) were fixed in 4% paraformaldehyde for tissue section preparation and intestinal morphology examination. The jejunum was cut and rinsed with sterile physiological saline. The contents of the cecum and jejunal mucosa were extruded and scraped into 2-mL aseptic centrifuge tubes, separately. Thereafter, for 16S rDNA amplicon sequencing and RNA extraction, the samples were placed in a − 80 °C refrigerator after being frozen in liquid nitrogen. To analyze SCFAs, ileal and cecal digesta were collected in small sample bags and placed at − 20 °C.

Analysis of growth performance

Daily records of feed amount, residual amount, and number of dead broilers were made during the experiment. The following formulae were used to calculate growth performance: average daily feed intake (ADFI), average daily gain (ADG), and feed conversion rate (FCR).

$$\mathrm{ADFI(g/d\cdot bird)} = \sum \left[(\text{daily feed amount} - \text{daily residual amount})/\text{number of broilers}\right]/\text{days}$$

$$\mathrm{ADG(kg/d\cdot bird)} = (\text{final average weight} - \text{initial average weight})/\text{days}$$

$$\mathrm{FCR} = \text{total feed intake}/\text{total weight gain}$$

Analysis of gut morphological

For morphological assessment, the fixed jejunal, ileal, and cecal tissues were dehydrated using ethanol and embedded in paraffin. The tissues were sectioned to 5-mm thickness and stained with eosin-methylene blue for histological analysis. Approximately 15 intact, well-oriented villus-crypt units from each section were randomly selected. Digital images allowed the measurement and calculation of the villus length (V, from the apex of the villus to the villus-crypt junction), crypt depth (C, from the villus-crypt junction to the root of

the crypt), and the ratio of villus length to crypt depth (V/C) from each intestinal section at × 40 magnification.

Analysis of serum biochemical values

The concentration of malondialdehyde (MDA) in sera was measured using an assay kit (A003-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on the spectrophotometer measurement of a red-complex produced during the reaction of thiobarbituric acid with MDA. Detailed steps were performed as previously described by Liu et al. (2010).

The activity of serum superoxide dismutase (SOD) was measured using an assay kit (A001-1; Nanjing Jiancheng Bioengineering Institute) by measuring the inhibiting rate of the enzyme to O_2^- produced by the xanthine morpholine (Deng et al. 2013).

RNA extraction and gene expression profiling

The total RNA from the jejunum was extracted, reverse-transcribed to cDNA, and stored at − 20 °C until the subsequent analysis. Real-time quantitative PCR was conducted to measure the expression of tight junction-related genes (those encoding zonula occludens 1 [ZO-1] and occludin) and inflammatory cytokines (interleukin-1β [IL-1β], interleukin-6 [IL-6], and tumor necrosis factor-α [TNF-α]). The β-actin gene was used as a reference. All primers used are listed in Supplementary Table S2.

16S rDNA sequencing of microflora in the cecal contents

Due to limited experimental funds, four of the 12 cecal samples were randomly selected for intestinal flora analysis. The V3-V4 hypervariable regions of the cecal bacterial 16S rRNA gene were amplified with the primer pair 338F (5′-ACTCCTRCGGGAGGCAGCAG-3′) and 806R (5′-ACTCCTRCGGGAGGCAGCAG-3′) and analyzed using a 454 high-speed sequencing platform (Shanghai Sangon Biotech Co., Ltd., Shanghai, China). After removing low-quality DNA sequences using the program, Mothur (V1.0; Schloss et al. 2009), the distance between the sequences was estimated. Filtered sequences were clustered for 97% similarity to determine operational taxonomic units (OTUs). The bacterial diversity after various treatments was explored and compared using the Silva database (https://www.arb-silva.de).

SCFA analysis

The concentrations of SCFAs (formic acid, acetic acid, propanoic acid, and butyric acid) in the ileal and cecal digesta were measured using high-performance liquid chromatography (HPLC; Agilent 1200; Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an ultraviolet detector and a ZORBAX SB-C18 column (4.6 mm × 250 mm × 5 μm; Agilent Technologies Inc.). Briefly, after the digesta samples were diluted with distilled water (1:2) and centrifuged at 8000 × g for 10 min, the supernatants were filtered through a 0.22-μm membrane, and the concentrations of SCFAs in the samples were determined using HPLC based on the standard curve. Specifically, the SCFAs in the filtrate were separated using a ZORBAX SB-C18 column through a mobile phase (acetonitrile: phosphoric acid solution 2.5:97.5, at a pH of 1.8) at a flow rate of 1.0 mL/min and temperature of 35 °C, and then detected at 210 nm wavelength.

Statistical analysis

All experimental data are presented as mean ± standard error (SE). Variance analysis of all results was performed using Excel (V2019, Microsoft Inc., Washington D.C., USA) and SPSS version 16 (SPSS Inc., Chicago, IL, USA). Differences among the five treatment groups were compared using Duncan's multiple comparison test. Significance was defined as $p < 0.05$, and statistically significant differences are indicated by different superscript letters.

Results

Growth performance

The effects of adding protease or *C. butyricum* to LP diets on ADFI, ADG, and FCR of broilers are shown in Table 2. During days 1–21, there were no significant differences in ADFI, ADG, and FCR between the LP and control groups; however, reducing the CP content in feed negatively affected ADG and FCR of broilers during days 1–42. When the diet was supplemented with antibiotic or protease, the FCR from days 1–21 and 1–42 was reduced. In addition, the ADFI in the LPH group was lower than that in the control group during days 1–42, whereas the ADG from days 1–21 and 1–42 in the antibiotic group were higher than that in the control group ($p < 0.05$). Interestingly, the FCR in the LPC group from days 1–42 was significantly lower than that in the LP group ($p < 0.05$), and the difference in FCR between the control and LPC groups was not significant. Furthermore, the ADFI of broilers among the five groups during days 1–21 was not affected by treatment ($p < 0.05$).

Intestinal morphology

Supplementary Fig. S1 shows the influence of various feeding groups on the intestinal structural integrity of broilers.

Table 2 Effects of supplementing low-protein diets with proteases or *C. butyricum* on the growth performance of broilers

Items	Control	LP	LPH	LPC	Antibiotic
1–21 d					
ADFI	41.09 ± 0.97	40.61 ± 0.74	40.13 ± 0.67	40.08 ± 0.75	40.95 ± 1.06
ADG	27.94 ± 0.69^{bc}	27.35 ± 0.63^{cd}	28.38 ± 0.74^{ab}	27.10 ± 0.47^{d}	28.78 ± 0.61^{a}
FCR	1.47 ± 0.06^{ab}	1.49 ± 0.04^{a}	1.42 ± 0.05^{c}	1.48 ± 0.02^{a}	1.42 ± 0.04^{c}
1–42 d					
ADFI	78.38 ± 3.19^{a}	77.03 ± 4.62^{a}	70.49 ± 4.09^{b}	74.56 ± 4.67^{ab}	77.90 ± 3.31^{a}
ADG	42.13 ± 1.32^{b}	39.59 ± 2.03^{c}	40.81 ± 2.23^{bc}	41.34 ± 1.53^{bc}	44.61 ± 2.16^{a}
FCR	1.86 ± 0.04^{b}	1.95 ± 0.08^{a}	1.73 ± 0.09^{c}	1.80 ± 0.06^{bc}	1.75 ± 0.04^{c}

Each value is the mean value of 6 replicates (two from each replicate). Values are expressed as means ± SEM

^{a,b,c,d}Means in the same row with no common superscripts differ ($p < 0.05$)

ADFI, average daily feed intake(g); *ADG*, average daily gain (g); *FCR*, feed conversion rate

Control, a basal diet; *LP* (low-protein diet),2% less crude protein than the control group; *LPH*, low- protein diet supplemented with 200 g/t HuPro protease; *LPC*, low-protein diet supplemented with 1.0×10^9 CFU/t *C. butyricum*; Antibiotic, a basal diet with 200 g/t oxytetracycline

Table 3 Effects of diet on the structure of intestinal villi in broilers

Groups		Control	LP	LPH	LPC	Antibiotic
21d						
Jejunum	V (μm)	711.73 ± 124.66^{ab}	609.63 ± 132.53^{b}	880.32 ± 158.08^{a}	836.53 ± 233.34^{a}	628.08 ± 62.30^{b}
	C (μm)	151.82 ± 46.53^{ab}	123.44 ± 32.73^{b}	119.64 ± 10.83^{b}	123.52 ± 20.24^{b}	173.74 ± 27.38^{a}
	V/C	4.88 ± 0.91^{b}	5.07 ± 0.88^{b}	7.37 ± 1.16^{a}	6.72 ± 1.16^{a}	3.68 ± 0.71^{c}
Ileum	V (μm)	549.93 ± 63.83^{b}	496.99 ± 49.47^{b}	515.62 ± 33.10^{b}	673.08 ± 94.65^{a}	523.35 ± 38.44^{b}
	C (μm)	127.23 ± 12.19^{a}	122.40 ± 10.27^{ab}	88.22 ± 9.73^{c}	109.94 ± 14.74^{b}	132.22 ± 12.22^{a}
	V/C	4.07 ± 0.24^{b}	4.06 ± 0.19^{b}	5.91 ± 0.85^{a}	6.15 ± 0.65^{a}	4.00 ± 0.58^{b}
Cecum	V (μm)	143.95 ± 25.52^{b}	142.74 ± 17.16^{b}	175.09 ± 25.81^{a}	182.40 ± 8.59^{a}	140.79 ± 11.51^{b}
	C (μm)	128.07 ± 20.29^{ab}	164.10 ± 43.30^{a}	141.02 ± 33.56^{ab}	109.61 ± 18.24^{b}	142.61 ± 29.28^{ab}
	V/C	1.13 ± 0.20^{bc}	0.94 ± 0.32^{c}	1.27 ± 0.15^{bc}	1.71 ± 0.32^{a}	1.03 ± 0.27^{bc}
42d						
Jejunum	V (μm)	1015.09 ± 176.38^{c}	967.99 ± 80.55^{c}	1249.87 ± 131.58^{a}	1206.31 ± 96.26^{ab}	903.92 ± 102.50^{c}
	C (μm)	185.74 ± 17.82^{b}	219.05 ± 29.76^{ab}	216.91 ± 11.28^{ab}	224.41 ± 13.71^{ab}	246.79 ± 54.33^{a}
	V/C	5.43 ± 0.59^{ab}	4.51 ± 0.86^{bc}	5.76 ± 0.49^{a}	5.40 ± 0.65^{ab}	3.79 ± 0.82^{c}
Ileum	V (μm)	754.30 ± 119.87^{bc}	814.58 ± 54.32^{abc}	988.82 ± 178.00^{a}	869.15 ± 184.01^{ab}	672.43 ± 78.85^{c}
	C (μm)	142.10 ± 27.63^{ab}	136.05 ± 19.07^{b}	150.15 ± 21.78^{ab}	129.02 ± 16.24^{b}	173.00 ± 31.22^{a}
	V/C	5.39 ± 0.83^{b}	6.09 ± 0.97^{ab}	6.60 ± 0.80^{ab}	6.75 ± 1.09^{a}	4.07 ± 1.10^{c}
Cecum	V (μm)	152.05 ± 36.18^{ab}	153.98 ± 40.53^{ab}	174.39 ± 23.15^{a}	179.87 ± 30.96^{a}	147.59 ± 19.09^{ab}
	C (μm)	165.19 ± 40.80^{a}	148.96 ± 37.43^{ab}	121.32 ± 13.93^{bc}	144.77 ± 39.58^{ab}	174.54 ± 33.77^{a}
	V/C	0.93 ± 0.13^{c}	1.04 ± 0.13^{c}	1.45 ± 0.24^{ab}	1.28 ± 0.22^{b}	0.88 ± 0.23^{c}

Each value is the mean value of 6 replicates (two from each replicate). Values are expressed as means ± SEM

^{a,b,c,d}Means in the same row with no common superscripts differ ($p < 0.05$)

Control, a basal diet; *LP* (low-protein diet), 2% less crude protein than the control group; *LPH*, low-protein diet supplemented with 200 g/t HuPro protease; *LPC*, low-protein diet supplemented with 1.0×10^9 CFU/t *C. butyricum*; Antibiotic, a basal diet with 200 g/t oxytetracycline

V, villus length (μm); *C*, crypt depth (μm); *V/C*, villus length to crypt depth

Almost all markers of intestinal structural integrity (V, C, and V/C) were negatively affected by the antibiotic treatment (Supplementary Fig. S1 and Table 3). On days 21 and 42, there was no significant difference ($p < 0.05$) between the LP and control groups in terms of intestinal structural integrity (V, C, and V/C of the jejunum, ileum, and cecum) (Table 3). After 21 days, the V/C ratio of the jejunum and ileum in the LPH and LPC groups was higher ($p < 0.05$) than that in the control and LP groups. In addition, compared with those in the control and LP groups, the C of the ileum and V

of the cecum were significantly decreased and increased in the LPH and LPC groups, respectively ($p < 0.05$). The V/C ratio of the cecum substantially increased ($p < 0.05$) in the LPC group compared with that in the control and LP groups. After 42 days, broilers fed LP diets supplemented with protease or *C. butyricum* had significantly higher ($p < 0.05$) jejunal V levels. The cecal V/C ratio in the LPH and LPC groups was significantly higher than that in the other groups, whereas the difference was not significant in the jejunum and ileum. Furthermore, the ileal V in the LPH group and ileal V/C ratio in the LPC group were significantly higher ($p < 0.05$) than those in the control group.

Serum anti-oxidant index

The levels of serum anti-oxidant markers (SOD and MDA) in broilers fed LP diets containing protease or *C. butyricum* are presented in Table 4. There was no significant difference ($p < 0.05$) in SOD activity or MDA concentration between the control and LP groups. The MDA concentration at 21 and 42 days in the LPC group was significantly lower ($p < 0.05$) than that in the control and LP groups. In addition, the 21-day SOD activity in the LPH group was significantly higher than that in the control and LP groups. However, the 42-day MDA concentration in the antibiotic group was significantly lower than that in the control and LP groups.

Expression of tight junction and immune-related genes in jejunal mucosa

The mRNA expression of tight junction proteins (ZO-1 and occludin) and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in different groups are shown in Fig. 1. Compared with the control group, the LP group showed a slight insignificant downward trend in the mRNA expression of ZO-1 and occludin (Fig. 1A and B). In contrast, a significant upward trend in the expression of IL-1β and IL-6 genes was observed (Fig. 1C and D). When HuPro protease was added to the LP diet, the mRNA expression levels of ZO-1 and occludin reached or even exceeded those of the control group, whereas the expression of IL-1β and IL-6 in the LPH group did not increase as much as that in the LP group (Fig. 1A–D). In particular, the LPH group exhibited lower TNF-α mRNA expression than the other groups (Fig. 1E). Notably, the mRNA expression of ZO-1 and occludin in the LPC group was slightly higher than that in the other groups (Fig. 1A and B). At the same time, the expression of IL-1β and IL-6 in the LPC group decreased sharply compared with that in the LP group, and there was no significant difference between the control and antibiotic groups (Fig. 1C, D and E). Interestingly, antibiotic had a negative effect on the mRNA expression of ZO-1 and occludin (Fig. 1A and B). Therefore, the LPC group almost presented the highest and lowest mRNA expression of tight junction proteins and pro-inflammatory cytokine-related genes, respectively, among the five experimental groups.

Concentration of SCFAs in the ileum and cecum

The SCFA concentrations in the ileum and cecum (formic acid, acetic acid, propanoic acid, and butyric acid) of the different diet groups are summarized in Fig. 2. The SCFA concentrations in the cecal digesta were considerably higher ($p < 0.05$) than those in the ileum (Fig. 2). Lowering the CP content in the diet did not have a significant effect ($p < 0.05$) on the concentrations of SCFAs in the ileum (formic acid, acetic acid, and propanoic acid) and cecum (propanoic acid, butyric acid, and acetic acid on day 42; Fig. 2), whereas the concentrations of cecal formic acid and acetic acid on day 21 in the LP group were significantly lower ($p < 0.05$) than those in the control group (Fig. 2B). The concentrations of SCFAs in the ileum and cecum of the LPC and antibiotic groups were higher than those in the other three groups. Particularly, the concentration of ileal acetic acid on day 42 in the LPC group increased from 64.88 μg/g to 265.30 μg/g compared with that in the control group (Fig. 2A). These

Table 4 Effects of supplementing low-protein diets with proteases or *C. butyricum* on serum antioxidant index in broilers

Groups	Control	LP	LPH	LPC	Antibiotic
21d					
SOD (U/ml)	610.86 ± 64.38^{c}	632.28 ± 74.30^{c}	836.32 ± 88.09^{a}	693.99 ± 66.64^{bc}	677.62 ± 90.39^{bc}
MDA (nmol/ml)	3.06 ± 0.22^{ab}	2.93 ± 0.50^{ab}	3.38 ± 0.63^{a}	1.98 ± 0.50^{c}	3.51 ± 0.94^{a}
42d					
SOD (U/ml)	599.32 ± 82.63^{ab}	685.08 ± 95.26^{a}	600.09 ± 82.92^{ab}	596.63 ± 57.14^{ab}	522.24 ± 63.82^{b}
MDA (nmol/ml)	3.15 ± 0.74^{ab}	3.60 ± 0.61^{a}	3.19 ± 0.82^{ab}	2.38 ± 0.59^{c}	2.57 ± 0.70^{c}

Each value is the mean value of 6 replicates (two from each replicate). Values are expressed as means ± SEM

[a,b,c,d]Means in the same row with no common superscripts differ ($p < 0.05$)

Control, a basal diet; *LP* (low-protein diet), 2% less crude protein than the control group; *LPH*, low-protein diet supplemented with 200 g/t HuPro protease; *LPC*, low-protein diet supplemented with 1.0×10^{9} CFU/t *C. butyricum*; Antibiotic, a basal diet with 200 g/t oxytetracycline

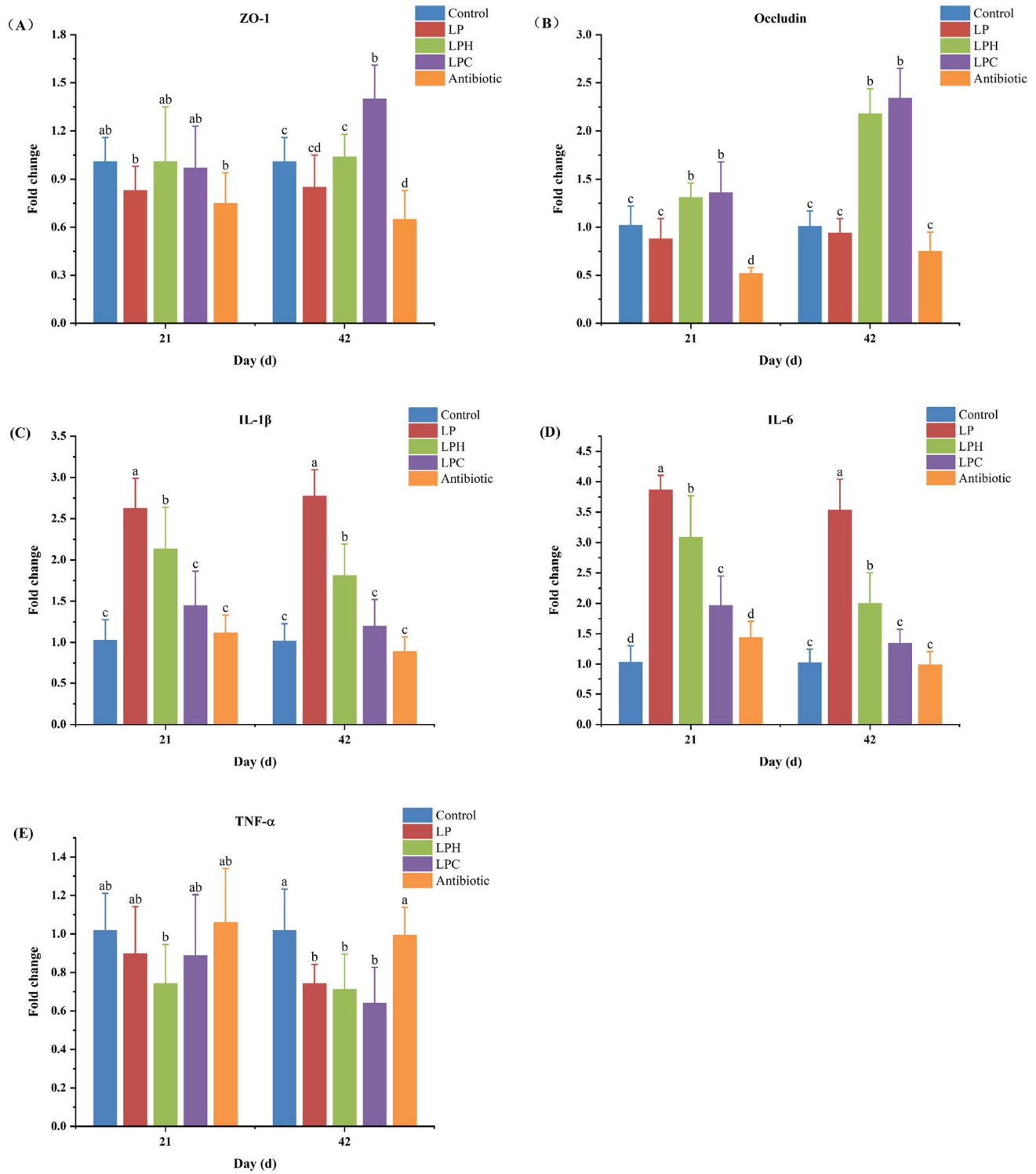
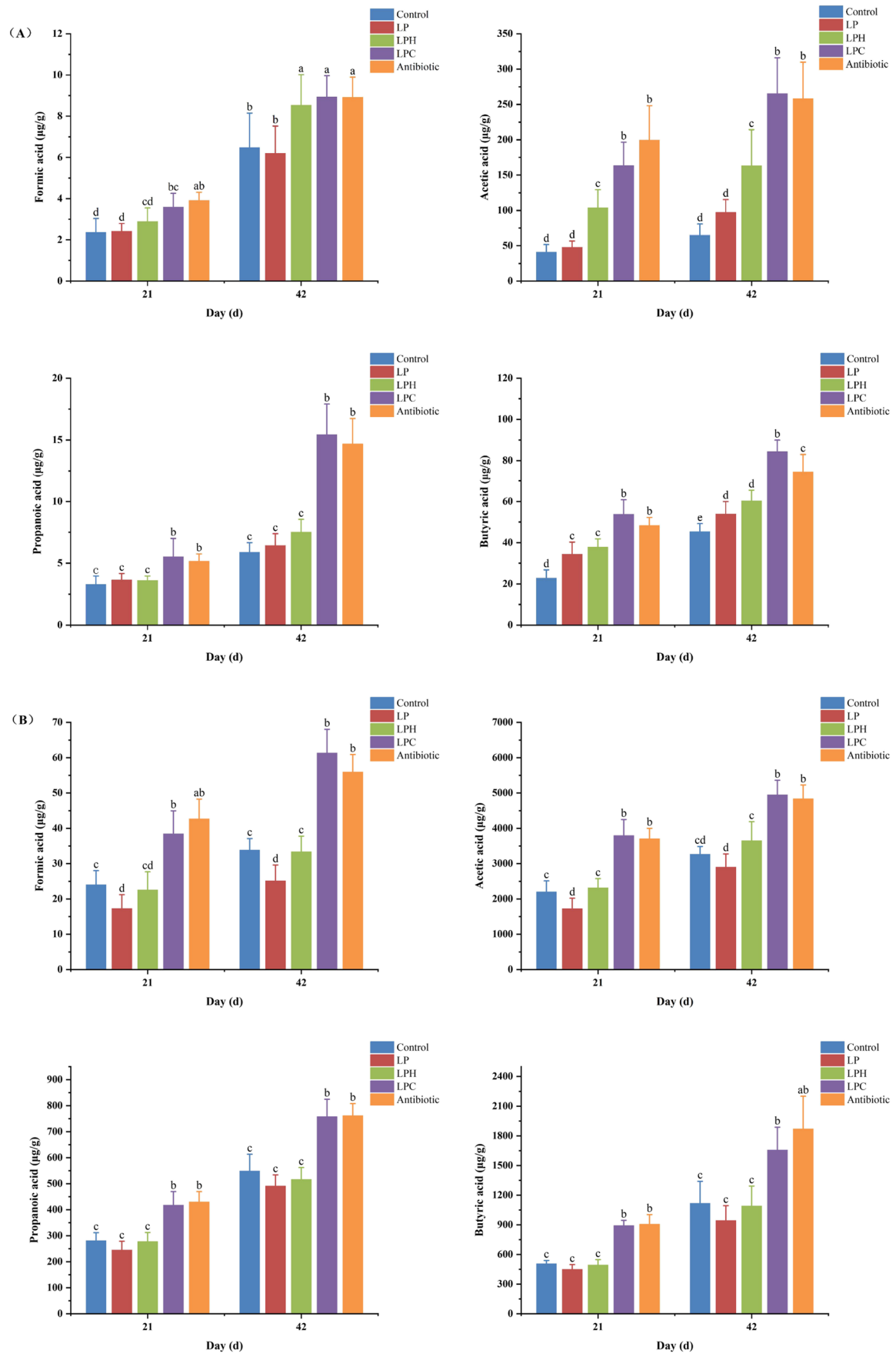


Fig. 1 The effects of supplementing low-protein diets with proteases or *C. butyricum* on the mRNA expression levels of tight junction proteins and pro-inflammatory cytokine related-genes. **A** and **B** mRNA expression levels of jejunal tight junction proteins related genes in broilers. **C**–**E** mRNA expression levels of jejunal mucosa pro-inflammatory cytokine related genes in broilers. Different lowercase letters above bars represent significantly different means ($p < 0.05$). IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; ZO-1, zonula occludens 1. Control, a basal diet; LP (low-protein diet), 2% less crude protein than the control group; LPH, low-protein diet supplemented with 200 g/t HuPro protease; LPC, low-protein diet supplemented with 1.0×10^9 CFU/t *C. butyricum*; Antibiotic, a basal diet with 200 g/t oxytetracycline


(A)
Formic acid (μg/g)
Acetic acid (μg/g)
Propanoic acid (μg/g)
Butyric acid (μg/g)
(B)
Formic acid (μg/g)
Acetic acid (μg/g)
Propanoic acid (μg/g)
Butyric acid (μg/g)
Control
LP
LPH
LPC
Antibiotic
Day (d)
21
42

◂**Fig. 2** Levels of SCFAs in the ileum (**A**) and cecum (**B**) of broilers' diets at day 21 and day 42. Significant differences are shown by bars labeled with various letters. [a,b,c,d]Means in the same row with no common superscripts differ ($p < 0.05$). Control, a basal diet; LP (low-protein diet), 2% less crude protein than the control group; LPH, low-protein diet supplemented with 200 g/t HuPro protease; LPC, low-protein diet supplemented with 1.0×10^9 CFU/t *C. butyricum*; Antibiotic, a basal diet with 200 g/t oxytetracycline

findings suggest that the addition of *C. butyricum* to an LP diet could effectively increase the concentration of SCFAs in the ileum and cecum (Fig. 2).

Cecal bacterial community

16S rDNA sequencing was performed to observe the alteration in the microbiota in the cecum digesta. Eventually, 1,728,758 valid sequences were obtained, with an average of 85,134 valid sequences per sample, and the average sequence length was 416 bp. The alpha diversity of the cecal bacterial community was assessed using Chao1 and Shannon indices. Under different diets, the diversity of the cecal microbial community in broilers varied. The Chao1 index in the LP, LPC, and antibiotic groups was significantly higher ($p < 0.05$) than that in the control and LPH groups (Fig. 3A). However, compared with that in the control and LP groups, the Shannon index in the LPH, LPC, and antibiotic groups decreased significantly ($p < 0.05$; Fig. 3A). A Venn diagram revealed that the five groups shared 913 bacterial OTUs. In the control, LP, LPH, LPC, and antibiotic groups, the number of unique OTUs was 2454, 3044, 2040, 3022, and 3158, respectively (Fig. 3B). These findings showed that the five groups differed remarkably in intestinal bacterial composition.

The relative abundance of cecal bacteria was determined at the phylum and genus levels. At the phylum level, *Firmicutes* and *Bacteroidetes* members were the primary bacteria found in the broiler cecum, accounting for approximately 52.5% and 36.7% of the total bacteria, respectively (Fig. 3C). Additionally, the LPH, LPC, and antibiotic groups had lower *Firmicutes*/*Bacteroidetes* ratios and higher *Bacteroidetes* proportion than the other groups. At the genus level, *Rikenellaceae RC9 gut* spp., *Alistipes* spp., *Clostridia vadinBB60* spp., and *Faecalibacterium* spp. were the most predominant bacteria (Fig. 3D). The relative abundance of *Rikenellaceae RC9 gut* spp. in the LPH, LPC, and antibiotic groups was higher than that in the control and LP groups, whereas the relative abundance of *Alistipes* spp. was lower in the LPC and antibiotic groups than in the other three groups. *Faecalibacterium* spp. was the predominant genus in the LP group, significantly more abundant than that in the other groups, whereas *Coprobacter* spp. was the predominant genus in the control group.

Correlation analysis results

The correlation between gut microbial populations and other critical parameters was predicted using Spearman's correlation matrix. As shown in Fig. 4, acetic, propanoic, and butyric acids in the ileum had significant negative ($p < 0.01$) effects on the abundance of *Coprobacter* spp. and *Alistipes* spp., but had positive effects ($p < 0.05$) on the abundance of *Rikenellaceae RC9 gut* spp. and *Helicobacter* spp. Similarly, the cecal acetic acid and ileal formic acid were negatively correlated with the abundance of *Coprobacter* spp. and *Alistipes* spp., but positively correlated with the abundance of *Lactobacillus* spp. and *Akkermansia* spp. ($p < 0.05$). The negative correlation between the abundance of *Alistipes* spp. and SCFAs is shown in Figs. 2, 3D, and 4. In addition, jejunal C was significantly negatively correlated ($p < 0.01$) with the abundance of *Synergistes* spp., but positively correlated ($p < 0.05$) with the abundance of *Rikenellaceae RC9 gut* spp. In contrast, the abundance of *Christensenellaceae R-7* spp. and *Clostridia vadin BB60* spp. was positively correlated with the expression of IL-6 and IL-1β, but negatively correlated with ADG ($p < 0.05$). Notably, the FCR had significant positive effects on the abundance of *Ruminococcaceae NK4A214* spp. ($p < 0.01$), as well as the *Bacteroides* spp., *Colidextribacter* spp. and *Clostridia UCG-014* spp. ($p < 0.05$). The abundance of *Desulfovibrio* spp. was positively correlated with cecal C and ADG but negatively correlated with IL-6 and IL-1β expression, ileal V, and cecal V/C ratio ($p < 0.05$). In addition, TNF-α was negatively correlated with the abundance of *Helicobacter* spp. ($p < 0.05$). A substantial negative correlation was found between the abundance of *Ruminococcus torques* spp. and ADFI ($p < 0.05$).

Discussion

In poultry nutrition, LP diets have major economic and environmental advantages, such as reduction in feed costs, nitrogen excretion, and phosphorus emissions, because feed is the main factor determining the total production cost, and CP is the most important nutritional index of feed (Amer et al. 2021). The addition of amino acids to LP feed to reduce the negative effects of LP diets on broilers has attracted increasing attention (Macelline et al. 2020; Kidd et al. 2021). However, only a few studies have explored whether adding enzymes or probiotics to LP feed can offset the negative effects of an LP feed. Therefore, we set the antibiotic group as a positive control (i.e., normal diet before antibiotic ban), whereas the experimental group supplemented HuPro protease or *C. butyricum* under the LP conditions in order to save breeding costs. By using these additives, we hoped to achieve the same effect as the positive control.

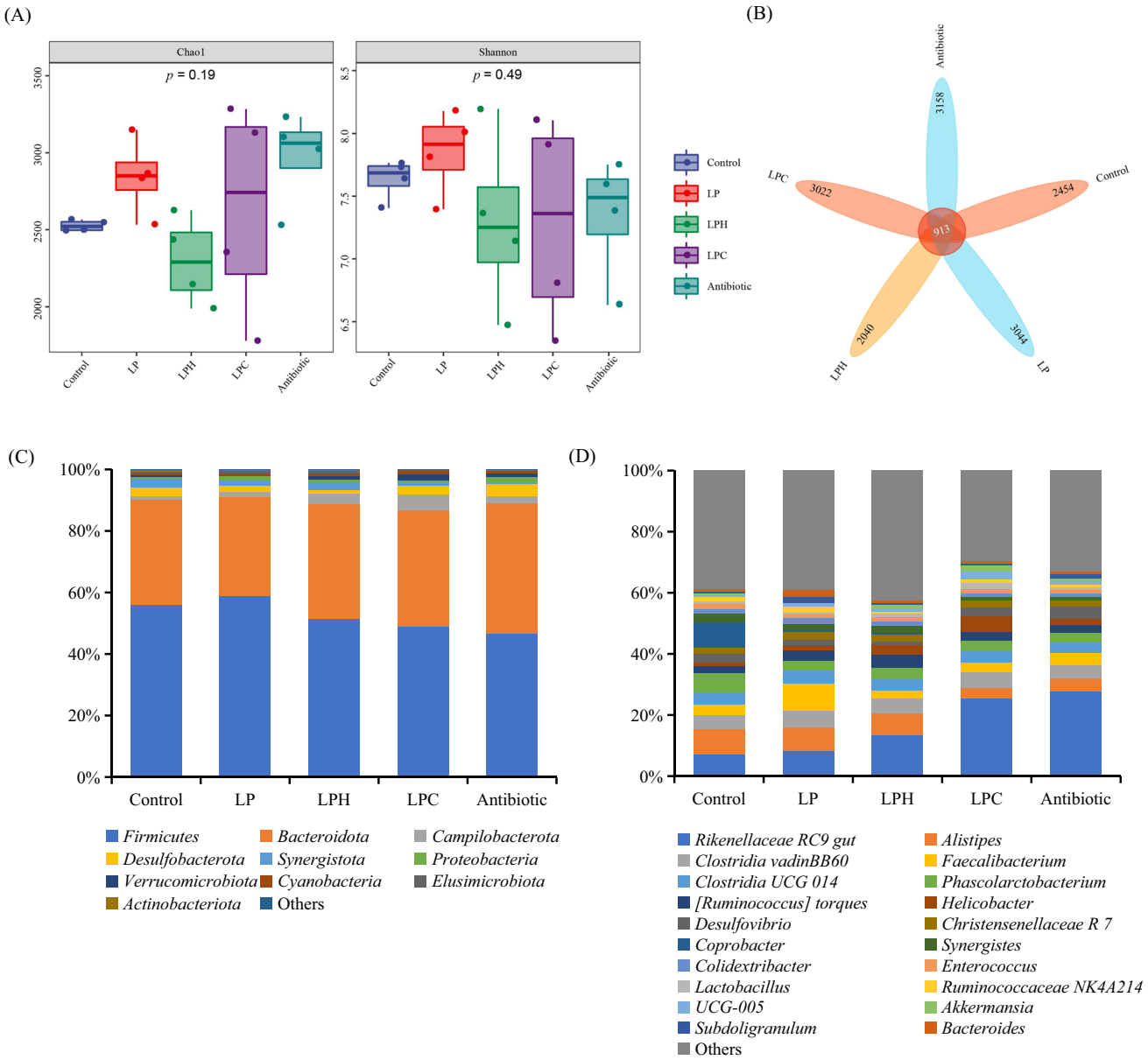


Fig. 3 The effects of the intestinal bacterial community on the cecal bacterial community diversity indexes of Chao1 and Shannon indexes (**A**). Venn diagram of the OTUs (**B**). The effects of intestinal bacterial communities on the relative abundances of cecal bacteria at the phylum level (**C**) and genus level (**D**). Control, a basal diet; LP (low-protein diet), 2% less crude protein than the control group; LPH, low-protein diet supplemented with 200 g/t HuPro protease; LPC, low-protein diet supplemented with 1.0×10^9 CFU/t *C. butyricum*; Antibiotic, a basal diet with 200 g/t oxytetracycline

This study demonstrated that lowering protein levels by 2% had no significant effect on the early growth stage of broilers but led to poor production performance (low ADG and high FCR) during the study period. This finding was consistent with previously reported results that LP diets retard the growth performance of broilers (Law et al. 2018). The lower FCR of the LPH and antibiotic groups revealed that these broilers ate the same quantity of feed but accumulated more meat. In this study, the LPH and antibiotic groups decreased FCR by decreasing ADFI and increasing ADG, respectively (Table 2). Jabbar et al. (2021) also showed that the addition of protease to LP diets decreases the FCR. Adding protease to LP diets significantly improved the apparent digestibility of CP and produced more amino acids and short peptides, thus increasing its absorption when passing through the small intestine. However, Oakley et al. (2014) found that adding exogenous protease to poultry diets did not significantly improve growth performance, which could be due to differences in diet structure, breeding management, and the source and dose of protease. In our study, adding

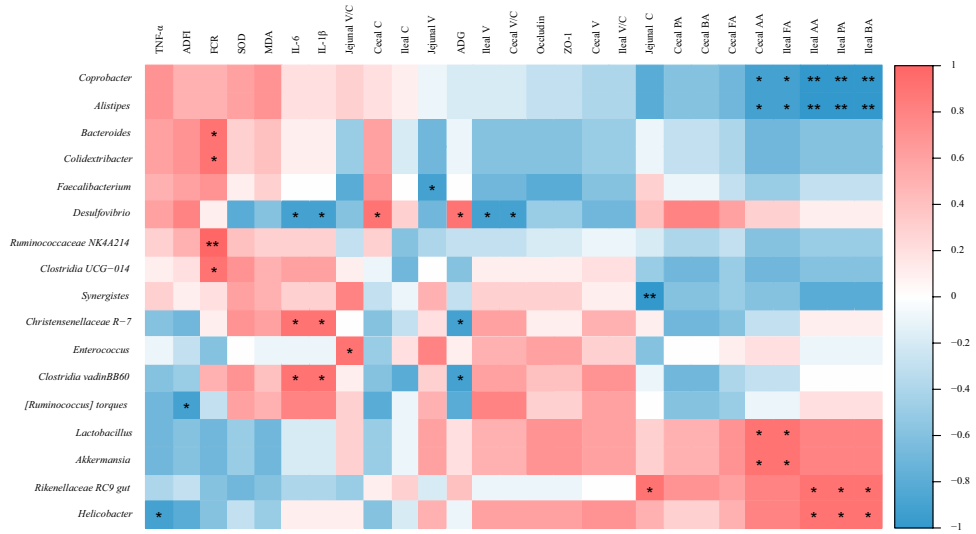


Fig. 4 The effects of various dietary groups on the differences in gut microbiota and their relationship to broiler phenotype. The correlation between diverse gut microbiota and broiler phenotypic. A positive correlation is represented by red, while a negative correlation is represented by blue. The strength of the correlation is shown by the color depth. Significant relationships are indicated by asterisks (*$p \leq 0.05$, **$p \leq 0.01$). Control, a basal diet; LP (low-protein diet), 2% less crude protein than the control group; LPH, low-protein diet supplemented with 200 g/t HuPro protease; LPC, low-protein diet supplemented with 1.0×10^9 CFU/t *C. butyricum*; Antibiotic, a basal diet with 200 g/t oxytetracycline

1.0×10^9 CFU/t *C. butyricum* to the LP diet reduced the FCR during days 1–42, and maintained the same level of ADG as in the control group. *C. butyricum* can improve gastrointestinal digestive enzyme activity and produce SCFAs, and thereby facilitate the release of nutrients from the diet (Zhang et al. 2016). As shown in Fig. 2, SCFA concentrations in the cecum and ileum in the LPC group were significantly higher than those in the other groups. Li et al. (2021) reported that the addition of *C. butyricum* at 1.0×10^9 CFU/kg improves ADG and decreases FCR of broilers during days 1–21 and 1–42. From this result, it can be concluded that the LP diet supplemented with 200 g/t HuPro protease replaced the typical diet supplemented with antibiotics promoting broiler growth, and the use of LP feeds also contributes to a reduction in environmental pollution. In addition, adding *C. butyricum* to LP diets can prevent the negative effects of LP diets on the growth performance of broilers; however, further investigation is needed to determine the optimum dosage of *C. butyricum* (Table 2).

The intestine is a vital digestive organ, and its proper development is essential for feed digestion and nutrient absorption in broilers. V, C, and their ratio are important indicators of intestinal health (Chamorro et al. 2019). Research has shown that the intestinal villi can expand the surface area of the intestinal epithelium and promote nutrient absorption (He et al. 2021). In addition, the fundamental function of the intestinal crypt is to secrete digestive enzymes, and as the crypt matures, its depth decreases (He et al. 2019). In brief, the turnover rate of new villus cells and the enhancement of digestion and absorption functions are represented by a decrease in C and an increase in V and V/C ratio. Compared with that of the control group, the morphological structure of the intestine (V, C, and V/C ratio) did not change significantly after the consumption of LP by broilers, which indicated that LP diets did not cause impaired intestinal morphology. The low ADG and high FCR in the LP group may be caused by insufficient nutrition intake (Tables 2 and 3). Ndazigaruye et al. (2019) also reported no significant difference in ileal morphology between the control and LP groups, whereas Yu et al. (2019) found that LP-fed piglets showed atrophy in the small intestine. Adding *C. butyricum* to LP diets improved the morphological structure of the jejunum, ileum, and cecum, indicating that intestinal absorption and barrier protection function were enhanced, thus reducing FCR and improving the growth performance of broilers compared with those in the LP group (Tables 2 and 3). This finding is consistent with the results of Zhang et al. (2016) and Cao et al. (2019). *C. butyricum* produces butyric acid, which is the most important SCFA for intestinal epithelial cells. It can increase the number of intestinal epithelial cells, increase the height of villi, and make crypts shallower, thus improving intestinal structure and enhancing the ability to absorb nutrients (Chen et al. 2018). The results shown in Fig. 2 also indicate that butyric acid concentrations in the ileum and cecum of broilers in the LPC group were significantly increased ($p < 0.05$).

SOD has anti-inflammatory, anti-aging, and anti-radioactivity properties as an oxygen-free radical scavenger.

MDA is one of the final metabolic products of the lipid peroxidation reaction, which can cross-link the amino groups of proteins and phospholipids, reducing the fluidity of the cell membrane (Hellberg et al. 2010). In this study, adding protease to LP diets increased the anti-oxidant capacity of 21-day-old broilers, and adding *C. butyricum* at a concentration of 1.0×10^9 CFU/t to LP diets removed harmful anti-oxidant substances. Li et al. (2021) found that dietary supplementation with 1.0×10^9 CFU/kg *C. butyricum* significantly reduces the MDA concentration and increases the SOD activity in the serum of 21-day-old broilers. Our findings indicated that adding *C. butyricum* had no influence on the SOD activity, which may be due to insufficient dosing.

The strength of the intestine barrier depends on structural integrity, tight junction proteins, and a stable microbiome (Wang et al. 2014). ZO-1 and occludin are essential tight junction proteins that regulate the function of the intestinal epithelial barrier and prevent macromolecular transmission (Ballard et al. 1995). In this study, protease or *C. butyricum* prevented the transmission of toxins and pathogens by increasing the expression of ZO-1 and occludin in the jejunal epithelial cells (Fig. 1). Our results correspond with the findings of Zhao et al. (2020), which showed that treating *Salmonella*-infected broilers with *C. butyricum* could increase the expression of ZO-1 in intestinal tissue. Moreover, Zuo et al. (2014) found that 300 g/t β-mannanase significantly enhanced the mRNA levels of occludin and ZO-1 in the ileum. Cytokines regulate cell growth and inhibit intestinal inflammation. Among these cytokines, pro-inflammatory cytokines, such as TNF-α and IL-1β, are key regulators of the inflammatory response. IL-6 is a potent pro-inflammatory cytokine in response to pro-inflammatory stimuli (Al-Sadi et al. 2009). In our study, LP diets increased the expression of pro-inflammatory factors IL-1β and IL-6; however, supplementation with protease or *C. butyricum* significantly reversed the upregulation of IL-1β, IL-6, and TNF-α in the jejunal mucosa of broilers by day 42 (Fig. 1). Several studies have shown that protease or *C. butyricum* supplementation improves immunological function in piglets and poultry (Park et al. 2020; Li et al. 2021; Yu et al. 2022). Taken together, our study and some previous studies demonstrate that the addition of *C. butyricum* to LP diets can achieve the effects of the antibiotic group (positive control) in maintaining intestinal barrier function and alleviating inflammatory response. The addition of HuPro protease also has a positive effect on these functions.

SCFAs can regulate the intestinal environment, improve immunity, and inhibit inflammation (Cani 2014). In the current study, the decrease in IL-1β and IL-6 expression and increase in growth performance of the LPC and antibiotic groups may be related to the increase in SCFAs in the cecum and ileum (Table 2, Figs. 1 and 2). Similar results were obtained in previous studies, indicating that *C. butyricum* induced higher intestinal SCFA concentrations and lower intestinal pH, thereby improving the immune system and growth performance of broilers (Liu et al. 2021; Han et al. 2018). A study found that butyric acid, an SCFA, can exert immunomodulatory and anti-inflammatory effects and promote the integrity of intestinal epithelial cells (Tedelind et al. 2007).

Intestinal microbes play a crucial role in controlling intestinal health, nutritional digestion, immunological function, feed utilization, and reducing fecal excretion. This positive effect may be achieved through the production of SCFAs (Valdes et al. 2018; Tremaroli and Backhed 2012). Our study aligns with others demonstrating that *Firmicutes* and *Bacteroidetes* are the predominant bacteria at the phylum level in the cecum of broilers (Li et al. 2021; He et al. 2021). As *Rikenellaceae RC9 gut* spp. process cellulose and hemicellulose (Zened et al. 2013), the FCR of the LPH, LPC, and antibiotic groups was reduced (Table 2 and Fig. 3). *Alistipes* spp. have been linked to several diseases, including colorectal cancer and segmental ileitis (Parker et al. 2020). In the present study, we found a lower abundance of *Alistipes* spp. in the LPC and antibiotic groups, indicating that both treatments suppressed the growth of pathogenic gut bacteria. Furthermore, the abundance of *Faecalibacterium* spp. was inversely associated with the expression of pro-inflammatory cytokines (IL-1β and IL-6) (Oakley and Kogut 2016). This could explain why the LP diet group had higher expression of IL-1β and IL-6 than the other groups in this study (Figs. 2 and 3). However, the mechanism by which proteases promoted growth in broilers in this study may involve hydrolysis of plant proteins in the feed into small peptides and amino acids rather than changes in the gut microbiome. The relative abundance of cecal bacteria did not differ significantly among the control, LP, and LPH groups (Fig. 3D). Notably, the level of SCFAs had significant negative effects on the abundance of *Coprobacter* spp. and *Alistipes* spp., but positive effects on the abundance of *Lactobacillus* spp., *Akkermansia* spp., *Rikenellaceae RC9 gut* spp., and *Helicobacter* spp. (Fig. 4). Although the relative abundance of *Lactobacillus* and *Akkermansia* spp. in the cecum of broilers is very low, they significantly influence the growth performance and immune response parameters of broilers. This may be because *Lactobacillus* spp. can convert mineral elements into ions that are easily absorbed by animals, promoting growth (LeBlanc et al. 2013). Similarly, *Akkermansia* spp. can promote the proliferation of intestinal epithelial cells and repair of the intestinal mucosa as well as provide energy for the growth of other colonizing symbiotic bacteria (Wlodarska et al. 2017).

In conclusion, the reduction in CP content in the feed had no significant effect on the early growth performance, serum anti-oxidant index, and SCFAs in the cecum and ileum of broilers but had negative effects on growth performance and immune response during the entire growth stage. However,

supplementation with *C. butyricum* or HuPro protease in LP diets can effectively counteract this negative effect, which is important for resource conservation and environmental protection. Specifically, the addition of 200 g/t HuPro protease to the LP diet had the same effect as the positive control (antibiotic group) in reducing FCR in broilers; this could reduce feed costs. Furthermore, LP diets supplemented with *C. butyricum* at 1.0×10^9 CFU/t had positive effects on the growth of broilers, which may result from reducing the levels of MDA and pro-inflammatory cytokines, improving the intestinal morphology, and increasing the concentrations of SCFAs in the cecum and ileum. However, it is worth noting that supplementing LP diets with protease or *C. butyricum* changed the gut microbial composition, for example, by increasing the abundance of *Rikenellaceae RC9 gut* spp. and decreasing the proportion of *Alistipes* spp. However, the effects of adding larger amounts of *C. butyricum* or the complex of *C. butyricum* and protease into LP feed in broilers require further study. Based on the functional comparison of these two supplements, this study provides a theoretical basis and support for the supplementation of *C. butyricum* or protease in LP feed and may influence the selection of growth additives in the poultry industry.

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Author contribution S.Z. and N.P. conceived and designed research. H.L., W.Z., and Y.Z. conducted experiments and contributed new reagents or analytical tools. T.W., and H.L. analyzed data. T.W. wrote the manuscript. Y.L., Y.H, and S.Z. revised the manuscript. All authors read and approved the manuscript.

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Data availability All raw sequencing data obtained in this study have been deposited at the NCBI SRA database (NCBI Bio Project PRJNA850881).

Declarations

Ethics approval All experiments were conducted according to the ethical standards of the Experimental Animal Center of the Huazhong Agricultural University (HZAUCH-2019–008). All efforts were made to minimize suffering.

Competing interests The authors declare no competing interests.

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