Highly nutritious diet resists Salmonella Typhimurium infections by improving intestinal microbiota and morphology in broiler chickens

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ABSTRACT Salmonella Typhimurium (*S.* Typhimurium) infection in broiler chickens threatens public health and livestock production. In this study, we explored the effects of highly nutritious (crude protein 21.8%, metabolizable energy 3.16 Mcal/kg) and lowly nutritious (crude protein 18.1%, metabolizable energy 2.98 Mcal/kg) diets on *S.* Typhimurium infection by altering the intestinal morphology and environment in broiler chickens. The highly nutritious diet significantly increased the body weight gain and reduced feed conversion ratio on day 1 to 21 ($P < 0.01$). The highly nutritious diets promoted the intestinal villus height, crypt depth, and their ratio to improve the intestinal epithelial maturation ($P < 0.05$). Highly nutritious diets significantly increased the expression of claudin-1, occludin, and *NF-κB* genes in the intestinal epithelium on the days of 14 and 21 ($P < 0.05$). *S.* Typhimurium activated the expression of *TLR4*, *MyD88*, and *NF-κB* genes to cause an inflammatory response. The *S.* Typhimurium can increase the activity of myeloperoxidase, which cause an inflammatory response. The *S.* Typhimurium significantly reduced the diversity indexes of the ileal microbiota ($P < 0.05$), increased the abundance of *Cyanobacteria* which can synthesize toxins. The highly nutritious diet group challenged with *S.* Typhimurium can increase the abundance of *Lactobacillus* in the ileum, which lead to improved intestinal health ($P < 0.05$). It is concluded that increasing the nutritional level of dietary is beneficial to improve the resistance to *S.* Typhimurium infection by altering the intestinal bacterial community.

Key words: broiler chickens, nutritional level, *Salmonella* Typhimurium, intestinal bacteria, ileal morphology

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INTRODUCTION

Salmonella infection is a major culprit of foodborne disease, causing about 118.3 million infections, and about 355,000 reported deaths in the world every year (Thompson et al., 2018). Among them, *Salmonella* Typhimurium (S. Typhimurium) and *Salmonella enteritidis* accounted for 99% of human salmonellosis (Lamas et al., 2018). The *S.* Typhimurium infection in broiler chickens can cause a serious threat to human health and economic losses, the main symptoms including diarrhea, mental depression, and low production performance (Dar et al., 2019). The resistance to *S.* Typhimurium infection through nutritional regulation is particularly important when antibiotics are gradually banned in the feed (Kappala et al., 2018). Previous studies have reported the addition of probiotics promoted the intestinal health by improving the intestinal bacterial community and the tight junction of the intestines (Bäumler and Sperandio, 2016). Increasing the abundance of *Lactobacillus* and *Bifidobacteria* in the gut can inhibit *Salmonella* infection (Willis et al., 2009; Olnood et al., 2015). The energy level of the diet is associated with the *Lactobacillus* abundance in the gut (Chen et al., 2018). Therefore, altering the intestinal bacterial community by adjusting the nutritional levels of the diet can be a strategy in suppressing *S.* Typhimurium infection.

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Maintaining intestinal health and growth performance remains an issue as the requirement increases for the restricted use of antibiotics in poultry production (Da Silva et al., 2016). The dietary energy concentration and protein levels are the most major consideration in broiler feed formula. The foreseeable advantages of low-protein diets include the reduction of feed costs and environmental pollution, such as nitrogen excretion and wet waste (Bodirsky et al., 2014). Studies have shown that reducing dietary protein level with no supplementing essential amino acids can exacerbate the effects of aflatoxin on broiler growth performance and nutrient digestion while increasing the intestinal permeability (Chen et al., 2016). The highly nutritious diet also affects congenital immunity; for example, the high energy diet can improve the immune suppression caused by stress in broilers (Hu et al., 2019; Patra et al., 2019). The nutrients requirement in the diet is different in broiler chickens under stress. However, it is not known whether dietary nutritional levels will have effects on intestinal barrier function, immune function, and permeability. Especially when antibiotics are reduced, the dietary level sits the ability to resist *Salmonella* infection.

Intestinal bacteria play an important role in resisting pathogenic infection, maintaining a steady-state of intestinal health and promoting nutrient absorption (Khan and Chousalkar, 2020). The nutritional level of the diet has a direct impact on the intestinal microbiota. The chyme provided nutrients for the growth of the microbiome in the gut, and the microbial metabolism can provide nutrients for the intestinal epithelial (Fang et al., 2019). Studies have shown that improving the dietary nutrition level can improve the relative abundance of *Lactobacillus* and increase the expression of the intestinal epithelial tight junction proteins of claudin-1 and occludin (Jahromi et al., 2016; Park et al., 2016). Reducing protein level can increase the relative abundance of *Proteobacteria* phylum and promote the gene expression of *NF-κB* associated with intestinal inflammation (Mukhopadhya et al., 2012). The *Lactobacillus* has a positive effect on maintaining a steady state in the intestines and is conducive to the protection against *S.* Typhimurium (Leblanc et al., 2010). Therefore, the regulation of dietary levels is of great significance to promote intestinal immunity and health.

Therefore, the purpose of this study was to systematically assess the resistance of the diet's nutritional level to the *S.* Typhimurium infection, and the effect of nutritional level on the growth performance, intestinal morphology, intestinal microbiota, and gene expression. So, it provides a theoretical basis for adjusting the nutritional level of the diet against *S.* Typhimurium infection by altering the gut microbiome.

MATERIALS AND METHODS

Experimental Design and Diet

All experimental procedures were approved by the China Agricultural University Laboratory Animal welfare and animal experimental ethical Committee. A total of 264 1-day-old female broiler chickens (Arbor Acres) were randomly assigned into 4 treatment groups, every treatment group had 6 floor pens with 11 chickens in each pen (1 m × 0.7 m). The chickens were vaccinated at hatch for Marek's disease, infectious bronchitis, and Newcastle disease. The lighting program of the chicken house was controlled at 23 h of light and 1 h of darkness. The temperature was controlled at 35°C for the first week and then gradually decreased to 25°C. The humidity was controlled at 60 to 70%. Each treatment groups received one of the following 2 × 2 diet strategies that highly nutritious diet group (**HN**), lowly nutritious diet group (**LN**), highly nutritious diet group challenged with *S.* Typhimurium (**HNST**), and lowly nutritious diet challenged with *S.* Typhimurium (**LNST**). The HN and LN pelleted dietary formulas are shown in Table 1. The HNST and LNST groups were orally gavaged with 1 mL *S.* Typhimurium stain CVCC541 (1×10^9 CFU/mL) per bird on the day 8. Birds in HN and LN groups were treated with the same volume of phosphate buffer solution as a placebo. The death of the broiler chicken was recorded daily. The feed intake and body weight gain were recorded weekly.

Sample Collection

On the days of 14 and 21, 6 broiler chickens in each treatment group were randomly selected, and their ileal contents near the cecum were collected. The intestinal mucosa tissue was isolated by scraping with the glass slide. The ileal contents and the intestinal mucosa tissue were stored at −80°C until the next step analysis. About 0.5 cm of the intestinal tract was fixed with the 4% paraformaldehyde solution for slicing to observe the intestinal morphology.

Ileal Morphology

The fixed intestinal tissue was embedded in paraffin after gradient dehydration. Then, the tissue was cut to a thickness of 5 μm and stained by hematoxylin–eosin. Ten complete intestinal morphology in each tissue slid were randomly selected to measure the villus height and crypt depth. The villus height was from the top of the intestinal mucous membrane to the opening of the intestinal gland. The crypt depth was from the opening of the intestinal gland to the base layer. The intestinal villus height and crypt depth were measure by microscope of SmartV350D and its image analysis system (Jieda Technology Development Co., Ltd., Jiangsu, China).

Total RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from the ileal mucosa tissue using the Trizol reagent according to the instruction (Invitrogen Life Technologies, Carlsbad, CA). The quality and quantity of the RNA were determined by Nano-

Table 1. The ingredients and nutrient composition of the diets (% as fed basis).

	Content[3]			Content	
Ingredients	HN	LN	Chemical composition[4]	HN	LN
Yellow corn	53.3	63.42	ME (Mcal/kg)	3.16	2.98
Soybean meal	28.5	29.15	Crude protein (%)	21.8	18.1
Corn gluten meal (60%)	7.83	0	Calcium (%)	1.00	1.00
Soybean oil	5.95	2.9	Nonphytate P (%)	0.45	0.45
Limestone	1.26	1.26	Tryptophan (%)	0.22	0.22
Dicalcium phosphate	1.95	1.95	Methionine (%)	0.51	0.50
Vitamin premix[1]	0.02	0.02	Lysine (%)	1.15	1.15
Mineral premix[2]	0.20	0.20	Threonine (%)	0.77	0.68
Salt	0.35	0.35			
DL-methionine	0.17	0.24			
L-lysine HCl	0.24	0.28			
Choline chloride	0.2	0.2			
Ethoxyquin	0.03	0.03			

[1]The trace mineral premix provides following per kg diet：Copper: 8 mg, Ferrum: 80 mg, Manganese: 100 mg, Selenium: 0.15 mg, Iodine: 0.35 mg.

[2]The vitamin premix provides following per kg diet: Vit A: 9,500 IU, Vit D3: 62.5ug, Vit E: 30IU, Vit K_3: 2.65 mg, Vit B_1: 2 mg, Vit B_6 6 mg, VitB12: 0.025 mg, Biotin: 0.0325 mg, Folic acid: 1.25 mg, Pantothenic acid: 12 mg, Nicotinic acid: 50 mg.

[3]HN, highly nutritious diet; LN, lowly nutritious diet.

[4]Crude protein and calcium were analyzed, and all other nutrient levels were calculated.

300 spectrophotometer (Yuanpinghao Biotechnology Co., Ltd., Beijing, China). The cDNA was synthesized by the Revert Aid First Strand Gene Synthesis Kit (Takara Biotechnology Co., Ltd., Dalian, China). The real-time PCR was carried out by an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) in a 20 μL reaction system, which consisted of 10 μL of SYBR Premix EX TaqTM II Kit (Takara Biotechnology Co., Ltd.), 500 nmol/L concentration of forward and reverse primers, 2 μL cDNA template, and supplemented to 20 μL with water. The PCR program was taken at 95°C for 10 min, followed by 40 cycles of that 95°C for 15 s and 60°C for 60 s. The primer sequences of the target genes are shown in Supplementary Table 1, and the *GAPDH* gene was used as the reference gene.

Permeability and Myeloperoxidase Activity of Ileal Mucosa

On the day 14, 6 broiler chickens in each treatment group were randomly selected. About 2 cm intestinal section was collected before the connection of the ileum and cecum. The intestinal section was soaked in a petri dish containing the Hanks Balanced Salt Solution (**HBSS**), the intestinal section was split by iris scissors and the contents were rinsed. The intestinal serosal layer was peeled and maintained the integrity of the mucous membrane layer. The mucous membrane was fixed in the Ussing Chamber, the mucous membrane side was added with the HBSS containing 2 mg/mL fluorescein isothiocyanate-dextran (**FITC**), the serosal side was added with HBSS. About 300 μL solution from the serosal side was collected for detecting the FITC concentration after 1 h.

On the day 21, 6 broiler chickens in each treatment group were randomly selected. These birds were gavaged with 1 mL of 10 mg/mL FITC solution. The blood was collected through wing veins after 3 h of filled, and the serum was isolated by 3,000 × g centrifugation for 10 min to detect the FITC concentration. The FITC concentration of samples and standards were measured at 485 nm and 525 nm wavelengths by a microplate reader. The permeability of the intestines was calculated according to previously reported methods (Vicuña et al., 2015). The activity of myeloperoxidase (**MPO**) in intestinal mucosa tissue was analyzed using the MPO assay kit (Nanjing Jiancheng Biological Company, Nanjing, China).

The DNA Extraction and 16sDNA Sequencing

The DNA of the ileal bacteria was isolated by the TIANamp Stool DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instruction. Each treatment group had 6 ileal content samples, every 2 samples from the same group were mixed with equivalent DNA to produce a representative sample, then every treatment group had 3 representative samples for sequencing. The V3-V4 hypervariable region of the 16sDNA was amplified by PCR. Every representative sample was labeled with a unique barcode *via* PCR. All PCR productions were mixed to create the library. The library was sequenced and generated paired-end 250 bp reads on an Illumina MiSeq platform by Novogene (Beijing Novogene Bioinformatics Technology Co., Ltd.). The raw data were upload to the Sequence Read Archive of NCBI, and the BioProject ID is PRJNA597067.

Data Processing

The sequencing data were analyzed using the QIIME, version 1.7.0 (Caporaso et al., 2010). In brief, a total of 564,801 raw reads were merged using FLASH and assigned to a sample according to the barcode (Zhang

et al., 2014), then the barcode and primer were removed. The sequence quality control was filtered by QIIME. Then 549,119 sequences were retained for downstream analyses, the mean and median reads were 45,760 and 40,323 tags per sample, respectively. The remaining quality-filtered reads were clustered (97% similarity threshold) into operational taxonomic units (Edgar, 2013). All tags were aligned and used to create a phylogeny with fasttree2 (Price et al., 2010). Alpha diversity, beta diversity of weighted UniFrac (Lozupone and Knight, 2005), and the principle coordinate analysis (**PCoA**) were estimated using QIIME2 diversity. Taxonomy was assigned to amplicon sequence variants using the QIIME2 feature classifier against the Greengenes 13_8 99% operational taxonomic unit reference sequences (Mcdonald et al., 2012).

Statistical Analysis

The 2-factor ANOVA analysis was carried out using the general linear model of SPSS17.0 software (IBM Corporation, Armonk, NY). The difference significance of the main effect and the interaction effect were carried out at the significance level of $P < 0.05$. If the interaction effect was a significant difference, the Duncan's was used to make multiple comparisons for each group.

The correlation between the intestinal other indexes and the relative abundance of intestinal bacteria were analyzed using the Spearman method by the corr package in the R software, whereas the correlation heat map was produced using the pheatmap package.

RESULTS

Feed Intake and Growth Performance

The effect of the nutritional level and *S.* Typhimurium infection on the feed intake and growth performance in broiler chickens are shown in Table 2. In the days 1 to 14, the highly nutritious diet significantly increased the body weight gain of the broiler chickens ($P < 0.05$). The *S.* Typhimurium significantly increased body weight gain and feed intake ($P < 0.05$). There was an interaction effect between dietary nutrition level and *S.* Typhimurium in feed conversion ratio ($P < 0.05$). In the day 1 to 21, the highly nutritious diets significantly increased feed intake ($P < 0.05$). The diet nutritional level of *S.* Typhimurium had a significant interaction effect on the body weight gain and feed conversion ratio ($P < 0.05$). The HNST group had the highest body weight gain that significantly higher than the other 3 groups ($P < 0.05$). The body weight gain in the HN group was significantly higher than that in the lowly nutritious diet ($P < 0.05$). The highly nutritious diet groups significantly reduced the feed conversion ratio ($P < 0.05$). Over 15 to 21 d, highly nutritious diets had tendency to reduce the mortality of the broiler chickens ($P = 0.078$).

Ileal Morphology

The effects of dietary nutritional level and *S.* Typhimurium infection on intestinal morphology are shown in Table 3. *S.* Typhimurium reduced the ratio of villus height to crypt depth (**V/C**). On the days 14 and 21, highly nutritious diets significantly increased the villus height, crypt depth, and V/C ($P < 0.05$).

Gene Expression of Ileal Epithelial Tissue

On the day 14, the effects of dietary nutritional levels and *S.* Typhimurium infection on gene expressions of the ileal epithelium are presented in Table 4. The highly nutritious diets significantly increased the gene expression of claudin-1, occludin*g*, and *NF-κB* but reduced the gene expression of the *IFN-γ* ($P < 0.05$). The *S.* Typhimurium significantly inhibited the expression of the claudin-1 gene and enhanced the expression of the *IFN-γ*, *TLR4*, and *NF-κB* gene ($P < 0.05$). The diet nutritional level and *S.* Typhimurium had an interaction effect on the gene expression of mucin-2 and *MyD88*. The expression of the mucin-2 and *MyD88* genes in the *S.* Typhimurium groups was significantly higher than that in the uninfected groups, whereas the LNST group was significantly higher than the HNST group ($P < 0.05$).

On the day 21, the effects of dietary nutritional level and *S.* Typhimurium infection on gene expression of

Table 2. The effects of diets and *S.* Typhimurium on the growth performance in broiler chickens.

	1–14 d			1–21 d			15–21 d
Treatment[1]	Body weight gain (g)	Feed intake (g)	Feed conversion ratio	Body weight gain (g)	Feed intake (g)	Feed conversion ratio	Mortality (%)
HN	234.56	312.46	1.36^{a}	507.91^{a}	825.40	1.57^{a}	0
LN	219.14	341.84	1.49^{b}	451.19^{b}	819.42	1.80^{b}	1.52
HNST	272.33	353.17	1.30^{a}	580.10^{c}	877.27	1.51^{a}	0
LNST	228.25	343.26	1.53^{b}	444.81^{b}	806.92	1.90^{b}	3.03
SEM	5.74	5.74	0.02	13.35	10.04	0.04	0.01
P-value							
Feed	0.001	0.247	<0.001	<0.001	0.038	<0.001	0.078
*S.*Typhimurium	0.005	0.040	0.559	0.035	0.183	0.914	0.542
Feed*S. Typhimurium	0.064	0.063	0.013	0.009	0.075	0.005	0.542

The different superscripts on a same column denote differences between means for $P < 0.05$.

[1]HN, highly nutritious diet group; LN, lowly nutritious diet group; HNST, highly nutritious diet group challenged with *S.* Typhimurium on 8 d of age; LNST, lowly nutritious diet challenged *S.* Typhimurium on the 8 d of age.

Table 3. The effects of diets and *S.* Typhimurium on ileal morphology in broiler chickens.

	Day 14			Day 21		
Treatment[1]	Villus height (μm)	Crypt depth (μm)	V/C[2]	Villus height (μm)	Crypt depth (μm)	V/C
HN	634.43	170.17	3.79	591.17	156.64	3.96
LN	539.13	157.09	3.44	518.29	151.13	3.36
HNST	612.40	169.46	3.59	594.07	162.37	3.69
LNST	536.47	159.39	3.36	495.92	146.46	3.41
SEM	11.92	2.14	0.04	11.14	2.75	0.06
P-value						
Feed	<0.001	0.005	<0.001	<0.001	0.0504	<0.001
S. Typhimurium	0.916	0.832	0.023	0.881	0.812	0.252
Feed*S. Typhimurium	0.499	0.689	0.255	0.343	0.332	0.090

[1]HN, highly nutritious diet group; LN, lowly nutritious diet group; HNST, highly nutritious diet group challenged with *S.* Typhimurium on 8 d of age; LNST, lowly nutritious diet challenged *S.* Typhimurium on the 8 d of age.
[2]V/C, the ratio of villus height to crypt depth.

the ileal epithelium are presented in Table 5. The highly nutritious diet groups increased the expression of claudin-1, occludin and *NF-κB* genes and significantly inhibited the expression of mucin-2 and *MyD88* genes ($P < 0.05$). The *S.* Typhimurium significantly reduced the expression of claudin-1 and occludin genes and increased the expression of the *TLR4*, *NF-κB*, and *MyD88* genes ($P < 0.05$). The diet nutritional level and *S.* Typhimurium had an interaction effect on the gene expression of *IFN-γ*, which in the HN group was significantly higher than that in the other 3 treatment groups ($P < 0.05$).

The Permeability and MPO Activity of the Intestine

On the day 14, the intestinal permeability in the LNST group was significantly lower than that in the other 3 groups, whereas the activity of MPO was significantly higher than in the other 3 groups (Figure 1). On the day 21, there were significant differences in intestinal permeability between the treatment groups. The LN group had the lowest intestinal permeability, whereas the LNST group had significantly higher permeability than the other 2 groups. The MPO activity in the LNST group was significantly higher than in the other 3 groups ($P < 0.05$).

Alpha Diversity of Intestinal Bacteria

There were significant differences in dietary nutritional levels and *S.* Typhimurium infections on the diversity indexes of the gut bacterial community (Figure 2). The *S.* Typhimurium infection significantly reduced the intestinal bacterial diversity indexes of observed_species, chao1, and Shannon ($P < 0.05$). The HN group significantly improved the intestinal bacterial diversity indexes of observed_species and Shannon. The diversity indexes of observed_species, chao1, and Shannon in LN group were significantly higher than those in the HNST group ($P < 0.05$). The diversity index of chao1 in the HNST group was significantly lower than the LNST group.

Taxonomic Analysis

The composition and relative abundance of the ileal bacterial community are shown in Figure 3. At the phylum level, *Firmicutes* and *Proteobacteria* were the main dominant bacteria, accounting for 44.9 and 41.3% of all the ileal bacteria, respectively (Figure 3A). The abundance of phylum *Firmicutes* was not significant among the 4 treatment groups. The relative abundance of phylum *Proteobacteria* in each treatment group was various, the HN group had the lowest abundance accounting for 36.2%. While the LN group had the highest abundance of *Proteobacteria* about 47.3%. Bacteria with a relative abundance of more than 1% at

Table 4. The effects of diets and *S.* Typhimurium on the gene expression of intestinal epithelium in broiler chickens on the day 14.

Treatment[1]	Claudin-1	Occludin	Mucin-2	*IFN-γ*	*TLR2*	*TLR4*	*NF-κB*	*MyD88*
HN	1.36	1.07	1.11[a]	1.04	1.12	0.92	1.06	0.81[a]
LN	0.79	0.74	0.85[a]	1.39	0.97	1.00	0.85	0.82[a]
HNST	0.81	1.00	1.54[b]	1.33	0.99	1.33	2.21	1.39[b]
LNST	0.31	0.79	2.08[c]	1.6	1.32	1.34	2.05	1.66[c]
SEM	0.11	0.04	0.11	0.06	0.05	0.07	0.15	0.09
P-value								
Feed	<0.001	0.001	0.057	0.001	0.473	0.641	0.004	0.024
S. Typhimurium	<0.001	0.467	<0.001	0.004	0.552	0.002	<0.001	<0.001
Feed*S. Typhimurium	0.784	0.320	<0.001	0.566	0.033	0.743	0.687	0.034

The different superscripts on a same column denote differences between means for $P < 0.05$.
[1]HN, highly nutritious diet group; LN, lowly nutritious diet group; HNST, highly nutritious diet group challenged with *S.* Typhimurium on 8 d of age; LNST, lowly nutritious diet challenged *S.* Typhimurium on the 8 d of age.

Table 5. The effects of diets and *S.* Typhimurium on the gene expression of intestinal epithelium in broiler chickens on the day 21.

Treatment[1]	Claudin-1	Occludin	Mucin-2	*IFN-γ*	*TLR2*	*TLR4*	*NF-κB*	*MyD88*
HN	1.09	1.16	1.14	0.87[a]	1.11	1.34	1.16	0.84
LN	0.80	0.80	1.32	1.79[b]	0.90	1.6	0.81	0.92
HNST	0.86	0.90	1.15	1.40[b]	1.05	1.62	1.24	1.02
LNST	0.65	0.74	1.46	1.75[b]	1.07	1.74	1.05	1.14
SEM	0.052	0.052	0.055	0.105	0.048	0.058	0.049	0.034
P-value								
Feed	0.004	0.003	0.029	<0.001	0.398	0.076	0.001	0.029
S. Typhimurium	0.019	0.029	0.490	0.008	0.657	0.041	0.037	<0.001
Feed*S. Typhimurium	0.515	0.192	0.522	0.022	0.287	0.471	0.243	0.588

The different superscripts on a same column denote differences between means for $P < 0.05$.
[1]HN, highly nutritious diet group; LN, lowly nutritious diet group; HNST, highly nutritious diet group challenged with *S.* Typhimurium on 8 d of age; LNST. lowly nutritious diet challenged *S.* Typhimurium on the 8 d of age.

the phylum level also contained *Cyanobacteria*, *Acidobacteria*, and *Actinobacteria*, accounting for 6.9, 1.7, and 1.4%, respectively. The *S.* Typhimurium group significantly increased the relative abundance of phylum *Cyanobacteria*.

At the genus level, the main dominant bacteria were *Lactobacillus* and *Helicobacter*, accounting for 37.1 and 31.7%, respectively (Figure 3B). The difference of genus *Lactobacillus* among the groups was significant, with the lowest relative abundance at 34.8% in the LN group and the highest abundance at 40.8% in the HNST group. The relative abundance of genus *Helicobacter* was widely varied among the treatment groups, the HN group had the lowest abundance of *Helicobacter* accounting for 16.8% and the LN group had the highest abundance at 41%.

Beta Diversity of Intestinal Bacteria

The PCoA showed that the ileal bacterial community of the high energy group was significantly different from the other groups, and the samples in high energy group clustered together (Supplementary Figure 1). The PC1 and PC2 of the PCoA were interpreted at 34.01 and 33.52%, respectively. There was also significant

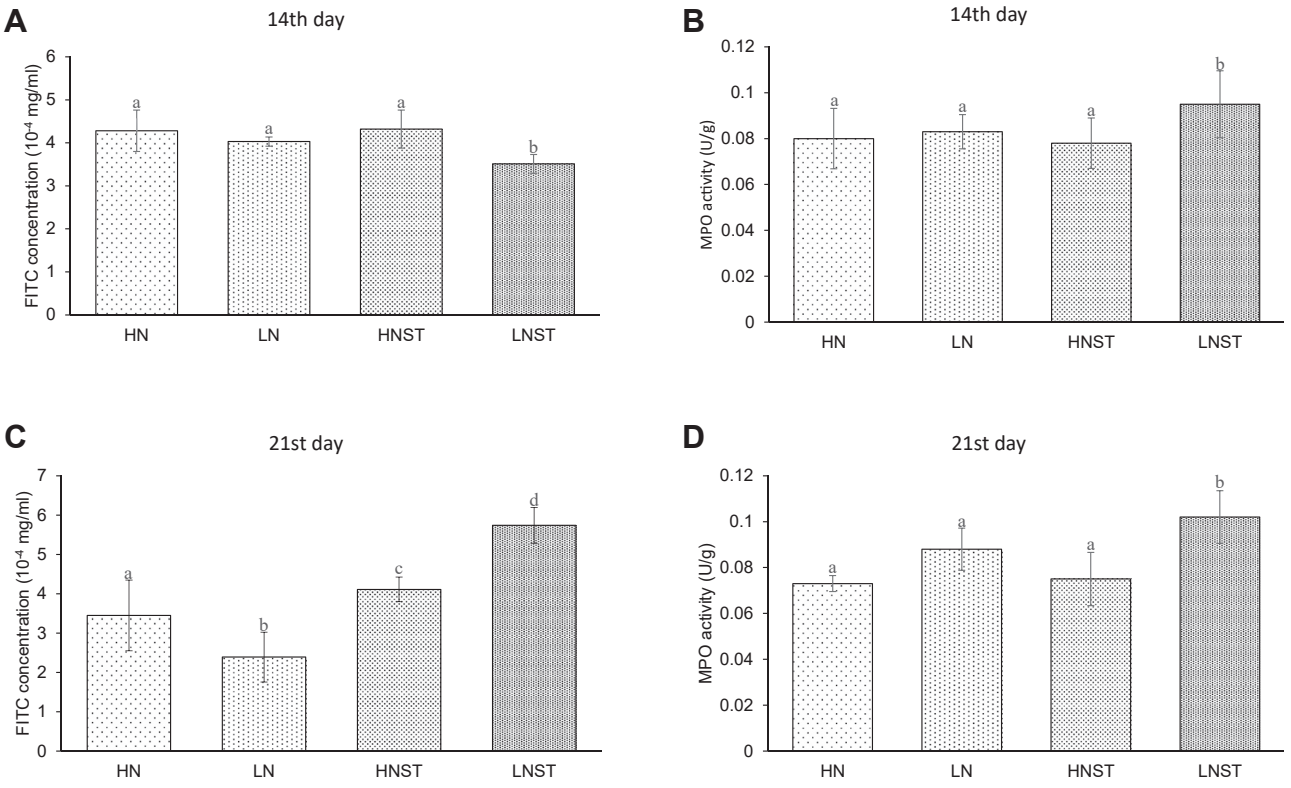

Figure 1. The effects of diets and *S.* Typhimurium on the mucosal permeability of fluorescein isothiocyanate (FITC) on the days of 14 (A) and 21 (C), and myeloperoxidase (MPO) activity on the days of 14 (B) and 21 (D) in broiler chickens. Small alphabetic letters show significance ($P < 0.05$). Abbreviations: HN, highly nutritious diet group; LN, lowly nutritious diet group; HNST, highly nutritious diet group challenged with *S.* Typhimurium on 8 d of age; LNST, lowly nutritious diet challenged *S.* Typhimurium on the 8 d of age.

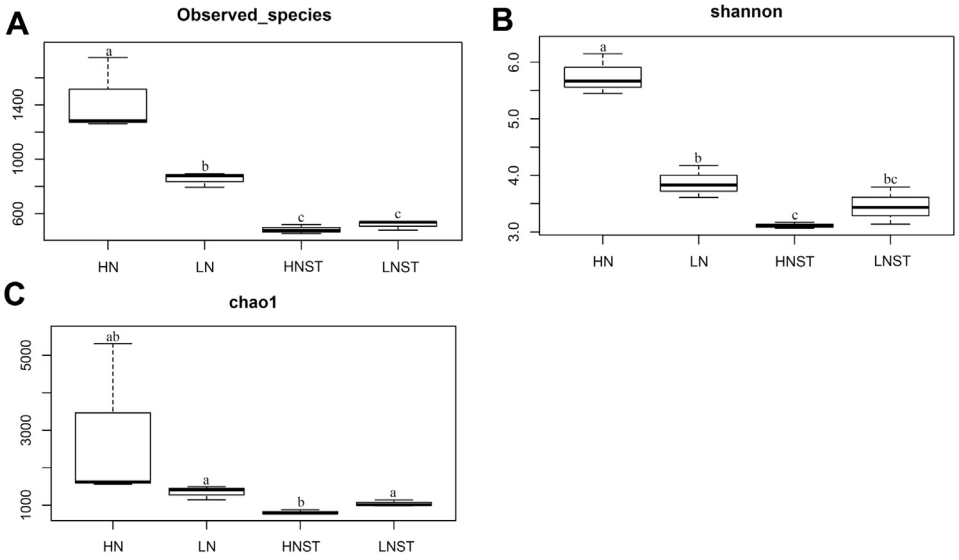

Figure 2. The effects of diets and *S.* Typhimurium on the ileal microbiota diversity indexes of observed_species (A), chao1 (B), and Shannon (C) on the 14th d. Small alphabetic letters show significance when $P < 0.05$. Abbreviations: HN, highly nutritious diet group; LN, lowly nutritious diet group; HNST, highly nutritious diet group challenged with *S.* Typhimurium on 8 d of age; LNST, lowly nutritious diet challenged *S.* Typhimurium on the 8 d of age.

clustering in *S.* Typhimurium groups and unfilled *S.* Typhimurium groups.

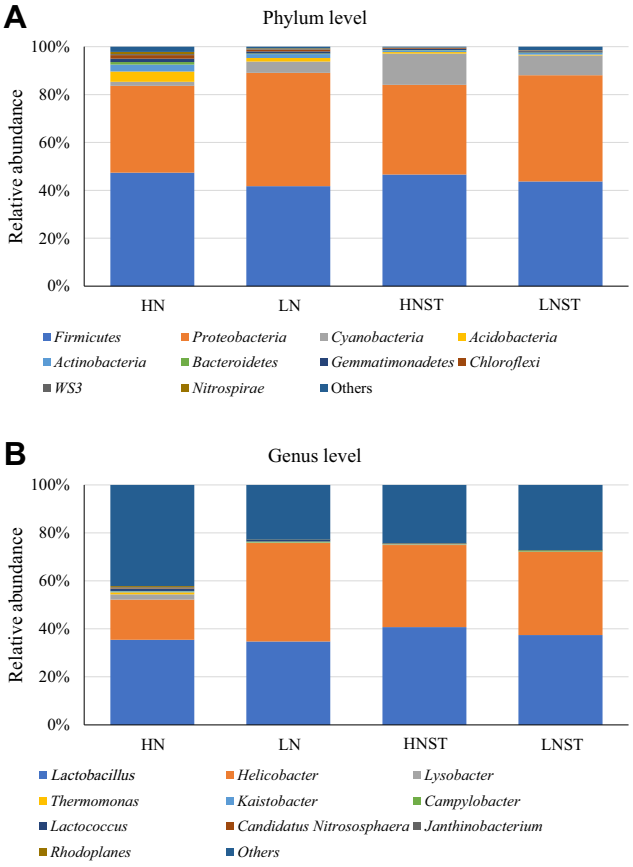

Figure 3. The effects of diets and *S.* Typhimurium on the relative abundances of ileal microbiota at the phylum level (A) and genus level (B) on the day 14. Abbreviations: HN, highly nutritious diet group; LN, lowly nutritious diet group; HNST, highly nutritious diet group challenged with *S.* Typhimurium on 8 d of age; LNST, lowly nutritious diet challenged *S.* Typhimurium on the 8 d of age.

Correlation Analysis

The relative abundance of intestinal bacteria at the genus level was correlated with other indicators of the intestine (Figure 4). *Lactobacillus* bacteria as the most abundant bacteria was not correlated with other indicators. *Helicobacter* bacteria had a significant positive correlation with IFN-γ, while had a significant negative correlation with the crypt depth and the expression of the occludin gene ($P < 0.05$). The relative abundance of *Campylobacter* bacteria was positively correlated with IFN-γ and significant negative correlation with occludin, villus height, and V/C ($P < 0.05$). It was interesting to note that *Lysobacter* bacteria was positively correlated with body weight gain, NF-κB, V/C, villus height, claudin-1, occludin, and significant negative correlation with IFN-γ and feed conversion ratio ($P < 0.05$).

DISCUSSION

In this study, the highly nutritious diet is beneficial to reduce the feed conversion ratio and body weight gain of broiler chicken because the HN groups in this study not only have high energy concentration but also contain high protein levels, compared with the LN diet groups. As the feed intake was not different, the diet with a high nutritional level is more beneficial to improve body weight gain and reduce the feed conversion ratio. Similar results have been reported before (Ale Saheb Fosoul et al., 2018; Khoddami et al., 2018). The nutritional level of diet and *S.* Typhimurium infection in the early stage tended to have an interaction effect on feed conversion ratio and body weight gain. The high-energy diets can improve the production performance when applied under S. Typhimurium infection conditions. But as the infection went on, the

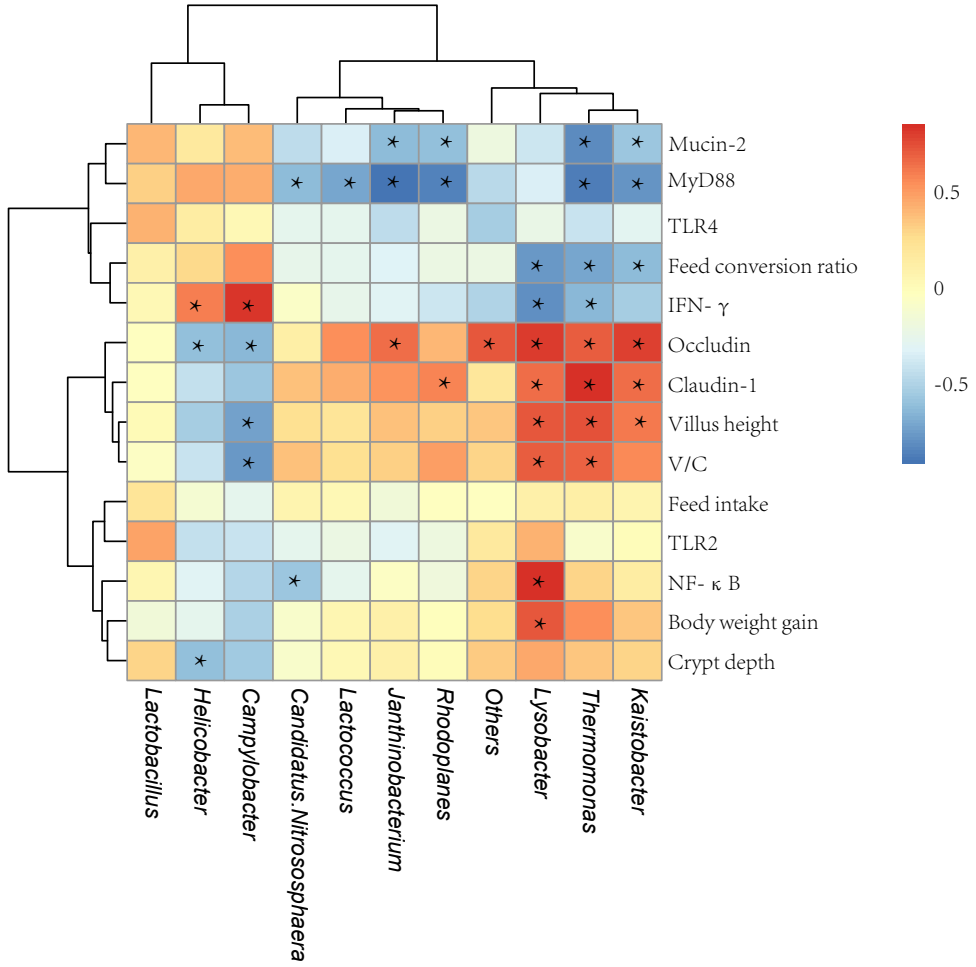

Figure 4. The correlations between the ileal microbiota and other indexes. The lattices were colored based on Spearman's rank correlation analysis. The red cells indicate positive correlation, and the blue color cells indicate negative correlation. * indicates a significant correlation ($P < 0.05$).

effect of the promotion gradually weakened. This suggested that the initial stage of *S.* Typhimurium infection does not have an adverse effect on the growth of broilers. Studies have shown that broilers at different ages and feeding strategies have different effects on resistance to *S.* Typhimurium invasion (Kim et al., 2018). In early growth stages, chickens challenge with *S.* Typhimurium often have serious consequences due to the immature intestinal microbiota and imperfect immune system (Withanage et al., 2004). As age older, the gut bacteria and immune system of broilers become mature, and their ability to against *S.* Typhimurium infections increases. The broiler feeding strategy is important for against *S.* Typhimurium infection, especially in the trend of antibiotics being gradually banned. This study shown that broiler chickens feed with a high nutritional level diet can protect against *S.* Typhimurium infection and help improve growth performance. This indicated that feeding a highly nutritious diet in broiler chicken had a positive effect on immunity. The LN diet did not protect broiler chickens from *S.* Typhimurium infection, which may be because of the fact that *S.* Typhimurium infection requires higher nutritious levels to compensate for the need for amino acids (Polansky et al., 2018).

A perfect and healthy intestinal morphology is of great significance for the health of broiler chicken and the improvement of growth performance. Higher villus height and lower crypt depth are beneficial to the absorption of nutrients in the intestines as it provides more surface area and higher absorption rate (Ni et al., 2012). This study showed that a highly nutritious diet can increase the villus height, crypt depth, and increase the ratio of villus height to crypt depth. This explained that a highly nutritious diet reduced the feed conversion ratio. *S.* Typhimurium remarkably reduced the ratio of villus height to crypt depth on the day 14, and this difference disappeared on the day 21, indicating that the adverse effects of *S.* Typhimurium infection decreased as the age increased. Previous studies have shown that increasing the energy concentration of diet is beneficial to intestinal development and improves intestinal health (Ale Saheb Fosoul et al., 2016). It was the reason that the *S.* Typhimurium did not cause serious consequences in the highly nutritious diet of broiler chickens. In this study, lowly nutritious diet inhibited the intestinal development of broilers, thus causing lowly nutritious

diets to negatively affect body weight gain and feed conversion ratio. Previous studies have shown that low-protein amino acid-supplemented diets do not adversely affect the intestinal morphology and growth properties of broilers (Amiri et al., 2019), which indicates that amino acid balance is important for the intestinal morphology development of broiler chickens and that a simple reduction of the protein level of the diet will have a negative effect on the growth of broilers.

The claudin-1 and occludin genes play an important role in maintaining the tight junction of intestinal epithelial cells (Brufau et al., 2016), and this study showed that highly nutritious diets were conducive to promoting tight junction between the intestinal epithelial cells on the days 14 and 21, which inhibited the invasion of pathogenic bacteria and the permeation of toxins. It is shown that high nutritional level diets can resist the invasion of pathogenic bacteria by increasing the tight junction (Kappala et al., 2018). *S.* Typhimurium infection can significantly inhibit the expression of claudin-1 gene on the day 14, inhibit the expression of claudin-1 and occludin genes on the day 21, indicating that *S.* Typhimurium can destroy the tight junction between intestinal epithelial cells. Previous studies have shown that highly nutritious diets promote intestinal health by meeting the increased demands for energy (Khoddami et al., 2018; Tickle et al., 2018), which were consistent with this study. The mucin-2 gene is of strict importance for ensuring the integrity of the intestinal mucosa (Sluis et al., 2006). This study showed that *S.* Typhimurium increased the expression of the mucin-2 gene on the day 14, but it had no difference on the day 21. *S.* Typhimurium can improve mucosal hyperplasia in the early stages of invasion, whereas excessive mucosa can inhibit nutrient absorption, which resulted in the lowest growth properties in the LNST group.

IFN-γ gene is of great significance for promoting immunity in broiler chickens (Pourabedin et al., 2016). However, the excessive gene expression, such as in the case of bacterial infection and inflammation, can cause autoimmune damage to the body, reducing animal growth performance and increasing nutritional consumption (Hakansson and Molin, 2011). *S.* Typhimurium infection and a lowly nutritious diet significantly increased *IFN-γ* gene expression in this study, suggesting that these stresses inhibited the absorption of nutrients in the intestines.

TLR2 and *TLR4* act as the receptor of lipopolysaccharide which is the specific antigen on cell membranes of gram-negative bacteria such as *S.* Typhimurium and *E. coli*(Cao et al., 2018), capable of synthesizing and producing excessive amounts of preinflammatory cytokines *NF-κB* (Qiutang and Verma, 2002; Vallabhapurapu and Karin, 2009). During the above process, *MyD88* plays the role of intermediate signal transmission (Kawai et al., 1999). In this study, The *TLR4/MyD88/NF-κB* signaling pathway can be activated the inflammatory response to *S.* Typhimurium infection. At the same time, a highly nutritious diet can improve the expression of *NF-κB* gene to improve the anti-inflammatory effect. The anti-inflammatory effect of the birds can consume the energy and protein to meet the additional metabolic demand, indicating that the lowly nutritious diet can inhibit the resistance of broilers to *S.* Typhimurium. These results explained that under the challenge of *S.* Typhimurium, highly nutritious diets can guarantee higher growth properties for broilers.

The permeability of the intestine is closely related to nutrients and resistance to pathogenic bacteria. Maintaining appropriate intestinal permeability is helpful to improve the absorption of nutrients and to prevent pathogen invasion in broiler chickens (Awad et al., 2012). The LNST group had the lowest permeability on the day 14 in this study, which showed that the LNST group was able to inhibit the absorption of nutrients and inhibit the invasion of pathogens such as *S.* Typhimurium. On the day 21, the LNST group had the highest FITC permeability, which may increase *S.* Typhimurium intrusion. The permeability of the HNST group was higher than that of the unfilled *S.* Typhimurium groups, which explained that the HNST group had the best growth performance. As a derived white cell enzyme, MPO can catalyze the production of many reactive oxidants. The MPO effectively removes H_2O_2 on the surface of *S.* Typhimurium, which was converted to a highly active HOCl (Nicholls and Hazen, 2005). The LNST group had significantly higher MPO enzyme activity than other groups, indicating that *S.* Typhimurium can significantly increase inflammatory responses in broiler chickens fed with low nutritional level diet, whereas broiler chickens fed with high-nutrient diets can reduce inflammation.

A complex and stable intestinal microbiota is important for maintaining intestinal health and promoting growth of the chickens (Ducatelle et al., 2018). A high intestinal bacteria diversity is beneficial to maintain intestinal stability. In this study, the challenge with *S.* Typhimurium significantly reduced the diversity of the intestinal bacterial community. It was shown that *S.* Typhimurium infection in the early stage can significantly inhibit the growth of other gut microbes and reduce the diversity of the community (He et al., 2019). Highly nutritious diets can significantly increase diversity indexes, indicating that high levels of nutrition can maintain intestinal health. Previous studies have shown that feed energy was negatively correlated with ileal bacteria class of Firmicutes (Stanley et al., 2013).

In terms of the composition of the microbiota, this study showed that *Firmicutes* and *Proteobacteria* were the main dominant bacteria in the ileum, which was consistent with previous studies that these 2 bacteria accounted for up to 85% of all bacteria in the bird gut (Choi et al., 2018). Lowly nutritious diet increased the relative abundance of *Proteobacteria* phylum which as the gram-negative microbiome contains a variety of pathogenic bacteria and produces lipopolysaccharides to cause the inflammatory response in the host (Mukhopadhya et al., 2012). The *S.* Typhimurium infection can increase the relative abundance of harmful phylum *Cyanobacteria*, which can synthesize toxins to produce adverse effects on animals (Zi et al., 2017). At

the genus level, the nutritional level has an important effect on the level of the genus. The highly nutritious diet can significantly reduce the genus *Helicobacter*, increase the other top10 genus bacteria except for the genus of *Helicobacter* and *Lactobacillus*. This also led to a significant classification of HN group with other groups in PCoA analysis. The HNST group had the highest abundance of genus *Lactobacillus*, which can improve the growth performance of broiler chicken and resist *S.* Typhimurium infections as probiotics (Jahromi et al., 2016; Park et al., 2016).

The study showed that there were correlations between intestinal bacteria and various indicators of broiler chickens. The relative abundance of intestinal bacteria in this study was significantly related to the gene expression, intestinal morphology, and growth performance of intestinal mucosa. This may be because the intestinal bacteria directly or indirectly are involved in the development of intestinal epithelial cells. *Lysobacter* bacteria were positively correlated with body weight gain, depending on their ability to produce antibiotics, dissolve pathogenic bacteria, and improve the intestinal morphology of broilers (Laborda et al., 2017). Therefore, it led to the *Lysobacter* was positively correlated with the body weight gain, V/C, villus height, and the gene expression of *NF-κB*, claudin-1, and occludin.

CONCLUSIONS

Broiler chickens fed with the highly nutritious diet can increase the weight gain and reduce feed conversion ratio compared with the lowly nutritious diet. The highly nutritious diet was beneficial to against *S.* Typhimurium infection by increasing *Lactobacillus* abundance, improving the ratio of villus height to crypt depth in intestinal morphology, and activating the *TLR4/MyD88/NF-κB* signal pathway. The lowly nutritious diet decreased the resistance to *S.* Typhimurium by increasing the intestinal pathogenic bacteria and improving MPO activity in broiler chickens. *S.* Typhimurium infection can significantly reduce the diversity index of the gut bacterial community and alter the structure of the microbiome.

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DISCLOSURES

The authors declare that there is no conflict of interest.

SUPPLEMENTARY DATA

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