The differences in intestinal growth and microorganisms between male and female ducks

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ABSTRACT There are great differences in physiological and biological functions between animals of different sexes. However, whether there is a consensus between sexes in duck intestinal development and microorganisms is still unknown. The current study used Nonghua ducks to estimate the effect of sex on the intestine by evaluating differences in intestinal growth indexes and microorganisms. The intestines of male and female ducks were sampled at 2, 5, and 10 wk from the duodenum, jejunum, ileum, and cecum. Then, the intestinal length and weight were measured, the morphology was observed with HE staining, and the intestinal content was analyzed by 16S rRNA sequencing. The results showed that male ducks have shorter intestinal lengths with higher relative weights/relative lengths. The values of jejunal villus height (**VH**)/crypt depth (**CD**) of female ducks were significantly higher at 2 wk, whereas the jejunal VH/CD was significantly lower at 10 wk. There was obvious separation of microorganisms in each intestinal segment of ducks of different sexes at the 3 time periods. The dominant phyla at different stages were Firmicutea, Proteobacteria, Bacteroidetes, and Actinobacteria. The duodenal Chao index at the genus level of male ducks was significantly higher at 10 wk than that of female ducks. Significantly different genera were found only in the jejunum, and the abundances of *Escherichia_Shigella*, *Pseudomonas*, *Clostridium_sensu_stricto_1*, *Sphingomonas*, and *Desulfovibrio* in male ducks were higher than those in female ducks, whereas the abundance of *Rothia* was lower, and the abundance of viral infectious diseases, lipid metabolism, metabolism of terpenoids and polyketides, parasitic infectious diseases, xenobiotic biodegradation and metabolism, cardiovascular disease, and metabolism of other amino acids in male ducks were higher than that in female ducks, whereas gene folding, sorting and degradation pathways, and nucleotide metabolism were lower. This study provides a basic reference for the intestinal development and microbial symbiosis of ducks of different sexes.

Key words: duck, sex, intestine, microorganism

INTRODUCTION

There are great differences in hormone secretion, energy metabolism, and immune responses as well as intestinal parameters (Chaloner and Greenwood-Van Meerveld, 2013; Steegenga et al., 2014) among animals of different sexes (Koohpeima et al., 2018). The sex of poultry is directly related to many economic traits. For example, male individuals always hold faster growth rates and higher feed returns in the production of meat poultry, whereas the economic benefits of egg production can be obtained by only raising female individuals. The intestinal tract is an important part of the body's defense system and the main site for the digestion and absorption of nutrients of feed (Garro et al., 2018). The functions of poultry intestines are mainly carried out in the small intestine, and the relative weight, length, and density of the small intestine are important indexes to measure the development of the intestine. It has been found that 1-day-old broilers have more significant advantages than hens in intestinal growth (Gonzales et al., 2003), and the intestinal weight of 7-wk-old Cobb broilers and 4-wk-old Ross 308 broilers also showed a similar performance (Marcato et al., 2006). Morphometric techniques are utilized to quantify alterations in microscopic intestinal parameters in poultry occurring with age in response to various rearing systems, diets, and

management practices and as a quantitative tool to supplement routine subjective histological evaluations of gut pathology (Awad et al., 2008; Nassiri Moghaddam and Alizadeh-Ghamsari, 2013). The determination of intestinal villus to crypt ratios is a common method utilized to evaluate effects (Abdelqader and Al-Fataftah, 2016). Conventionally domesticated animals have higher small intestine weights because of their thicker walls, longer villi, and deeper crypts, which allow infiltration of immune and connective tissue, than do wild animals (Coates, 1980).

More than 1×10^6 microbial genes have been found in the poultry gastrointestinal tract, which is $40 \sim 50$ times that of the whole chicken genome, whereas $90 \sim 95\%$ of cecal microorganisms cannot be cultured in the laboratory environment (Yang et al., 2017). Poultry intestinal microorganisms are related to the structure of diet, age, sex, and individual specific conditions, and the composition of microbial flora in different intestinal segments also varies (Mao et al., 2018a; Jang et al., 2019). Thirty species of intestinal microorganisms significantly affected by host sex, such as *Lactobacillus*, *Lactococcus,* and *Actinomyces*, were found in high body weight line families of chickens ($P < 0.05$), and 17, such as *Lactobacillus*, *Acinetobacter,* and *Brachybacterium*, were found in low body weight line families ($P < 0.05$) (Zhao et al., 2013).

It can be noted that there are multiple ways to control intestinal growth and microbial proliferation. Although many studies have addressed the effects of sex on intestinal development and microorganisms, only limited data are available on intestinal development and microbiota in duck models. Ducks have good environmental adaptability, strong disease resistance, a low sebum rate, and good meat quality and have been highly popular with consumers (Choi et al., 2015). Studying the differences in intestinal development and microorganisms in ducks of different sexes can enrich the basic biological data of ducks and provide reference values for related research.

MATERIALS AND METHODS

Laboratory Animals and Sample Collection

The Nonghua duck used in this experiment was provided by the poultry experimental farm of Sichuan Agricultural University. Thirty healthy male ducklings and 30 healthy female ducklings were mixed in a unified way to ensure that each duck ate and drink similarly at liberty during the experiment, and all the test samples received routine immunization with no antibiotic treatment. A commercial duckling diet was supplied ad libitum for 0 to 3 wk, and the normal growth feed for ducks was supplied after that (Table 1). The ducks were transferred to a shed for online flat breeding and gradually transferred to natural light conditions. The temperature maintained during the first 3 d was approximately 31°C, and this was gradually lowered to ambient temperature by the 7th d. Ten ducks of each sex were

Table 1. The ingredients of the diet given to the ducks at different stages.

Ingredients	Weeks	
	$0 \sim 3$	$3 \sim 10$
Crude protein (%)	19.50	17.00
Crude fat (%)	3.64	4.00
Crude fiber (%)	3.59	3.88
Crude ash (%)	6.53	6.05
Calcium (%)	0.90	0.80
Total phosphorus (%)	0.73	0.70
Available phosphorus (%)	0.42	0.38
Metabolizable energy (Mcal/Kg)	2,850.00	2,800.00
Total lysine (%)	1.00	0.80
Total methionine (%)	0.42	0.38
Total cystine (%)	0.32	0.29
Total threonine (%)	0.74	0.63
Total tryptophan (%)	0.27	0.23
Total sulfur amino acids (%)	0.74	0.67

n = 60.

randomly selected and weighed at 2, 5, and 10 wk (Table 2) and then killed by cervical dislocation after fasting for 12 h. The intestines were sampled for measurements. The duodenum is located on the right side of the abdominal cavity at the opening of the bile duct and pancreatic duct and from the yolk sac to the pylorus is the jejunum. The ileum only refers to the segment where the mesentery connects with the cecum. The opening of the cecum is behind the junction of the ileum and rectum, and the body narrows gradually to form an apex. The intestinal contents were dipped into liquid nitrogen for transportation and stored at −80°C (Thermo, Waltham, MA) in the laboratory, and remaining contents were subsequently treated as nonhazardous waste. All animal handling procedures were approved by the Sichuan Agricultural University Animal Welfare Committee (Ya'an, China).

Measurement of Intestinal Growth

Fixed ends of the duodenum, jejunum, ileum, and cecum were placed on a glass plate moistened with distilled water after removal of the pancreas, fat, and other tissues attached to the intestinal tract. Then, the plate was straightened slightly, and the length was measured with a ruler when the intestinal tract recovered and no longer retracted. Filter paper was used to dry the intestinal segments after the intestinal contents were squeezed out, and the intestines were weighed by electronic balance. Multiple measurements were taken, and the average value was used. The relative length (**RL**, cm/kg) and relative weight (**RW**, g/kg) of the

Table 2. Weight of male and female ducks at different stages.

Weeks	Male	Female	P value
2	0.42 ± 0.03	0.45 ± 0.03	0.20
5	1.66 ± 0.22	1.74 ± 0.13	0.22
10	3.06 ± 0.16	2.80 ± 0.24	0.05

M refers to male ducks, F refers to female ducks. The P value represents the significance between the values in the 2 lines preceding it. n = 10.

intestine were calculated according to the following formula.

RL = intestinal length (cm)/live weight (kg)

RW = intestinal weight (g)/live weight (kg)

The middle parts of the duodenum, jejunum, and ileum were cut into 1-cm pieces, washed with normal saline, fixed in 4% polyformaldehyde solution (Solarbio, Beijing, China), embedded in paraffin, sectioned, and stained with hematoxylin dyeing solution (Solarbio). Microscopic images were taken at 200 × (Nikon, Tokyo, Japan). Three replicates were made for each intestinal segment, 3 different visual fields were used for each replicate, 3 complete and straight villi and crypts were chosen for each photo for measurement, and finally, the average values were taken for comparison. Villus height (**VH**), crypt depth (**CD**), and intestinal wall thickness (**WT**) of the duodenum, jejunum, and ileum were measured and recorded by Image-Pro Plus 6.0 software, and the ratio of VH to CD was calculated. The vertical distance from the top of villus to the opening of the crypt was defined as VH (μm), from the bottom of the crypt to the transition area between the crypt and villus was defined as CD (μm), and the longest straight line perpendicular to the tangent line of intestinal wall was defined as IWT (μm).

DNA Extraction and 16S rRNA Gene Sequencing

A Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) was used to extract the total bacterial DNA of the cecum according to the manufacturer's instructions. The ratios of 260 nm/280 nm and 260 nm/230 nm were used as indicators of both DNA quality and quantity. The extracted DNA was stored at $-80°C$ until further processing. 5′-ACTCCTACGGGAGGCAGCA-3' (forward primer) and 5′-GGACTACHVGGGTWTC-TAAT-3' (reverse primer) were used to combine the adapter sequences and barcode sequences to obtain the bacterial 16S rRNA gene V3-V4 region. Ten microliters of buffer, 10 μL of high GC enhancer, 10 μM of each primer, 0.2 μL of Q5 high-fidelity DNA polymerase, 1 μL of dNTPs, and 60 ng of genomic DNA were mixed for PCR amplification. The thermal cycling conditions were as follows: denaturation at 95°C for 5 min, reaction at 95°C for 1 min 15 times, 50°C for 1 min, 72°C for 1 min, and extension at 72°C for 7 min. PCR products were then second-round processed after purification by Agencourt Ampure XP beads (Beckman Coulter, Irving, TX) with 8 μL of ddH2O, 20 μL of 2 × Phμsion HF MM, 10 μM each primer, and 10 μL of PCR products from the first-step PCR. The thermal cycling conditions were as follows: 98°C for 30 s, 98°C for 10 s for 10 cycles, 65°C for 30 s min, 72°C for 30 s, and extension at 72°C for 5 min. PCR products were quantified using the Pico-Green dsDNA Assay Kit (Invitrogen, Carlsbad, CA), followed by gel purification using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and then requantified by PicoGreen. The Illumina HiSeq 2,500

platform (2 × 250 paired ends) was used for sequencing. Sequences overlapping for more than 10 bp were assembled by FLASH (version 1.2.11), filtered via Trimmomatic (version 0.33), and considered high-quality tag sequences after removal of chimeras by UCHIME (version 8.1).

Data Statistical Analysis

QIIME software (v.1.9.1) was used to remove the primers, barcodes, and low-quality sequences, and FLASH (v.1.2.11) was used to merge high-quality paired-end reads into tags, which were clustered into operational taxonomic units at 97% sequence identity using USEARCH (v.10.0). The alpha diversity and beta diversity of the filtered samples were calculated using Mothur (v.1.41.1) and QIIME (v.1.9.1), respectively, and were visualized for partial least squares discrimination analysis by the R language tool (version 3.6.0). Venn diagrams were used to show the number of shared genera. With linear discriminant analysis effect size analysis (v.1.5.3), the nonparametric factor Kruskal-Wallis rank sum test and Wilcoxon rank sum test were used to detect the differences between the 2 groups, and the biomarker was obtained based on an LDA >4. SPSS software (v.12.0) was used to analyze the data, and a t-test was used to analyze significant differences in the sample data. The data are expressed as the mean $\pm$ SD. Statistically, $P < 0.05$ represents a significant difference, and $P < 0.01$ represents an extremely significant difference.

RESULTS

The Effect of Sex on Intestinal Development

There was no significant difference in body weight between the male and female ducks at 2, 5, and 10 wk ($P >$ 0.05). No significant difference in intestinal relative length (**RL**), relative weight (**RW**), or RW/RL was found at 2 wk ($P > 0.05$). The RL of the ileum of female ducks was significantly higher than that of male ducks ($P < 0.05$), whereas the RW/RL values of the duodenum, jejunum, and cecum were significantly lower at 5 wk ($P < 0.05$). The RW of the duodenum and cecum and the RW/RL of the jejunum of female ducks were significantly lower than those of male ducks at 10 wk ($P < 0.05$) (Table 3).

The HE staining results of each intestinal segment during the experiment are shown in Figure 1. At 2 wk, the values of jejunal VH/CD and VH and ileal WT of female ducks were significantly higher than those of male ducks ($P < 0.01$). The VH and CD of the duodenum and the VH ileum of female ducks were significantly higher than those of males at 5 wk ($P < 0.01$), and the CD of the ileum was significantly higher ($P < 0.05$), whereas jejunal WT was significantly lower than that of male ducks ($P < 0.05$). The values of duodenal VH and jejunal VH and WT of male ducks were significantly higher than those of female ducks at 10 wk ($P < 0.01$),

Table 3. Intestinal growth-related indicators of ducks at different stages.

Weeks	Intestinal segment	Sex	Relative length (cm/kg)	Relative weight (g/kg)	Relative weight/relative length (g/cm)
2	Duodenum	M	45.79 ± 5.22	6.10 ± 0.76	0.13 ± 0.02
		F	43.59 ± 4.62	5.38 ± 1.03	0.12 ± 0.03
		P value	0.46	0.20	0.47
	Jejunum	M	115.62 ± 17.52	13.56 ± 2.93	0.12 ± 0.02
		F	114.97 ± 6.88	13.26 ± 2.90	0.12 ± 0.02
		P value	0.93	0.87	0.81
	Ileum	M	117.25 ± 16.78	13.18 ± 2.56	0.11 ± 0.03
		F	109.24 ± 9.00	12.99 ± 1.30	0.12 ± 0.02
		P value	0.33	0.87	0.75
	Cecum	M	43.67 ± 5.49	2.38 ± 0.73	0.05 ± 0.02
		P value	40.47 ± 5.68	1.90 ± 0.37	0.05 ± 0.01
		P value	0.34	0.29	0.41
5	Duodenum	M	13.87 ± 2.09	4.14 ± 0.88	0.30 ± 0.02
		F	15.28 ± 1.05	3.52 ± 0.77	0.23 ± 0.06
		P value	0.17	0.22	0.04
	Jejunum	M	37.29 ± 5.33	9.20 ± 2.03	0.25 ± 0.02
		F	41.35 ± 3.64	8.13 ± 1.32	0.20 ± 0.04
		P value	0.16	0.31	0.02
	Ileum	M	32.48 ± 3.24	7.36 ± 2.24	0.23 ± 0.06
		F	37.15 ± 1.76	7.14 ± 0.62	0.19 ± 0.02
		P value	0.01	0.82	0.23
	Cecum	M	13.95 ± 3.13	1.65 ± 0.26	0.12 ± 0.01
		F	15.97 ± 1.41	1.59 ± 0.19	0.10 ± 0.01
		P value	0.18	0.65	0.01
10	Duodenum	M	9.41 ± 0.52	2.44 ± 0.47	0.26 ± 0.04
		F	9.07 ± 1.43	1.94 ± 0.27	0.22 ± 0.06
		P value	0.59	0.05	0.21
	Jejunum	M	25.13 ± 2.61	5.37 ± 0.85	0.21 ± 0.02
		F	25.23 ± 1.76	4.60 ± 0.62	0.18 ± 0.02
		P value	0.94	0.11	0.04
	Ileum	M	24.19 ± 2.50	4.95 ± 0.63	0.20 ± 0.02
		F	24.23 ± 2.84	4.60 ± 0.46	0.19 ± 0.02
		P value	0.98	0.30	0.22
	Cecum	M	10.49 ± 1.24	1.30 ± 0.09	0.13 ± 0.01
		F	10.44 ± 0.97	1.11 ± 0.20	0.11 ± 0.02
		P value	0.95	0.05	0.09

M refers to male ducks, F refers to female ducks. The P value represents the significance between the values in the 2 lines preceding it. n = 3.

and jejunal VH/CD was significantly higher ($P < 0.05$), whereas the CD of ileum was significantly lower, than that of female ducks ($P < 0.05$) (Table 4).

The Effect of Sex on Intestinal Microorganisms

A total of 6,096,426 pairs of reads were obtained by sequencing the contents of the intestines from 60 ducks, and 5,101,854 clean tags were generated after splicing and filtering, with at least 22,390 clean tags produced by each sample and 4,873 clean tags on average.

The number of operational taxonomic units from the intestinal content obtained by clustering was used as the vertical coordinate to draw the rarefaction curves of multiple samples, and each curve trend tended to be flat with an increase in the number of randomly selected sequencing pieces, suggesting that the sequencing quantity was sufficient to reflect the species diversity (Figure 2A). Fifty-five microorganisms were found in both sexes at the genus level (Figure 2B). Firmicutea, Proteobacteria, Bacteroidetes, and Actinobacteria were the dominant phyla (Figure 2C), and *Candidatus, Arthromitus*, *Bacteroides*, *Helicobacter,* and *Streptococcus* were the dominant genera (Figure 2D). The percentages of bacterial phyla and genera per each segment,

age and sex are clearly summarized in Supplementary Table 1. The value of the duodenal Chao index at the genus level of male ducks was significantly higher than that of females at 10 wk ($P < 0.05$), whereas there was no difference at the genus level during the whole experiment (Table 5). The samples of each intestinal segment from male and female ducks showed a separate cluster distribution at 2, 5, and 10 wk (Figure 2E).

Linear discriminant analysis effect size analysis was carried out on the samples at the genus level to identify differential enrichment of microorganisms between the 2 sexes (Figure 3A). The results showed that significant differences were found only in the jejunum, and the abundances of *Escherichia_ Shigella* (5.53 vs. 2.54%), *Pseudomonas* (2.77 vs. 0.37%), *Clostridium_ sensu_ s-tricto_ 1* (3.16 vs. 1.27%), *Sphingomonas* (2.37 vs. 0.47%), and *Desulfovibrio* (2.70 vs. 0.94%) in male ducks were higher than those in female ducks, whereas the abundance of *Rothia* (0.62 vs. 6.09%) was lower ($P < 0.05$). Further KEGG analysis showed that the top 3 functional pathways of microbial abundance in all intestinal segments, which were carbohydrate metabolism (14.80~17.22% vs. 13.00~16.47%), global and overview maps (11.56~14.07% vs. 11.97~14.37%), and amino acid metabolism (8.09~12.03% vs. 8.53~12.16%), were the same (Figure 3B), and 9 pathways were differentially enriched in male and female
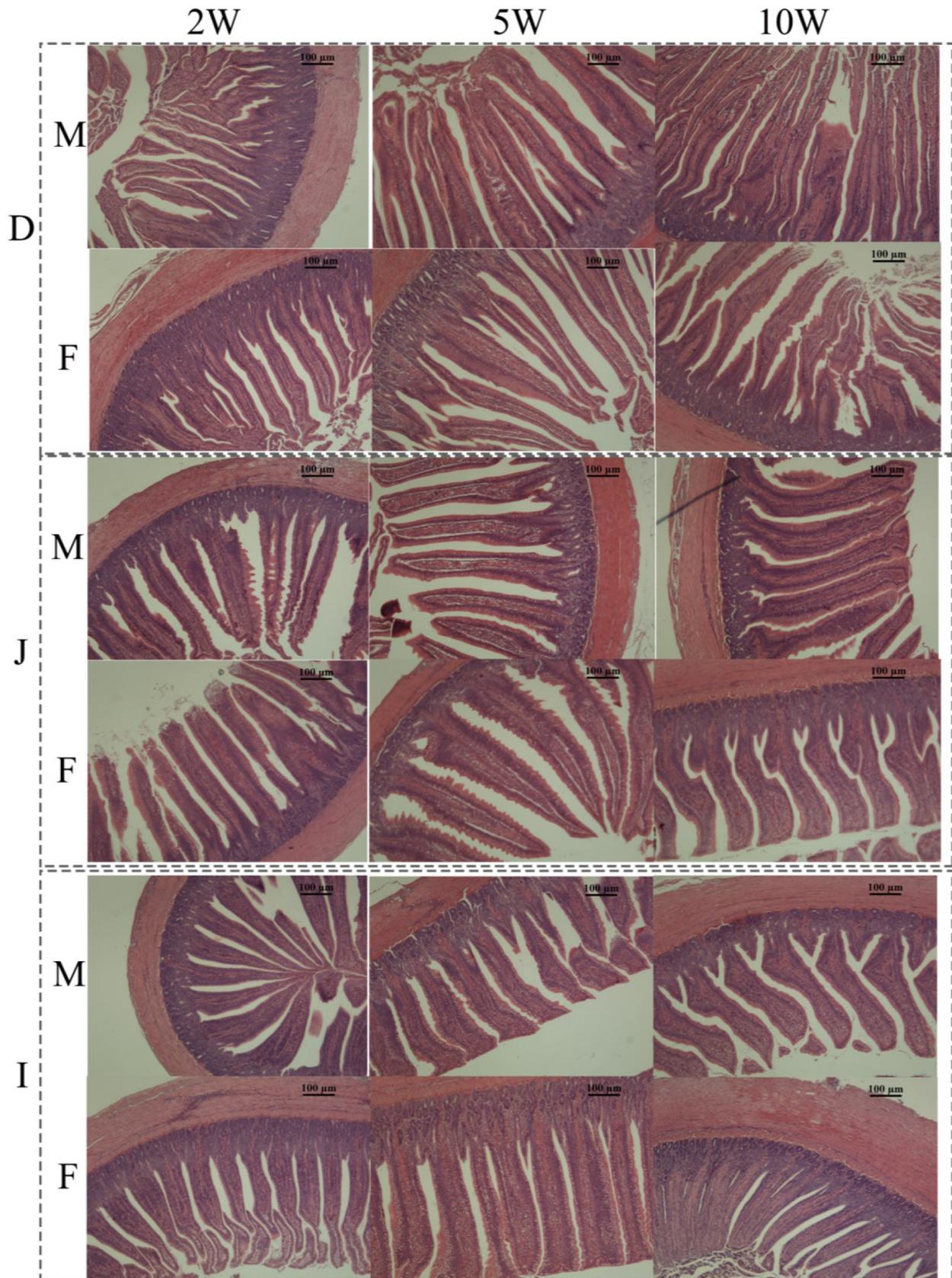
Figure 1. Morphology structure of small intestine after HE staining. D, J, and I in the first vertical row on the left represent duodenum, jejunum, and ileum, respectively. The second vertical M and F represent male duck and female duck, respectively. 2 W represents 2 wk of age, 5W represents 5 wk of age, and 10W represents 10 wk of age. n = 3.

Table 4. Morphological structure indexes of the small intestines of ducks at different stages.

Weeks	Intestine	Sex	Villus height (μm)	Crypt depth (μm)	Villus height/crypt depth	Wall thickness (μm)
2	Duodenum	M	1,124.85 ± 284.67	233 ± 34.69	4.93 ± 1.44	548.76 ± 149.83
		F	1,161.43 ± 163.41	217.54 ± 45.77	5.57 ± 4.82	517.58 ± 89.72
		P value	0.54	0.12	0.070	0.34
	Jejunum	M	870.83 ± 172.43	231.21 ± 36.18	3.87 ± 1.08	514.48 ± 164.48
		F	1,091.06 ± 270.01	235.49 ± 34.73	4.82 ± 1.69	496.37 ± 128.10
		P value	0.00	0.55	0.00	0.56
	Ileum	M	785.89 ± 177.83	268.98 ± 55.54	3.06 ± 0.98	437.23 ± 151.06
		F	847.91 ± 150.95	271.11 ± 45.26	3.24 ± 0.90	538.87 ± 143.87
		P value	0.10	0.85	0.41	0.00
5	Duodenum	M	1,661.61 ± 186.46	320.39 ± 63.51	5.34 ± 1.26	482.28 ± 150.51
		F	1,816.46 ± 210.42	366.97 ± 75.72	5.15 ± 1.22	341.21 ± 118.98
		P value	0.00	0.01	0.53	0.11
	Jejunum	M	1,466.48 ± 318.77	286.68 ± 54.87	5.31 ± 1.49	407.88 ± 85.86
		F	1,612.36 ± 119.79	293.93 ± 53.36	5.68 ± 1.22	348.74 ± 74.47
		P value	0.09	0.66	0.39	0.04
	Ileum	M	1,135.75 ± 130.44	222.68 ± 54.59	5.35 ± 1.25	345.79 ± 95.97
		F	1,277.65 ± 147.49	261.82 ± 69.24	5.11 ± 1.10	398.72 ± 158.93
		P value	0.00	0.03	0.44	0.27
10	Duodenum	M	1,630.87 ± 225.74	314.52 ± 39.56	5.30 ± 1.15	710.51 ± 84.30
		F	1,420.04 ± 201.94	298.57 ± 46.95	4.85 ± 0.96	691.59 ± 224.52
		P value	0.00	0.24	0.18	0.81
	Jejunum	M	1,175.15 ± 263.10	224.34 ± 23.31	5.31 ± 1.38	486.76 ± 136.94
		F	971.44 ± 119.88	227.22 ± 54.86	4.48 ± 1.06	338.43 ± 136.96
		P value	0.00	0.84	0.04	0.01
	Ileum	M	957.83 ± 189.54	252.73 ± 65.61	3.83 ± 0.98	514.62 ± 146.83
		F	1,005.63 ± 284.12	281.21 ± 49.81	3.81 ± 1.66	583.30 ± 184.24
		P value	0.39	0.03	0.95	0.08

M refers to male ducks, F refers to female ducks. The P value represents the significance between the values in the 2 lines preceding it. n = 5.

ducks and only distributed in the jejunum (Figure 3C). The viral infectious diseases, lipid metabolism, metabolism of terpenoids and polyketides, parasitic infectious diseases, xenobiotic biodegradation and metabolism, cardiovascular disease, and metabolism of other amino acids pathways of the male duck were more highly enriched than those of the female duck, whereas the abundance of gene folding, sorting, and degradation pathways and nucleotide metabolism pathways of females were more highly enriched than those in males.

DISCUSSION

Intestinal development directly affects the digestion, absorption, and metabolism of nutrients, which is very important for health and growth potential (Nitsan et al., 1991). Intestinal transit tends to be slower in females because steroidal hormones promote intestinal hypomotility and inhibit gastric emptying by acting as a smooth muscle relaxant (Liu et al., 2006); additionally, progesterone could also decrease gastrointestinal motility through an inhibitory effect on motilin (Cheng et al., 2010). However, a completely different angle, other than a hormonal explanation for these sex-based differences, is provided in this study. Previous studies have shown that postprandial changes in intestinal volumes are higher in males than females by using single photon emission computed tomography (Bouras et al., 2002), and the volume of postmortem fluid in the intestine was higher in men than in women after standardization by body weight (Gotch et al., 1957), indicating that sex has a certain influence on the capacity of the intestine. Generally, there are differences in the speed of

development between males and females during animal development, so we first compared the weights of ducks at different times to ensure that all the differences only originated from sex, and the results showed no difference between them. According to the results of this experiment, greater relative length of the intestine in the female duck may lead to longer intestinal emptying time, whereas the greater relative weight/relative length of the male duck intestine could contribute to more effective peristalsis, explaining the difference in digestion time between different sexes from another point of view.

The digestive and absorptive capacity of the intestinal tract for nutrients depends on the comprehensive action of the pancreas, intestinal enzyme activity, intestinal surface area, and intestinal nutrient transport carriers (Li et al., 2017; Takahama and Hirota, 2018), and the surface area of intestinal villi is the key factor limiting the growth of poultry (Osuka et al., 2017; Wismann et al., 2018). The nutrients in the intestines can be easily absorbed because of the tight blood capillaries with thin vascular walls in villi (Kato et al., 1999). The VH is adjusted correspondingly with the change in animal function and intestinal nutrient demand under normal circumstances, which is realized by the incremental rate and intestinal cell turnover rate of intestinal crypt cells (Blackmore et al., 2017). In the early stage of the experiment, female ducks had a stronger capacity for nutrient absorption because of their higher VH, and a thicker intestinal wall could also accelerate the passage of food from the intestine. In addition, the VH/CD, which can best reflect the intestinal digestion capacity, was also higher in female ducks. However, the intestinal morphology of female ducks no longer indicated greater

Figure 2. Species distribution and diversity of intestinal microorganisms. (A) The multisamples rarefaction curves of male and female ducks. (B) The Venn map of duck intestinal microorganisms at different wk. (C) Distribution of microorganisms in different intestinal segments. (D) β analysis of intestinal microorganisms in ducks of different sexes. In A, B, C, and D, M represents male duck, F represents female, 2 represents 2 wk of age, 5 represents 5 wk of age, and 10 represents 10 wk of age, and D, J, and I in the first vertical row on the left represent duodenum, jejunum and ileum, respectively. All analyses are based on genus level. n = 5.

values of these parameters at 10 wk, and the microbial diversity of females was significantly lower than that of male ducks, suggesting that the developmental peak of the male duck intestine lags behind that of the female duck, whereas its total developmental potential is

higher. However, a more robust experiment is required to confirm this finding.

No bacteria were detected in any part of the gastrointestinal tract in hatching chickens, but a large number of *Streptococcus faecalis* and *Escherichia coli* could be

Table 5. Chao 1 index of intestinal microorganisms.

Weeks	Intestinal segment	Phylum			Genus		
		Male	Female	P value	Male	Female	P value
2	Duodenum	9.60 ± 2.51	9.60 ± 1.95	1.00	131.97 ± 27.14	134.65 ± 14.27	0.85
	Jejunum	11.40 ± 2.07	9.75 ± 1.71	0.24	148.47 ± 33.65	130.69 ± 10.85	0.35
	Ileum	9.10 ± 2.41	8.40 ± 1.34	0.59	117.59 ± 11.11	114.36 ± 14.64	0.71
	Cecum	7.20 ± 1.64	7.20 ± 0.45	1.00	86.28 ± 13.05	84.80 ± 7.01	0.83
5	Duodenum	12.20 ± 2.95	12.60 ± 3.65	0.85	158.13 ± 30.81	136.89 ± 39.43	0.37
	Jejunum	13.80 ± 0.84	11.20 ± 2.45	0.06	166.70 ± 22.98	130.43 ± 38.52	0.11
	Ileum	11.60 ± 1.82	11.87 ± 1.65	0.81	142.96 ± 9.31	134.51 ± 27.38	0.53
	Cecum	9.00 ± 1.22	8.60 ± 0.89	0.57	107.96 ± 10.98	111.27 ± 16.71	0.72
10	Duodenum	12.40 ± 0.89	12.00 ± 0.71	0.46	158.25 ± 5.04	141.20 ± 7.46	0.00
	Jejunum	12.80 ± 0.84	12.30 ± 1.48	0.53	139.42 ± 19.13	150.16 ± 19.32	0.40
	Ileum	12.70 ± 0.97	13.10 ± 1.14	0.57	160.42 ± 20.18	161.80 ± 14.47	0.90
	Cecum	9.60 ± 0.87	9.40 ± 0.89	0.73	106.03 ± 7.37	117.27 ± 15.99	0.19

The P value represents the significance between the 2 values in each row. n = 5.

Figure 3. LEfSe analyses and KEGG analyses of duck intestinal microorganism. (A) LEfSe analyses of jejunal microorganism. (B) Histogram of microbial functional pathways. (C) Differential functional pathways of jejunum microorganisms. M represents male duck, F represents female, 2 represents 2 wk of age, 5 represents 5 wk of age, and 10 represents 10 wk of age, and D, J, and I in the first vertical row on the left represent duodenum, jejunum, and ileum, respectively. All analyses are based on genus level. n = 5. Abbreviation: LEfSe, linear discriminant analysis effect size analysis.

isolated from all parts of their gastrointestinal tract by day 3, and microbial communities were established in their small intestines within approximately 2 wk (Lan et al., 2005). There was little difference in the genera of microorganisms in the duck intestine from week 5 to 10, indicating that the microbial community in the intestines of ducks may be stable at approximately 5 wk of age, which is consistent with previous studies on ducks of the same species (Ran et al., 2020). The dominant bacteria in the intestine were Firmicutes, Proteobacterium, Bacteroidetes, and Actinobacteria at the phylum level, and among them, Firmicutes accounted for the largest proportion, which was consistent with the results in Turkey, Landers, and broiler (Li et al., 2017; Zhao et al., 2017). Oviedo-Rondón and Hume demonstrated a positive correlation between the diversity and stability of gut microbial populations and improved broiler performance and health, which in turn improved feed conversion and nutrient utilization in birds (Oviedo-Rondón and Me, 2013). The diversity of intestinal microorganisms in male ducks was significantly higher than that in female ducks at the 10th wk, which is also similar to the results from chickens (Marcato et al., 2006). Sex-related differences in intestinal microbial communities have been observed in many animals, such as mice, macaques, and humans (Mueller et al., 2006; Schloss and Handelsman, 2006; Mckenna et al., 2008), and the

same situation was found in this experiment. *Escherichia-Shigella* belongs to the phylum Proteobacteria and can become pathogenic bacteria under stressful conditions (Lutful Kabir, 2010). *Escherichia-Shigella* plays an important role in amino acid utilization in animals (Mao et al., 2016), but other reports have shown that this group is related to necrotizing enterocolitis and can also cause lung injury in broilers by activating NLRP3 inflammatory corpuscles (Liu et al., 2020). When ducks suffer from viral infectious diseases, such as flower liver disease, parvovirus disease, and gosling plague, they are usually depressed, anorexic, and have diarrhea, shortness of breath, and dehydration, before finally dying of exhaustion (Shehata et al., 2016; Xie et al., 2017). One of the pathogenic substances of *Escherichia-Shigella* is plasma coagulase (Barreto et al., 2016), and the main symptoms of infection are consistent with the above symptoms. *Pseudomonas* is a gram-negative bacterium that is widely distributed in normal skin, intestine, and respiratory tract tissues (Mahajan-Miklos et al., 1999; Tan et al., 1999) and can affect the health of many eukaryotic animals, including *Caenorhabditis elegans* (Apidianakis and Rahme, 2009). *Clostridium_sensu_stricto_1* is more abundant in animals with endometritis (Wang et al., 2017), but it can also be used to ferment protein (Zhou et al., 2016), and the proportion of Clostridium_sensu_stricto_1 in the pig

ileum significantly decreases as the protein concentration decreases (Fan et al., 2017). *Sphingomonas* and *Desulfovibrio* are often found in highly metal-contaminated environments (Mao et al., 2018b). Intestinal bacteria influence physiological and pathological processes throughout the body, including the bioavailability and meta-ballism of macronutrients and micronutrients as well as metabolites. The composition of intestinal microorganisms was different in different sexes, and their functional pathways were also different. The common parasitic diseases in ducks are coccidiosis, echinococcosis, and Baiguan disease (Olsen, 2009), infection with which is usually hidden, often causing tissue damage, malnutrition, and cell damage, endangering animal health, causing large amounts of duck death, hampering the growth, development, and reproduction of the duck population and bringing extremely heavy economic losses (Asfaw et al., 2019). Different microbiota are able to modulate plasma lipid and protein levels. Fu et al. found that gut microbiota composition explained 6% of triglyceride variation, 4% of HDL variation, and 1.5% of LDL variation (Fu et al., 2015). Despite several benefits to the host, intestinal microbes also decrease fat digestibility by deconjugating bile acids (van der Klis and Jansman, 2002). In addition, reliable evidence indicates that intestinal microbiota may play an essential role in the metabolism of many alimentary compounds, such as choline, phosphatidylcholine, and carnitine, leading to the generation of potentially proatherosclerotic compounds, such as trimethylamine-N-oxide (Wang et al., 2011). The highly heterogeneous intestinal composition of microbes may also be related to the production of the metabolic precursor of trimethylamine-N-oxide, trimethylamine (Romano et al., 2015). The higher abundance of *Escherichia_Shigella*, *Pseudomonas*, *Clostridium_sensu_stricto_1*, *Sphingomonas,* and *Desulfovibrio* in the jejunum of male ducks was consistent with the higher abundance of disease-related and metabolism-related metabolic pathways.

The intestinal microbiota interact among each other, with their host and with the diet of the host, whereas commensal bacteria play a pivotal role in host health and metabolism, and pathogenic bacteria cause direct or indirect harmful effects (Jha and Berrocoso, 2015). It has been reported that microbiota can provide nutritional compounds to the host in the form of short-chain fatty acid (**SCFA**), which stimulate gut epithelial cell proliferation and differentiation and increase villus height, thereby increasing the absorptive surface area (De Vadder et al., 2014), suggesting that there is a close relationship between intestinal microflora and intestinal growth. In this experiment, the difference in the intestinal microbial metabolic pathway between the male and female ducks was only found in the jejunum, and the difference in VH/CD between the 2 sexes was also only found in the jejunum. It can be inferred that the difference in intestinal development and microorganisms between male and female ducks may mainly occur in the jejunum, and more follow-up tests are needed to support this conclusion.

CONCLUSION

There are differences in the intestinal development and microorganisms of ducks of different sexes. Male ducks have shorter intestinal lengths and higher relative weight/relative length values. Obvious separation of microorganisms was found in each intestinal segment of ducks of different sexes over 3 time periods. Differences in villus height/crypt depth, microbial abundance, and metabolic pathways were only found in the jejunum because of the higher abundance of *Escherichia_Shigella*, *Pseudomonas*, *Clostridium_sensu_stricto_1*, *Sphingomonas,* and *Desulfovibrio* and the lower abundance of *Rothia* in male ducks than in female ducks.

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DISCLOSURES

The authors declare no conflicts of interest

SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.psj.2020.10.051.

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