

The differences of intestinal development and microorganism between male and female ducks

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Abstract

Background There are great differences in physiological and biological functions between animals of different genders. However, whether there would be a consensus in duck intestinal development and microorganisms is still unknown. **Methods** Male and female ducks were weighed at 2, 5 and 10 weeks, respectively, then the duodenum, jejunum, ileum and cecum were sampled and measured, and the content were analyzed by 16S RNA. **Results** The results showed that male duck hold shorter intestinal length with higher density and jejunum VH/CD, and the similarity, evenness and diversity of the total intestinal microorganism of male duck were higher, with 99.66% OTUs shared by both gender, while *Rikenellae*, *Prevotella* and *Nocardia* were only in male duck. *Proeobacteria* hold higher abundance than *Bacteroidetes* in female intestine, while the value of *Firmicutes/Bacteroides* was lower than male duck. Whats more, the different functional pathway of microorganisms was only showed in jejunum, among which the metabolic and disease pathways of male ducks were higher, while gene pathways was lower.

Conclusions The intestines of male ducks have higher development, nutrient absorption, fat deposition and metabolic capacity while their disease resistance was relatively weak, providing a basic reference for intestinal development and microorganism symbiosis of duck in different genders.

Introduction

Intestinal tract is an important part of the body defense system and the main place for animal to digest and absorb the nutrients of feed [1, 2]. The functions of poultry intestines are mainly carried out in the small intestine, which is also the longest part of the digestive tract[3]. The nutrients in small intestinal content can be easily absorbed due to the tight blood capillaries with thin vascular wall in villi [4], and lymphatic capillaries, unstriated fibers and neural net also play a certain role through accelerating blood flow by villi flex and swing. The cells at the top of the villi are differentiating from the crypt basal epithelial, and the premature mice with poor weight hold lower villus height by comparing with the premature mice with normal weight, [5]. The height of intestinal villi and the depth of crypt can reflect the body's ability to absorb nutrients and the maturation rate of crypt cells, respectively, and the value of villi height/ crypt depth (VH/CD) comprehensively reflect the nutrient absorption capacity of small intestine.

Intestinal microorganism is mainly distributed in the chyme flowing in the intestinal cavity and adheres to the intestinal and related mucosa. There are lots of species and microbial communities in the intestinal tract, which are rich in genes involved in carbohydrate, microbial, amino acid and short chain fatty acids (SCFAs) metabolism. The dynamic balance of interdependence and mutual restriction between host and microorganism exist in a healthy intestinal state, and most of the microorganisms exchange energy, transmit information and symbiosis with each other, while diseases may occur when the population convert to maladjustment[6]. About 35% of the enzymes of microorganisms can be used by the host, which plays an important role in nutrient metabolism regulation, intestinal mucosal immune activation, host behavior regulation, intestinal epithelial cell repair and resistance to pathogenic microorganisms[7, 8]. More than 1×10^6 microbial genes have been found planted in poultry gastrointestinal tract, which is 40 ~ 50 times of the whole chicken genome, while 90 ~ 95% of cecal microorganisms can not be cultured

in the laboratory environment, and these microorganisms play an essential role in the absorption of diet nutrients and the immune metabolism[9]. Poultry intestinal microorganism is related to the structure of diet, age, gender and individual specific conditions, and the composition of microbial flora in different intestinal segments also varies from others[10, 11].

There are great differences in hormone secretion, energy metabolism and immune response among animals of different genders[12], including intestinal health[13, 14]. It has been found that the diversity of intestinal microorganisms in female Octopus was significantly lower than that in males[15], and the relative abundance of anaerobes decreased with the facultative anaerobes increasing in female LTscIKO mice during the development of HCC, while *Paraprevotella*, *Parapervotella* and *Prevotella* increased in males [16]. Besides, 30 species of intestinal microorganisms affected by host gender significantly has been found in high body weight line families of chickens ($P < 0.05$), such as *Lactobacillus*, *Lactococcus* and *Actinomyces*, and 17 found in low body weight line families ($P < 0.05$), such as *Lactobacillus*, *Acinetobacter* and *Brachycharacter*[17]. Although many studies have addressed the effects of endogenous and exogenous factors on the intestinal development and microbiota composition, only limited data are available on the intestinal development and microbiota in animal models of different genders. The study on the development and microorganisms of intestine of male and female ducks is helpful to provide basic reference for the research on the intestinal growth and microorganism symbiosis and co metabolism rules of duck in different genders, and has certain theoretical and practical significance for the in-depth study on the characteristics of ducks.

Methods

Laboratory Animals

The Nonghua sheldrake used in this experiment was provided by the poultry raising experimental farm of Sichuan Agricultural University. 30 healthy male ducklings and 30 healthy female ducklings were managed in a unified way to ensure each duck intake and drink similarly at liberty and under unified management during the experiment. 10 ducks of each gender were randomly selected at 2, 5 and 10 weeks, killed by cervical dislocation after fasting for 12h, then sample the intestine and its content, and subsequently treated as non-hazardous waste. All animals handling procedures were approved by Sichuan Agricultural University Animal Welfare Committee.

Measurement of Intestinal Growth

Take out the duodenum, jejunum, ileum and cecum of each duck successively, fix one end of them on the glass plate moistened with distilled water after removing the pancreas, fat and other attachments attached to the intestinal tract, then straighten it slightly and measure the length with a ruler when the intestinal tract recovers to no longer retract. The filter paper was used to dry the intestinal segments after the intestinal contents were squeezed out, and the intestines were weighed by electronic balance.

intestinal density (ID) = intestinal weight (g) / intestinal length (cm)

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

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

The middle parts of duodenum, jejunum and ileum were cut 1 cm respectively, washed with normal saline, and fixed in 4% polyformaldehyde solution, then embedded in paraffin, sectioned and stained with HE. The microscopic images were taken at 50x and 100x. Three replicates were collected for each sample, and three different visual fields with complete and straight direction villus were collected for each replica. Villus height (VH), crypt depth (CD) and intestinal wall thickness (IWT) of 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 is VH(mm), from the bottom of the crypt to the transition area between the crypt and villus is CD(mm), and the lineal length of the longest and widest intestinal wall is IWT(mm).

Place the obtained contents of intestines in the marked EP tubes and put them into liquid nitrogen immediately. The 16S rRNA gene library was constructed by extracting total microbial DNA from intestinal contents and amplified 16S rRNA sequences of all microorganisms. PCR products were purified using Agencourt Ampure XP beads(Beckman, USA) accounting to the instructions of manufacturer and quantified using the PicoGreen dsDNA Assay kit (Invitogen, USA), equally combined and followed by gel purification using a QIAquick Gel Extraction Kit (Qiagen, USA), and then requantified by PicoGreen. The prepared DNA library was then sequenced using the MiSeq platform (Illumina, USA). An OTU table was generated using the Uparse clustering method (97 % cutoff), and all samples were rarefied to the same sequencing depth by resampling OTUs prior to downstream analysis.

Data Statistic Analysis

Mothur software and QIIME software were used to evaluate the Alpha diversity and Beta diversity of the samples, and the figures was drawn by R language tool. SPSS 21.0 software (IBM, USA)was used to analyze the data and T-test was used to analyze the significance of the sample data. The data were expressed in the form of Mean \pm S.D. . Statistically, $P < 0.05$ represents significant difference and $P < 0.01$ is extremely significant difference.

Results

The effect of gender on intestinal development

There was no significant difference in RL, RW and ID between the male and female ducks at 2 weeks ($P > 0.05$) by comparing the development of intestine between the two genders. The RL of jejunum and ileum in female ducks was significantly higher than that in male ducks at 5 weeks ($P < 0.05$); nevertheless, the ID of duodenum and jejunum in female ducks were great significantly ($P < 0.01$) and significantly lower ($P < 0.05$) than that in male ducks, respectively. Both duodenal RW and jejunal ID of the male ducks hold significantly higher value than that of the female duck at 10 weeks ($P < 0.05$) (**Figure 1**).

There was no significant difference in the intestinal morphological structure of duodenum, jejunum and ileum between male and female ducks in appearance during the whole experiment (**Figure 2**). Then the result was further studied by relevant data and it showed that the VH, VH/CD of the jejunum and the IWT of the ileum in females were significantly higher ($P<0.01$) at 2 weeks. Both VH and CD of both duodenum and jejunum of female ducks were significantly higher than that in males, while the IWT of the jejunum was significantly lower ($P<0.05$) at 5 weeks. The VH of duodenum and jejunum in male ducks was great significantly higher than that in female ducks ($P<0.01$) at 10 weeks, while the CD of ileum was significantly lower ($P<0.05$), and only the VH/CD of jejunum was significantly higher than females ($P<0.01$) (**Figure 3**).

The effect of gender on intestinal microorganisms

A total of 6 096 426 pairs of reads were obtained by sequencing the contents of the intestine 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 4873 clean tags on average.

The number of intestinal OTUs of male and female ducks obtained by clustering was used as the vertical coordinate to draw the multy samples rarefaction curves, and each curve trend tended to be flat with the increase of the number of randomly selected sequencing pieces, suggesting the sequencing quantity was enough to reflect the species diversity. The highest species richness of intestinal microflora in both sexes was appeared in 10 weeks with the lowest in 2 weeks, and the intestinal microflora richness of male ducks was higher than that of females except 2 weeks. In addition, the abscissas of the end point of the multy samples rarefaction curves of the female ducks in the three time periods were all larger than males (**Figure 4A**). However, only the microbial abundance in the duodenum of male ducks was significantly higher than females at 10 weeks by comparing the Chao1 index (**Table 1**). The distribution of female duck was obviously separated both at the first and second principal component according to the PAC analysis, while the distribution of male duck is more closely clustered at the second principal component (**Figure 4B**).

Table 1 the chao 1 index of of small intestine and cecum in different genders

Weeks	intestinal segment	male	female
2W	duodenum	286.230±20.148	297.532±14.616
	jejunum	300.105±23.838	313.345±22.172
	ileum	280.910±22.306	286.390±29.149
	cecum	256.107±10.081	259.757±4.645
5W	duodenum	336.735±38.497	312.733±44.254
	jejunum	347.383±40.3585	318.577±49.648
	ileum	361.174±26.420	379.543±42.546
	cecum	353.042±10.258	363.824±10.109
10W	duodenum	445.436±23.253	356.181±14.174*
	jejunum	362.096±23.688	399.347±39.741
	ileum	467.633±46.671	442.956±39.338
	cecum	372.919±16.818	376.630±4.671

The ** above the two bars represents the great significant difference between the two samples ($P < 0.01$), * represents the significant difference between the two samples ($P < 0.05$). The picture below is the same.

889 OTUs were identified from intestinal contents of two genders of ducks, of which 99.66% were both in male and female, while the *Rikenellaceae* and *Prevotella* and *Nocardia* were unique to male ducks (**Figure 4C**). The dominant intestinal microflora of both genders were *Firmicutes*, *Proeobacteria*, *Bacteroidetes* and *Actinobacteria* based on the analysis at phylum level. However, the abundance of *Bacteroidetes* (17.2%) male duck was higher than that of *Proeobacteria* (14.4%), while that *Proeobacteria* (25.3%) of female duck was higher than that of *Bacteroidetes* (15.4%) (**Figure 4D**). The ratio of *Firmicutes* to *Bacteroidetes* was calculated and it turned to be out that the value is significantly higher in the male than that in the female at 10 weeks as well as the weight of the male was significantly higher at the same time ($P < 0.05$) (**Table 2**).

Table 2 the value of *Firmicutes*/*Bacteroidetes* and weight of duck

Items	gender	2W	5W	10W
F/B	Male	3.585±2.871	1.717±0.827	1.864±0.586
	Female	2.282±1.000	1.386±0.340	1.385±0.158*
Weight	Male	0.431±0.026	1.800±0.143	3.002±0.091
	Female	0.457±0.031	1.766±0.122	2.721±0.153**

LEfSe analysis was carried out on the samples at different microbial classification levels with LDA score > 4 to determine which microorganisms caused above differences in the intestinal microflora. The results showed that there were different microorganisms in the other three intestine segments except ileum between male and female ducks. The differences of *Firmicutes* at phylum level, *Clostridia* at Class level, *Clostridiales* at order level and *Lachnospiraceae* and *Ruminocaceae* at family level existed in duodenum, and all the abundance of the them was higher in the samples of male ducks. Only the abundance of *Rothia* at genus level in the female ducks jejunum was higher than that of male ducks, while 22 other microorganisms were lower at different classification levels, such as *Pseudomonas*, *Sphingomonas* and *Desulfovibrio* at genus level. The male ducks had higher *Proteobacteria* abundance at phylum level than the female in the cecum (**figure 5**). Further analysis showed that the differential pathways were only distributed in the jejunum. The viral infectious diseases, lipid metabolism, metabolism of terpenoids and polyketides, parasitic infectious diseases, xenobiotics biodegradation and metabolism, cardiovascular disease and metabolism of other amino acids of the male duck was higher than that of female duck, while the abundance of gene folding, sorting and degradation pathway and nucleotide metabolism of female was higher than that of male (**figure 5**).

Discussion

Intestinal development directly affects the digestion, absorption and metabolism of nutrients, which is very important for health and growth potential. The increase peak of small intestine length of broilers is earlier than their daily intake and daily gain, and the relative weight can reach the peak only after the relative growth speed of digestive organs reaches the peak, suggesting that the quality and index of intestine can reflect the biological and ecological conditions and functions of animals in the growth and development stage [18]. Intestinal transit tends to be slower in female for the steroidal hormones would promote intestinal hypomotility and inhibit gastric emptying by acting as a smooth muscle relaxant [19], and progesterone also could decrease gastrointestinal motility through an inhibitory effect on motilin [20]. However, a completely different angle is provided in this experiment rather than hormone. Previous studies have shown that postprandial changes in intestinal volumes are higher in males than females by using a single photon emission computed tomography [21], and the volume of post-mortem fluid in the

intestine were higher in men than in women after standardization by body weight[22], indicating that different gender had certain influence on the capacity value of intestine. According to the results of this experiment, greater RL of the female duck lead the longer intestinal emptying time, while the greater ID of the male duck contribute the more effective peristalsis, suggesting that the digestion cycle of male duck is shorter than females.

The digestion and absorption ability of the intestine to nutrition depends on the joint action of pancreas, enzyme activity, surface area and nutrient transport carrier [23, 24], and the surface area of intestinal villi is the key factor limiting the growth of poultry[25, 26]. Another structure of the mucosal layer, crypt, transports nutrient molecules from the digestive tract to the blood for use, and the value of CD can reflect the renewal speed of the small intestine epithelium[27]. Therefore, the higher CD value of the female ducks mean faster update of epithelial cells in intestine. VH is adjusted correspondingly with the change of animal function and intestinal nutrient demand under normal circumstances, which is realized by the increment rate and intestinal cell turnover rate of intestinal crypt cell [28]. The intestinal epithelium is a complex multicellular system, closely related to the growth potential. Sexbased differences of infection and inflammation were found in the intestine of 2-week-old C57BL/6 mice [14]. In this study, the digestive capacity of the male duck's jejunum was lower than females in the early stage due to VH/CD, and then higher than females in the later stage with the development of VH, which is similar with intestinal microbial diversity, suggesting there is consistency between intestinal development and intestinal microorganisms in ducks.

The balance and stability of intestinal microorganisms play an important role in maintaining the health of the body. Gender-related differences in intestinal microbial communities have been observed in many animals, such as mice, macaques and humans[29–31]. Samples from same gender were found clustered together by Principal Component Analysis of T-RFLP and DGGE profiles from either human flora associated rats or specific pathogen free rats[32]. Androgens could impact intestinal microbial composition in lupus-susceptible mice and protect males against the development of lupus[33]. Besides, males have higher serum SCFAs level come from the microbial fermentation by comparing with females, such as butyrate, propionate and acetate, which have a myriad of anti-inflammatory effects[34, 35]. Likely causes not only include differing sex hormone levels in males and females but also influenced by the intestinal microbiota.

The evenness and diversity of the intestinal microflora of the female duck were higher by comparing with the male duck, and the distribution of the intestinal microflora were significantly separated. Proteobacteria is the largest family of bacteria, including many pathogens and free-living species, such as E.coli, Salmonella and many bacteria that can carry out nitrogen fixation, covered with lipopolysaccharide which can cause strong pro-inflammatory effect and secrete pro-inflammatory factors including interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) [34], and γ Proteobacteria increase significantly in intestinal in NAFLD and NASH patients[36]. Bacteroides was initially known for its pathogenicity, and then found it was a large number of normal bacteria in the body, which played an important role in the host's nutrition absorption, fat accumulation, gastrointestinal flora balance and body

immunity. The difference of the abundance of Proteobacteria and Bacteroides between the male and female ducks suggested stronger metabolism ability weaker anti-inflammatory ability in male ducks. It has been found that the abundance ratio of Firmicutes to Bacteroides in intestinal microorganisms can reflect the degree of obesity to a certain extent [37, 38], and the relative proportion of Bacteroides in obese and thin people is low [39], and the decrease of Bacteroides was accompanied by the increase of Firmicutes [40]. In this experiment, the difference of the intestinal Firmicutes/Bacteroides and body weight between the male and the female ducks appeared at 10 weeks, suggesting that the fat deposition potential of male ducks was stronger females.

Microorganisms in the intestine affect the health and growth performance of the host through participating in different functional pathways[41]. There was no significant difference in functional pathways although there were significant differences between male and female ducks at 2, 5 and 10 weeks. Only jejunum showed significant differences of function pathways between the two sexes, which was consistent with the value of VH/CD. Lipid metabolism is an important and complex biochemical reaction [42, 43], processed into substances needed by the body through digestion, absorption, synthesis and decomposition with the help of various enzymes and bile salts to ensure the normal operation of physiological functions [44, 45]. It has been found that the upper part of small intestine was the main place where fat digested and hydrolyzed to glycerol and fatty acids [46]. The higher abundance of lipid metabolism pathway in male duck's jejunum in this experiment may be related to the higher nutritional absorption capacity of male ducks. Nevertheless, The disease pathway of the male duck is significantly higher by comparing with the female duck, which noteworthy in today's intensive aquaculture and it is necessary to strengthen the vaccine requirements and prevention and control measures for the male duck. Different from the male duck, the females have higher abundance of gene aspect. Nucleotide is the precursor of synthetic biological macromolecular RNA and DNA, which plays a dominant role in the growth, development, reproduction and heredity of organisms[47]. Congenital whey aciduria would be caused by the damage of pyrimidine nucleotide metabolism, and ventilation, Lesch Nyhan syndrome and immune deficiency by the damage of purine nucleotide metabolism[48, 49]. It has been reported that the male to female ratio of gout patients is 20:1 and the incidence of obesity is higher in male, which is consistent with the high abundance of nucleotide metabolic pathway and the weak ability of fat deposition in this experiment.

Conclusion

The differences in duck intestinal development and microorganisms between different genders were found in this study. Compared with female ducks, males had shorter digestion cycle because of shorter intestines and stronger peristalsis, which allowed them to take in more energy in the same time. The intestinal digestive capacity and microbial diversity of female ducks were higher in the early stage, while lower than that of male ducks in the later stage. The difference of intestinal morphology structure mainly occurred in jejunum, which was also the only intestinal segment with different microbial function pathways. The abundance of intestinal metabolism and disease pathways in male ducks is higher, while the abundance of gene pathway is lower than females. This study showed that the male ducks hold

stronger metabolism ability weaker disease resistant under the common growing environment, indicating it is necessary to strengthen the fattening of female ducks and the immunity of male ducks in the actual rearing process, providing a basic reference for intestinal development and microorganism symbiosis of duck in different genders.

Abbreviations

Abbreviation	Full name
VH	Villi height
CD	Crypt depth
SCFAs	Short chain fatty acids
HCC	Hepatocellular Carcinoma
ID	Intestinal density
RL	Relative length
RW	Relative weight
IWT	Intestinal wall thickness
OTUs	Operational taxonomic units
PCA	Principal Component Analysis
LEFse	Linear Discriminant Analysis effect size
LDA	Linear Discriminant Analysis
T-RFLP	Terminal restriction fragment polymorphism
DGGE	Denaturing gradient electrophoresis
IFN- γ	Interferon- γ
TNF- α	Tumor necrosis factor- α
NAFLD	Non-alcoholic fatty liver disease

Declarations

Ethics approval and consent to participate

The experimental protocol utilized in this research were complied with the Chinese guidelines for animal welfare and approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan Agricultural University (DKY-B20141401).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

Not applicable.

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Authors' contributions

JW, SH and LL put forward the idea and method of the experiment. BH, LH, JH and LC participated in the incubation, breeding and sample collection of the experimental animals. HX and RZ performed the HE staining of three intestinal segments. XC, CH and BK participated in the 16S RNA analysis of intestinal contents. HL and HH analyzed and interpreted the collected data. LL was the supervisor of the project. XC was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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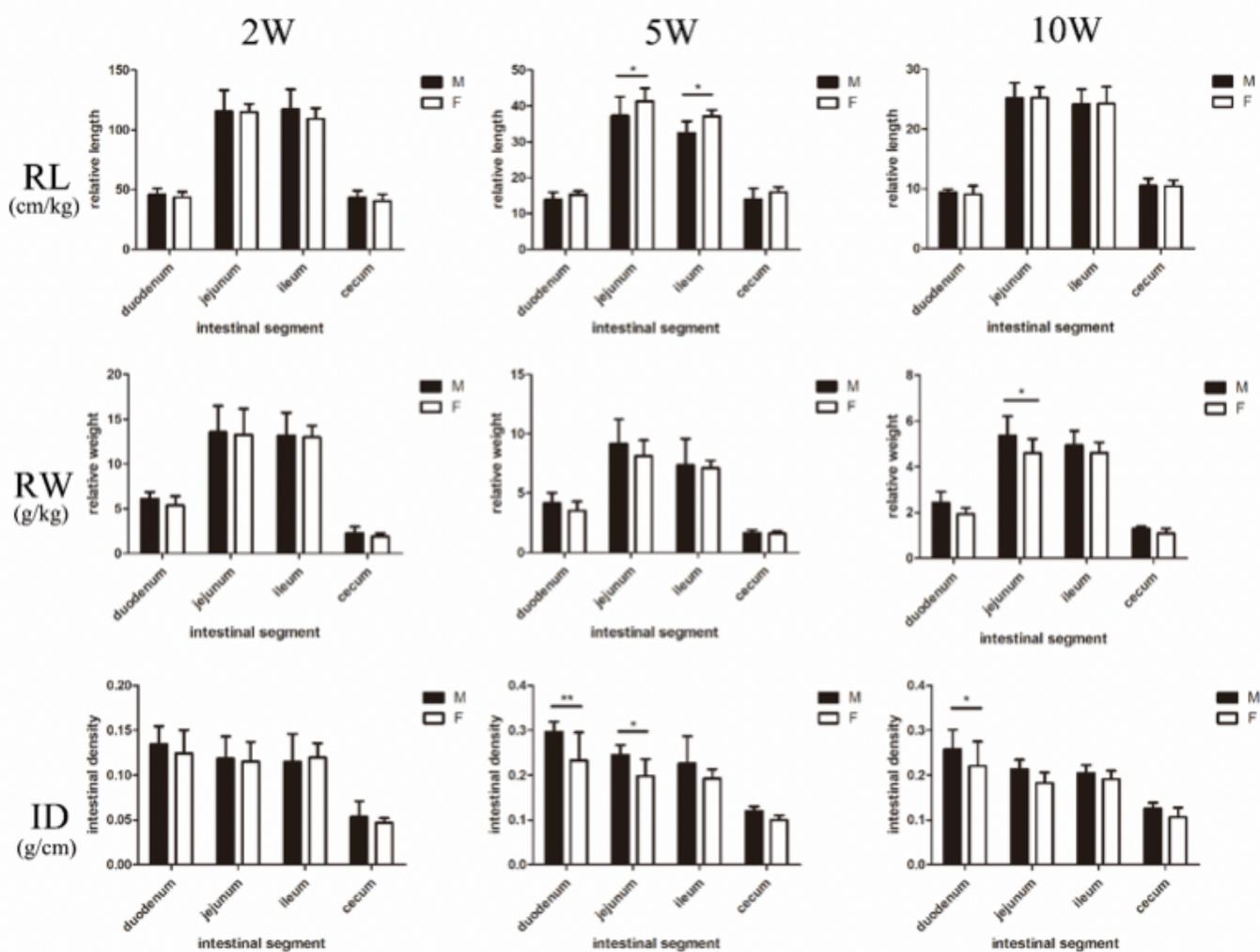
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Figures



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Figure 1

the development of the intestine in the male and female duck. RL, RW and ID in the figure represent the relative length, relative weight and intestinal density of intestine, respectively. 2W, 5W and 10W represent three time periods. The ** above the two bars represents the great significant difference between the two samples ($P < 0.01$), * represents the significant difference between the two samples ($P < 0.05$). The picture below is the same



Figure 2

Structure and morphology 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.

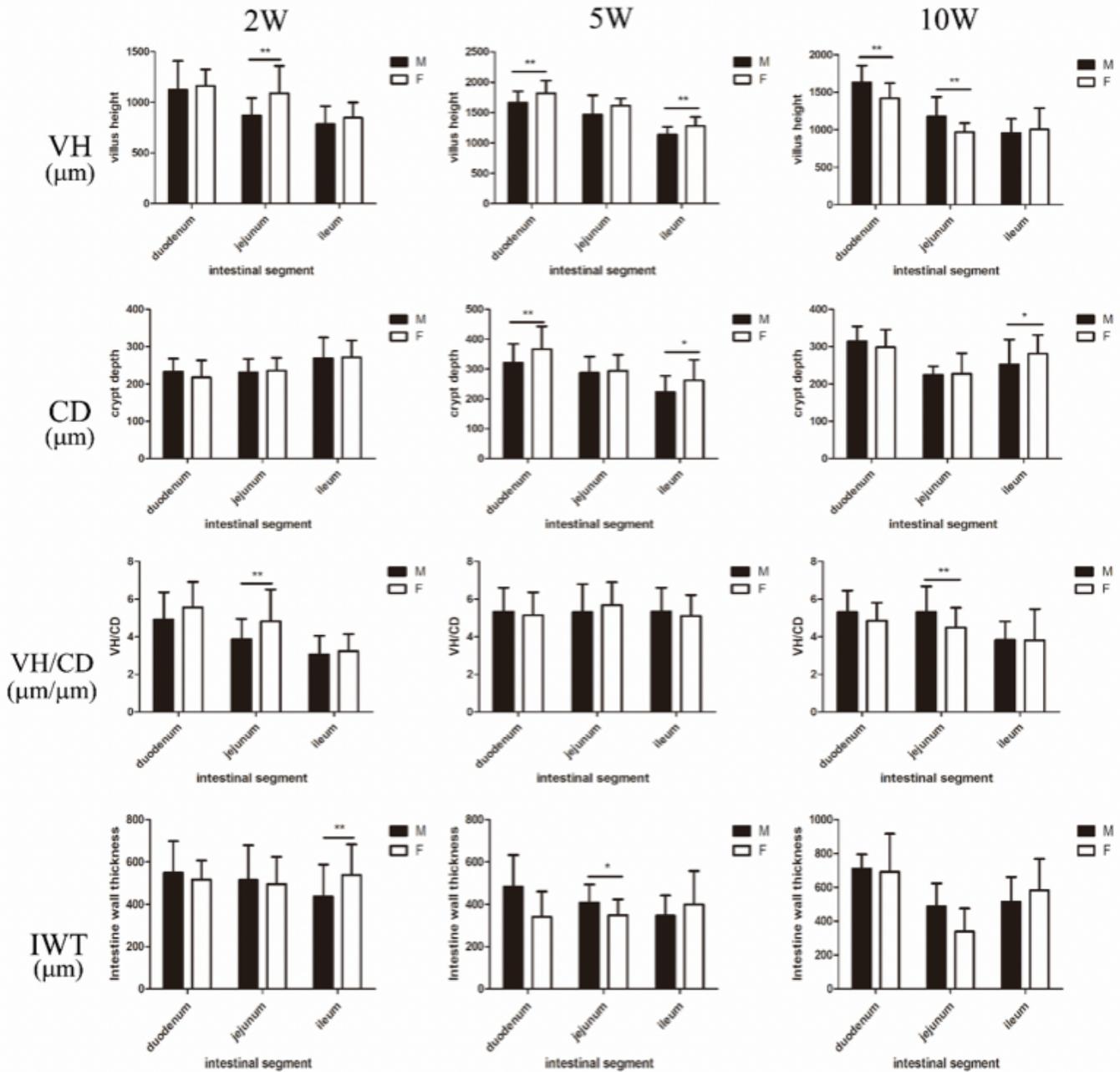


Figure 3

Morphological structure of small intestine in male and female ducks. VH, CD and IWT in the figure respectively represent the villi height, crypt depth and the intestine wall thickness.

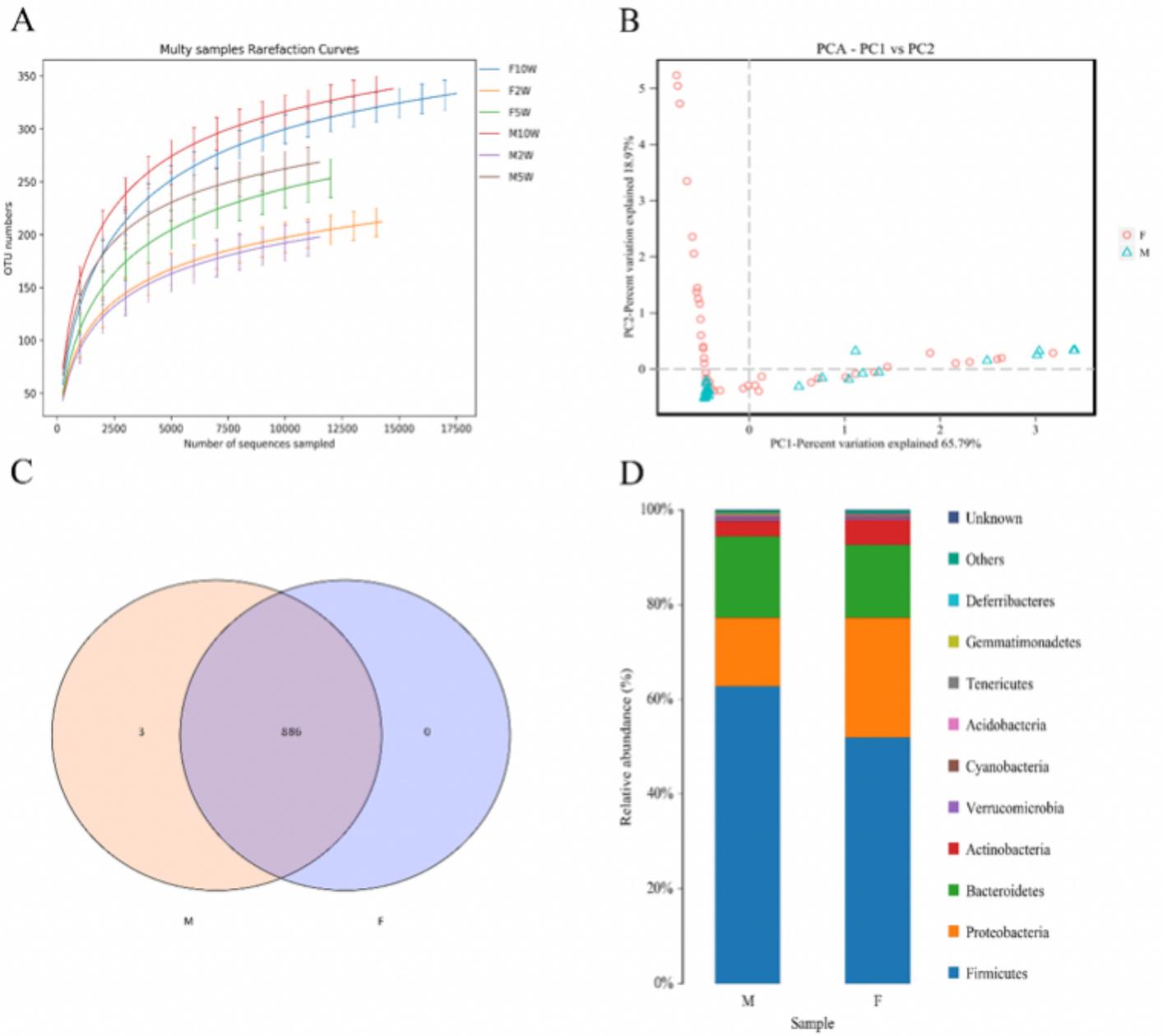


Figure 4

The effects of gender on duck intestinal microorganism. A: The multy samples rarefaction curves of male and female ducks at 2, 5 and 10 weeks. B: The PCA analysis of total intestinal microorganism in two genders. C: Detected OTUs of intestine microorganisms in male and female ducks. D: Species distribution of total microorganisms. The figure shows the top 10 microorganisms in proportion

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