# An altered gut microbiota in duck-origin parvovirus infection on cherry valley ducklings is associated with mucosal barrier dysfunction

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ABSTRACT Duck-origin parvovirus disease is an epidemic disease mainly caused by duck-origin goose parvovirus (**D-GPV**), which is characterized by beak atrophy and dwarfism syndrome. Its main symptoms are persistent diarrhea, skeletal dysplasia, and growth retardation. However, the pathogenesis of Cherry Valley ducks infected by D-GPV has not been studied thoroughly. To perceive the distribution of D-GPV in the intestinal tract, intestinal morphological development, intestinal permeability, inflammatory cytokines in Cherry Valley ducks, and expression of tight junction protein, the D-GPV infection was given intramuscularly. Illumina MiSeq sequencing technology was used to analyze the diversity and structure of ileum flora and content of short-chain fatty acids of its metabolites. To investigate the relationship between intestinal flora changes and intestinal barrier function after D-GPV infection on Cherry Valley ducks is of great theoretical and practical significance for further understanding the pathogenesis of D-GPV and the structure of intestinal for a in ducks. The results showed that D-GPV infection was accompanied by intestinal inflammation and barrier dysfunction. At this time, the decrease of a large number of beneficial bacteria and the content of short-chain fatty acids in intestinal flora led to the weakening of colonization resistance of the intestinal flora and the accumulation of potentially pathogenic bacteria, which would aggravate the negative effect of D-GPV damage to the intestinal tract. Furthermore, a significant increase in Unclassified S24-7 and decrease in Streptococcus was observed in D-GPV persistent, indicating the disruption in the structure of gut microbiota. Notably, the shift of microbiota was associated with the transcription of tight-junction protein and immune-associated cytokines. These results indicate that altered ileum microbiota, intestinal barrier, and immune dysfunction are associated with D-GPV infection. Therefore, there is a relationship between the intestinal barrier dysfunction and dysbiosis caused by D-GPV, but the specific mechanism needs to be further explored.

Key words: D-GPV, gut microbiome, intestinal barrier dysfunction, immune dysfunction

#### INTRODUCTION

Duck-origin parvovirus (**D-GPV**) is a novel goose parvovirus which has caused serious side-effects on the health of ducks and contributed toward great economic loss to the breeding industry since 2015 in China (Chen et al., 2015b). Cherry Valley ducklings, mule ducklings, and all experimentally infected ducks were  $\frac{2021 \ Poultry \ Science \ 100:101021}{https://doi.org/10.1016/j.psj.2021.101021}$ 

highly sensitive to D-GPV infection and showed pathological symptoms similar to naturally infected ducks, such as the protruded tongue, beak atrophy, diarrhea, growth retardation, and fractured legs (Chen et al., 2015a, 2016a; Ning et al., 2018). Meanwhile, significant changes in diagnostic pathological lesions have not been found in sick or dead ducks. The range of morbidity is 10-100%, and mortality was as low as 0-10% because of greater resistance of age (Chen et al., 2015a, 2016a,b). Hereafter, a strain of beak atrophy and dwarfism syndrome from Cherry Valley ducklings was reported, which identified as the GPV variant strain (D-GPV QH-L01), and it was closely associated to SYG26-35 GPV strains (Chen et al., 2017). Previous studies have been reported that epithelial cell abscission and inflammatory cell infiltrates were observed in duodenum and

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ileum after D-GPV infection (Chen et al., 2017; Liu et al., 2018). In addition, alimentary infection is one way of the D-GPV transmission (Chen et al., 2016b), which elaborates that the virus passes from the lumen of the intestine via epithelial cells of the intestine, which is inhabited by gut microbiota and represents a physical and immunological barrier (Linden et al., 2008; Turner, 2009). Therefore, the gastrointestinal abnormalities in D-GPV infection seems to be associated with the disturbance in epithelial integrity. However, the association of D-GPV infection with gastrointestinal abnormalities is not clearly fully understood. However, we have previously preliminarily explored the association between D-GPV injury and cecal flora (Luo et al., 2019), and here we would like to investigate whether D-GPV has a similar association with the ileum flora.

The microbiota plays an integral role in modulating host health (Cho and Blaser, 2012; Gilbert et al., 2016). Particularly, the formation of normal intestinal microbial colonization is important for the postnatal barrier maturation for protection against pathogenic bacteria (Huang et al., 2013). In addition, gut microbiota stimulates the maturation of the mucosal innate immune system (Hooper et al., 2012; Tremaroli and Bäckhed, 2012; Kim et al., 2016) and regulates immune homeostasis by several different mechanisms (Artis, 2008; Slack et al., 2009). However, most of the studies have been reported that the alterations of gut microbiota and changes in intestinal physical and immunological barrier functions are associated with the pathogenicity of many pathogens (Yan et al., 2013; Dillon et al., 2014; Li et al., 2018). Most of the bacteria are symbiotic; however, after translocation through the mucosa or under specific conditions, such as immunodeficiency caused by virus infection, commensal bacteria can cause infection (Marchetti et al., 2008; Petrova et al., 2013; Glavan et al., 2016). Certain gut bacteria may stimulate toll-like receptor to activate an immune response to protect the virus (Ichinohe et al., 2011), but not all symbiotic bacteria are responsible for host protection.

Poultry have billions of species of bacteria in their guts, including pathogenic, nonpathogenic, normal flora, and opportunistic bacterial species (Adegunloye, 2006). One of the main sites of pathogen exposure is the gastrointestinal tract. Thus, homeostatic association between host and gut microbiota can be threatened by the infection in such a highly reactive environment. Considering the potential relationship between viral infection and gut microbiota (Wilks and Golovkina, 2012; Lynch, 2014) and the vital role of the immune system in regulating the gut microbiota (Hepworth et al., 2013; Sun et al., 2015), it is really important to understand the changes in the composition of the microbiota after D-GPV infection and how these changes affect the pathogenic mechanism of the disease-causing pathogen.

So far, there have been no detailed and comprehensive studies on the ileum microflora in Cherry Valley ducks infected with D-GPV. In this study, we investigated the bacterial community composition characteristics of ileum from Cherry Valley ducks during various times after viral infection by examining whether there is a potential link between the gut microbiota, intestinal epithelial barrier, and mucosal immune system in D-GPV infection. This study provides a preliminary basis to explore the relationship between D-GPV and the microbiota of Cherry Valley ducks.

### MATERIAL AND METHODS

## Experimental Animals and Duck-Origin Parvovirus

The QH-L01 strain of D-GPV was isolated in Sichuan province, China, from the liver of Cherry Valley duckling flock having beak atrophy and dwarfism syndrome (Chen et al., 2017). Fourty Cherry Valley ducklings were gained from the breeding facility of the Institute of Poultry Sciences, Sichuan Agricultural University, and they were confirmed to be D-GPV antigen-free by PCR detection at the age of 2 d. According to our prior study, the titer of QH-L01 strain was estimated at  $10^{6.54}$  EID<sub>50</sub>/0.2 mL for intramuscular challenge (Luo et al., 2019).

## Animal Experiments

Fourty Cherry Valley ducklings, 2 d of age, were randomly divided into a control group and infection group, kept in the animal house with a negative pressure isolator, and given commercial food and water ad libitum. At the age of 2 d, the infection group was inoculated with D-GPV QH-L01 at  $10^{6.54}$  EID<sub>50</sub>/0.2 mL. An equivalent volume of the sterile nutrient solution was inoculated into the control group. The clinical signs of disease and the bodyweight of all ducklings were measured daily. From each of the groups, 10 ducklings were euthanized and autopsied on 6 and 15 d postinfection (dpi) upon completion of the experiments, respectively. All the animal trials were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University and followed the standard protocols provided by National Institute of Health.

#### Sample Collection

The PCR analysis of cloacal swabs detected that all the infected ducklings were positively infected as described in the previous studies (Chen et al., 2017; Luo et al., 2019). The blood samples were collected in a pyrogen-free vacuum anticoagulant vessel via the cervical vein from every duckling at 6 and 15 dpi before euthanizing to separate the plasma for plasma D (-)-lactate and lipopolysaccharide (LPS) detection. Half of the ileum and its contents were removed aseptically from each duck to store at  $-80^{\circ}$ C to process these samples for short-chain fatty acids (SCFAs) analysis, RNA isolation, and total microbial DNA analysis. The remaining half of the ileum samples were preserved in 4% formaldehyde for fixation and embedding process in paraffin wax. These embedded samples were cut into 5- $\mu$ m-thick sections and stained with hematoxylin (**H**) and eosin (**E**), and the virus antigen was detected with immunohistochemical (**IHC**) staining. The paraffin section of ileum was stained with glycogen, and the number of goblet cells per 100 epithelial cells was calculated.

## Immunohistochemical and Histopathological Analyses

IHC staining was performed with slight modifications as compared to conventional protocol. The monoclonal antibody of rabbit against GPV VP3 (Beijing Bioss Biotechnology Co., Ltd., China) was diluted in 1:200, as the primary antibodies. After the incubation of overnight at 4°C in primary antibody, it was washed in phosphate buffered saline solution thrice. Thin sections were incubated at 37°C for 30 min with mouse antirabbit secondary antibody (Boster Biological Technology Co., Ltd., China). The protocols of the immunoassay kit were followed to perform all the procedures. The negative staining cells were blue, while the positive staining cells appeared dark-brown, indicating the presence of D-GPV antigen under the light microscope (Nikon 80i). Ileum samples were concurrently stained with H&E, before catching under a light microscope to assess the histopathology to alleviate the histopathology of ileum samples under the light microscope, before H&E staining was performed.

### Enzyme-Linked Immunosorbent Assay

The plasma D (-)-lactate and LPS have been confirmed as a plasma biomarker of intestinal barrier injury both in mammals and poultry (Sun et al., 2001). The plasma samples were assessed for D (-)-lactate and LPS levels according to the recommended procedures in enzyme-linked immunosorbent assay kit manual (Shanghai Enzyme-linked Biotechnology Co., Ltd., China).

#### RNA Isolation and cDNA Preparation

Total RNA was extracted from selected ileum tissues by using RNAiso Plus reagent (Takara Biomedical Technology (Beijing) Co., Ltd.). The integrity and quantity of the RNA in each sample were determined by a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). cDNA was prepared by using a PrimeScript RT reagent Kit with gDNA Eraser in accordance with the manufacturer's instructions (Takara Biomedical Technology, Beijing, Co., Ltd.). Finally, the samples were stored at  $-80^{\circ}$ C until the need for next use.

#### Real-time Quantitative PCR

The expressions of tight junction proteins and immune-related intestinal mucosal cytokines in ileum were detected by the  $2^{-\Delta\Delta CT}$  method with relative real-time quantitative PCR (**qRT-PCR**). The specific primers used in **qRT-PCR** analysis of gene are given in Table 1. The qRT-PCR was performed using CFX96 Real-Time Detection System (Bio-Rad, Hercules, CA). Candidate gene expression was normalized to GAPDH that was used as standard for constitutively expressed endogenous control gene.

#### Bacterial 16S rRNA Gene Sequencing

Bacterial DNA was extracted for broad range of amplification, and 16S rRNA genes sequence analysis was performed using the previously described method (De et al., 2015). Briefly, DNA was extracted from ileum and its contents by using the Fast DNA SPIN extraction kit (MP Biomedicals, Santa Ana, CA) using manufacturer's instructions. The quality and quantity of extracted DNAs were measured with a NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis, respectively. Bacterial 16S rRNA genes from V3-V4 region were amplified using the forward and reverse primers, and their sequence are 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), respectively. Amplicons of PCR were purified using Agencourt AMPure beads (Beckman Coulter, Indianapolis, IN). After quantification of DNA concentration by using a PicoGreen dsDNA Assav Kit (Invitrogen, Carlsbad, CA), amplicons were pooled in equal quantity. Pairend 2  $\times$  300-bp sequencing was performed at Illumina MiSeq (http://www.personalbio.cn) platform with a MiSeq Reagent Kit v3 purchased from Shanghai Personal Biotechnology Co., Ltd. (China). The Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0, Rob Knight, Boulder, CO) pipeline was used to process the sequencing data, as mentioned previously in a protocol (Caporaso et al., 2010). Sequence analyses were mainly performed using R packages (v3.2.0)and QIIME for statistical analysis and bioinformatics (Caporaso et al., 2010), and all steps necessitated comparison of sequences with a reference database, for example, GreenGenes database (Desantis et al., 2006). Clustering of reads that passed quality controls were further processed into operational taxonomic units through an open-reference strategy with 97% threshold identity (Edgar, 2010).

## Determination of SCFA in lleum

The SCFAs of ileum contents were extracted with a slight modification as described by Lee et al. (Lee et al., 1999). Ileum content samples of weight 1 g were used and diluted at a ratio of 1:4 to 1:8 (w/v) in sterile distilled water. The samples were then vortexed for 1 min, and the homogenate was centrifuged at 5,000 rpm for 10 min. The filtration of SCFA-containing supernatant was performed by using a cellulose acetate membrane with a pore size of 0.22  $\mu$ m. SCFA analysis was performed using high-performance liquid chromatography as described by Huda-Faujan et al. (2010).

 Table 1. List and sequences of primers used in this study.

| Gene name | Sense primer $(5'-3')$   | Anti-sense primer $(5'-3')$ |
|-----------|--------------------------|-----------------------------|
| GAPDH     | TGCTTGCTGCTCCTCCTCAT     | TGGCTACCACTTGGACTTTGC       |
| ZO-1      | CACCTCAGAATAAGCCAGCCAT   | TTGTAAAGCGGTTGTAAGAAGGAG    |
| Occludin  | GCAGGATGTGGCAGAGGAATA    | TGCGCTTGATGTGGAAGAGTT       |
| Claudin-3 | CGGCGTCATCTTCCTGCTCT     | CCAGCTCCCTCTTCTGCGATT       |
| IL-22     | TTCCTGGCATCCCTGACCTC     | ATTCTTTCCATTCTCTCCCAACTGT   |
| MHC-I     | GCATTGAGTGGCTGAGGAAACA   | AGCTGATGGAGATGGGTCGC        |
| MHC-II    | GTCACTACCTCAATGGCACCG    | AAGGCTTGCCCAGTTCCGT         |
| IFN-α     | ACCAACGACACGCAGCAAGC     | TGTGGTTCTGGAGGAAGTGTTGGAT   |
| IFN-β     | GCAACCTTCACCTCAGCATCA    | ATCCGCCGTATTAGCGTGTC        |
| IFN-γ     | AGTAATTCGGATGTAGCTGATGGC | TCTTTAGCATTTCCAAGTACAGGGT   |
| IL-6      | TCTGGCAACGACGATAAGGC     | AATGAAGTAAAGTCTCGGAGGATGA   |
| CD8α      | CCTGCTTGCTGCTTCTCATTG    | TTGGCACCTTGGGATTCATT        |

#### Statistical Analysis

According to  $\beta$  diversity measurements, the structure and composition of the duck ileal microbiome population were significantly correlated with the age (or infection times) and D-GPV infection. In unweighted UniFrac Principal Coordinates Analysis (PCOA), samples from D-GPV-infected ducks in different times were isolated from controls on one axis (left to right), and samples from infected 6 d were separated from 15 d on a distinct axis of variation (top to bottom). In order to reveal the relationship between the presence or absence of bacterial groups and their phylogenetic correlations, we used the UniFrac index based on the phylogenetic tree to perform principal coordinate (PCOA) analysis on the samples. In unweighted Uni-Frac principal coordinates analysis, a point in the multidimensional space represents a sample based on the composition of the bacterial population in each sample. In order to examine the overall difference in bacterial composition over time between D-GPVinfected ducks and control ducks, the distance between each sample was calculated using Bray-Curtis similarity and unweighted UniFrac.

The statistical calculations were carried out with tests by using the SPSS software package 20.0. The Shapiro-Wilk test was performed to check the normality of distribution of variables. The normal distribution of variables was found by using the independent samples t test procedure. Nonparametric statistics were accomplished with no adjustments for multiple comparisons becaue of the exploratory nature of the study. The Statistical comparisons of bacterial communities were performed with R software (http://www.r-project.org/). The overall statistical differences between the gut microbiomes of negative control and D-GPV-infected ducks were assessed at the phylum, family, and genus levels by using the QIIME software package (http://giime.org/). Principal coordinates analysis was applied to see the differences between the infected and uninfected samples vs. days. Statistical differences in operational taxonomic units at each level of taxonomy were analyzed by Wilcoxon test. Pearson rank correlation was performed to evaluate associations between 2 variables. Significance was declared at P < 0.05. For principal coordinates

analysis, distance matrices were calculated with the Bray-Curtis ecological dissimilarity index (vegdist function of vegan R package) and applied to the cmdscale function in R.

#### RESULTS

## D-GPV Targeted Intestinal Epithelial Cells and Caused Intestinal Structure Injury

Daily weight gain of D-GPV-infected Cherry Valley ducklings gradually decreased from 3 to 15 d compared with that of saline-treated controls, which occurred with mild diarrhea over the same period (Figure 1A). Most of the D-GPV-infected ducks were found to be mesenteric hyperemia, and the contents of the intestines were blackened with latent blood. The epithelial cells of ileum mucosa became necrotic and exfoliated; however, the epithelial cells of lamina propria became proliferated after D-GPV infection (Figure 1B). Moreover, the crypt depth of ileum was significantly increased, while villus height and the ratio of villus height to crypt depth were significantly decreased compared with the control group (Figure 1C). These results suggested that D-GPV affected the development of intestinal morphology and decreased the intestinal villi height and the ratio of villi height to crypt depth which resulted to create an obstacle for digestion and absorption.

In order to detect whether intestinal damage of QH-L01 infected cherry valley ducks was a feature, we first detected virus load from the ileum (Figure 1D). The peak of virus load was observed at 6 dpi (days after infection), and mucosal and glandular epithelial cells were strongly invaded by the D-GPV antigen. However, the antigen was mainly distributed in the basal area, the basal membrane of epithelial cells, and the lamina propria cells at 15 dpi (Figure 1E).

The number of goblet cells in the ileum of the infected group was significantly reduced at 6 and 15 dpi compared with the control group (Figure 1F). However, insignificant difference was observed between peripheral index of the immune organs in each treatment (Figure 1G). Together, these data indicated that D-GPV infection may affect only severe mucosal immune injury in the intestine.



Figure 1. The location and histopathological changes of D-GPV antigen in ileum. (A) Daily weight growth rate and diarrhea score after D-GPV infection in Cherry Valley ducklings. (B) Hematoxylin and eosin staining show the histological features in the D-GPV infection group (i) and control group (c) at 6 dpi and 15 dpi. The red arrows indicate necrosis and abscission of mucous epithelial cells. The black arrows indicate proliferation of lymphocytes in lamina propria. (C) After infection, the ileum crypt depth increased obviously, and the villi height and villi height and crypt depth were significantly decreased. (D) Detection of virus load in ileum. (E) Positive virus signals were detected on the surface of epithelium cells and glandular epithelial cells at 6 d after infection (dpi). Positive virus signals were mainly detected in basal and basement membrane of cells at 15 dpi. (F) Glycogen staining and statistical analysis of goblet cells in ileum (\*P < 0.05 and \*\*P < 0.01). The black arrows indicate goblet cells. (G) Index of Immune organs.

## D-GPV Infection Damages Intestinal Tight Junction and Increases Intestinal Permeability

As tight junctions are functional junctions between epithelial cells, which have a vital function in maintaining material transport and osmotic balance, we wanted to explore whether D-GPV infection would affect the expression of tight junction proteins. ZO-1, a peripheral membrane protein, was significantly downregulated (P < 0.05) at 6 dpi and 15 dpi (Figure 2A). Occludin and claudin-3, the major transmembrane proteins related to the cell membrane permeability, were both significantly downregulated at 6 dpi (P < 0.01) and 15 dpi (P < 0.05) (Figure 2A). The results suggested that tight junctions in the mucosal epithelial cell were injured after D-GPV infection. The plasma levels of D (-)-lactate and LPS were significantly increased at 6 dpi (P < 0.01) and 15 dpi (P < 0.05) compared with the control group as shown in (Figures 2B and 2C). Noteworthy, the plasma levels of D (-)-lactate and LPS also changed in the control group over time; however, the increased tendency in D-GPV infected group was more significant at 6 dpi (P < 0.01). The increase of D (-)-lactate and LPS as plasma biomarker hinted at the raise of intestinal permeability and the injury of intestinal barrier function. Moreover, the damage was more severe after 6 d of D-GPV infection. The plasma levels of serum immune factors TNF- $\alpha$ , IL-6, and IL-1 $\beta$  were increased at both 6 dpi and 15 dpi





compared with the control group (Figures 2D–2F). However, the expression of IL-1 $\beta$ , IL-6, IL-17a, IL-22, IFN- $\alpha$ , IFN- $\beta$ , and TNF- $\alpha$  significantly increased, while IL-4 and IL-10 significantly decreased (Figures 2G and 2H).

## Effect of D-GPV Infection on the Diversity and Structure of Ileum Microflora

Multiple  $\alpha$  diversity index showed that there were significant differences in microbial population richness and diversity of ducks in different periods during the

growth period. Compared with the control group, the ACE, Chao, and Simpson abundance indices of D-GPV-infected ducks did not reflect the richness and diversity of the samples. But the Shannon analysis of D-GPV-infected ducks showed a significant decrease in the sample diversity at 15 dpi (Figure 3A). There was a significant and positive correlation between viral load, ACE, and Chao1 in the tissue. Combined with Figure 3B, it can be observed that in the detoxification period (I6), ACE and Chao1 were positively correlated with viral load. In the stable period (I15), the



Figure 2. D-GPV Infection damages intestinal tight junction and increases intestinal permeability. (A) The tight junction gene expression of ZO-1, occludin, and claudin-3 at 6 dpi and 15 dpi. (B) The plasma levels of D (-)-lactate at 6 dpi and 15 dpi. (C) The plasma levels of LPS at 6 dpi and 15 dpi. (D) The plasma levels of TNF- $\alpha$  at 6 dpi and 15 dpi. (E) The plasma levels of IL-6 at 6 dpi and 15 dpi. (F) The plasma levels of IL-1 $\beta$  at 6 dpi and 15 dpi. (G) The expression of inflammatory cytokines at 6 dpi. (H) The expression of inflammatory cytokines at 15 dpi. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001.

correlation was negative. In general, the diversity of the microbial populations increased significantly on all indicators as the ducks matured without infection. Nevertheless, there was no significant difference between D-GPV–infected groups at maturity. Collectively, these data indicated that the increase in the diversity of the microbial population was hindered by the prolongation of D-GPV infection.

The proximity of the 2 points in PCOA indicates that the bacterial population composition is similar between samples. The halo around each point described the change in the point position observed in the threedimensional space after the folding knife resampling at the specified sparse level (Figure 3C). The top 20 dominant bacterial groups are listed in Figure 3D, among which the positive correlation with viral load is Unclassified\_Lachnospiraceae (r = 0.632, P = 0.004) and Cupriavidus (r = 0.465, P = 0.045) Megamonas (r = -0.482, P = 0.036) and Oscillospira (r = -0.476, P = 0.040) were negatively correlated (Figure 3E).



Figure 3. D-GPV infection decreased the diversity of ileum microflora and changed its structure. (A) Abundance and diversity of microorganisms in ileum of uninfected and D-GPV-infected ducks. Values are displayed in minimum to maximum, and the mean value of each group is calculated. \*Statistical tests were performed using Mann-Whitney *U*-test, and ns indicated no significant differences. (B) The correlation between  $\alpha$  diversity and viral load. (C) Principal coordinate analysis of unweighted UniFrac distances. Throughout the growth process, samples are colored by different groups—Orange, control-6 d; Green, D-GPV infected-6 d; Blue, control-15 d; Red, D-GPV infected-15 d. The axes are scaled according to the percentage change in the interpretation of each principal coordinate. (D) The first 20 dominant bacterial groups. (E) Dominant bacterial groups associated with viral load. (F) Cluster analysis based on unweighted UniFrac distance UPGMA. \*\*P < 0.01 and \*\*\*P < 0.001.

The results showed that D-GPV infection and age (or infection times) had an obvious clustering relationship with individual ileum samples, suggesting that the main change of D-GPV–infected ducks compared with control ducks was the composition of ileum microorganism. Using Bray-Curtis similarity, unweighted arithmetic mean matching method was used to stratify the samples, further confirming that the clustering pattern of D-GPV–infected ducks was different from that of the control group (Figure 3F).

Differences between groups were statistically significant in the multivariate analysis of variance (Adonis) test based on differences (unweighted UniFrac distances, DF = 3.999 simulations, F = 5.3672, R<sup>2</sup> = 0.32792, P = 0.001). Pairwise comparison of each group was statistically significant (pairwise adonis, unweighted UniFrac distances, DF = 1.999 simulations, F = 3.1393–6.9043, R<sup>2</sup> = 0.24–0.91, P = 0.001).

# Comparisons of Intestinal Microorganisms at Different Levels

Three dominant phyla, for example, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, were detected in the ileum of ducks from control and infected groups (Figure 4A). The remaining phyla each accounted

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for <5% of the total abundance. There was no significant difference (P > 0.05) between uninfected and D-GPV-infected ducks at the phylum level. However, when we individually compared uninfected and D-GPV-infected ducks with the relative abundance of the phyla, the average relative abundance of *Tenericutes* at 6 dpi and *Actinobacteria* at 15 dpi were obviously enriched in D-GPV infection group, while the average relative abundance of Firmicutes decreased at 15 dpi (Figure 4B). This suggested that some beneficial bacteria could significantly inhibit the growth of certain pathogens.

There was a significant difference (P < 0.01) of bacteria at the family level in the overall relative abundances between uninfected and D-GPV-infected ducks (Figure 4C). Specifically, the *Streptococcaceae* of *Firmicutes* phylum was significantly inhibited, and *S24-7* of *Bacteroidetes* phylum was significantly enriched after D-GPV infection. The relative abundance of *Streptococcaceae* (P = 0.001) and *Enterococcaceae* (P = 0.046)

was statistically lower in D-GPV-infected ducks, whereas *Erysipelotrichaceae* (P = 0.027) and S24-7 (P = 0.036) were higher in the infection group relative to control group at 6 dpi. However, the relative abundance of *Streptococcaceae* (P = 0.019) was statistically lower in D-GPV in infected ducks, while S24-7 (P = 0.015) was higher than that in uninfected ducks at 15 dpi.

As shown in Figures 4D and 4E the relative abundance of *Candidatus Arthromitus* of the *Firmicute* phylum was significantly (P = 0.001) lower in D-GPV-infected ducks, while *Unclassified Lachnospiraceae* and *Streptococcus* were significantly (P = 0.001) higher than those of uninfected ducks at 6 dpi. There was a significant reduction of *Lactobacillus*, *Unclassified\_Clostridiales*, and *Faecalibacterium* observed within *Firmicutes* phylum at 15 dpi in D-GPV-infected ducks. There was a significant shift in *Bacteroidetes* phylum: The relative abundance of *Unclassified\_S24-*7 was significantly decreased at 15 dpi in conjunction with an increase in relative abundance at 6 dpi within



Figure 4. D-GPV infection alters the composition of intestinal microbiota. (A) Stacked bars represent the average relative abundance of the top 10 most abundant phyla within ileum. "Others" refers to bacteria that have not been identified to belong to any known phylum. The Adonis function of Vegan Community Ecology R package was used for statistical analysis, and there was no significant difference existed at the phylum level between control and D-GPV infection groups. (B) Comparison of the relative abundance of the Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria phyla within control and D-GPV–infected ducks. Values are displayed as a fraction of the total bacteria detected within each sample. Lines represent the median value. The Mann-Whitney *U*-test was used for statistical analysis. (C) The average relative abundance of the first 20 families in ileum of the control group and D-GPV–infected group was indicated by the stacked bars. The mean relative abundance of the 2 groups was statistically different. "P < 0.05, "P < 0.01. (D) Figure of the Mann-Whitney U-test assessment showing a statistically significant difference in the relative abundance of individuals of different genera between the control group and D-GPV–infected ducks. Values are shown as a fraction of the total bacteria detected within each individual. Lines represent the median value. (E) Comparison of relative abundance of different genera in Firmicutes, Proteobacteria, and Bacteroidetes between control group and D-GPV infection group.

the *Bacteroidetes* phylum in D-GPV–infected ducks. Besides, D-GPV–infected ducks also had a significant increase in the relative abundance of *Proteobacteria* phylum at 6 dpi. Conclusively, along with the extension of infection time, the effect of D-GPV on the rate and order of colonization of intestinal microorganisms was more significant.

## Associations Between lleum Microbiota, Tight Junction Protein, Cytokines, and Plasma LPS Level

We focused on the analysis of 29 most predominant bacterial genera with a relative abundance  $\geq 0.1\%$  in each sample (Figure 5) and have observed significant



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Samples

associations in major genera that were statistically associated with ZO-1, claudin-3, LPS, IL-22, IFN- $\gamma$ , IL-6, IFN- $\alpha$ , MHC-I, MHC-II, and CD8 $\alpha$ ; Unclassified\_S24-7, Unclassified\_Lachnospiraceae, Streptococcus, Sutterella, Prevotella, and Ruminonococcus were altered after D-GPV infection. Specifically, Streptococcus genus significantly reduced after D-GPV infection at both 6 dpi and 15 dpi, and it was positively associated with MHC-I and CD8 $\alpha$ , indicating that the decrease of Streptococcus was relative to the reduction of cellular immune function. Noteworthy, the enrichment of gram-negative bacteria Unclassified\_S24-7 after D-GPV infection at both 6 dpi and 15 dpi was negatively associated with IFN- $\gamma$  and, conversely, positively associated with LPS. Furthermore, tight-junction protein, especially claudin-3, was significantly positively correlated with many genera producing SCFA, including *Bacteroides*, *Unclassified\_Lachnospiraceae*, and *Dorea* producing gas from carbohydrates.

## The Difference of SCFA Content Was Associated With Ileum Microorganism

The production of SCFAs (short-chain fatty acids) was related to the metabolic process of the intestinal microorganisms. As part of our study, the content of SCFA in the ileum was detected by the high-performance liquid chromatography method, and the ileum concentration of



Figure 4. Continued.

acetic acids, propionic acids, and butyric acids was determined and compared between uninfected and D-GPV-infected ducks at 6 dpi and 15 dpi. The content of acetic acids was lower at 6 dpi and 15 dpi of D-GPV infection, while the content of butyric acid was lower at 15 dpi (Figure 6). There was no significant difference in propionic acids between D-GPV infection groups and controls. The decrease of acetic acid might link to the abatement of *Streptococcus*, a primary acetogen. Moreover, the decreased relative abundance of *Lactobacillus*, *Butyricimonas*, *Faecalibacterium*, *Prevotella*, and *Ruminococcus* was associated with the decrease of butyric acids at 15 dpi.

## DISCUSSION

The gut microbiota has a positive impact on health and profoundly influences the normal structural and functional advancement of the mucosal immune system. It is known that gut has prime importance to the health of poultry; however, fewer studies have focused on the complexity of gut microbial community with the virus infection. In the present study, we compared the structure and composition of ileum microbiota of cherry valley ducklings and investigated the interaction between intestinal barrier dysfunction and intestinal microorganism under D-GPV infection during various times. Consequently, we explored the new intersections among microbiology and virology which will help to categorize the key mechanisms affecting viral infections.

We has a realization that the intestinal epithelium is not merely a static barrier to the external environment but can function effectively to prevent the invasion of pathogens. The key to the maturation of the postnatal intestinal barrier is the establishment of normal intestinal microbial values (Huang et al., 2013). The complex relationship between microbiota and host immune system attributed to the establishment and maintenance of intestinal homeostasis (Slack et al., 2009; Hooper et al., 2012). In the present study, D-GPV was detected



Figure 5. Correlation analyses between the top 29 most abundant genera, the levels of tight junction protein, cytokines expression, and plasma LPS levels. According to the Pearson correlation coefficient, tight-junction protein expression was positively (shaded in blue) and negatively (shaded in red) correlated with immune-related cytokines and plasma LPS levels in heatmap. \*P < 0.05, \*\*P < 0.01. Clustering was performed based on genera associations with the tight-junction protein, immune-related cytokines, and LPS. Genera are color-coded based on their respective phylum. Genera that were altered in D-GPV-infected ducks relative to uninfected ducks are underlined.

in the epithelial cells and in lymphocytes from the lamina propria of ileum in cherry valley ducks. While D-GPV infection leads to altered mucosal immunity as well as significant structural disruption in the gastrointestinal tract, we investigated whether D-GPV-infected ducks had specific microbiota associated with tight junction protein expression, various cellular immune-associated cytokines, proinflammatory cytokines, and plasma LPS indicative of systemic inflammation, microbial translocation, and epithelial damage. The Pearson rank correlation coefficients of relative abundance among various genera were summarized in the heat map with intestinal epithelial damage parameters such as ZO-1, occluding, and claudin-3; immune response proteins such as MHC-I, MHC-II, and CD8a which associated with antigen presentation; and immunomodulationrelated cytokines such as IL-6, IL-22, IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$ , as well as LPS. Notably, not all genera clustered together in each phylum, suggesting that associations between genera and immune cytokines or



intestine damage do not depend solely on higher taxonomic rank. Goblet cell is a secretory cell in the intestinal mucosal epithelium, which is specially used to synthesize and secrete gelatinous mucin and plays an important regulatory role in mucous barrier and mucosal immunity. The decreased number of goblet cells after D-GPV infection reflects the low immune function of the intestinal mucosa.

Tight junction is the main connection between intestinal epithelial cells which plays an important role to regulate the permeability of intestinal epithelial cells (González-Mariscal et al., 2008) and hinder the small molecules, cells, and microbes to pass through the paracellular space (Steed et al., 2010; Shen, 2012) by comprising occludin, claudins, and 3 ZO proteins (Aijaz et al., 2006). It has been reported in previous studies that viruses could hijack different components of any organ to complete their infectious cycles (Torres-Flores and Arias, 2015). In order to achieve dissemination, many viruses infect the epithelial cells and take advantage of receptors or coreceptors (readily available in the apical region of epithelial cells) or open tight junctions by facilitating the degradation of multiple tight-junction proteins. According to the histopathological examination, increase of plasma D (-)-lactate and LPS and decrease in expression of claudin-3 after D-GPV infection contributed toward the increase of intestinal mucosal permeability. As the structural basis of intestinal barrier function and disruption of tight junction caused intestinal barrier dysfunction and increased intestinal permeability, it provided an opportunity for pathogens to invade the organism. So, we considered that D-GPV infection has caused intestinal barrier dysfunction. However, future studies are needed to elucidate whether the claudin-3 tight-junction protein is associated with D-GPV receptor or replication.

After D-GPV infected the ileum, the expression of various inflammatory cytokines significantly increased, which further activated the different pathways, such as NF- $\kappa$ B to downregulate the expression of tight-junction proteins, and hereafter contributed toward increase in intestinal permeability. Therefore, D-GPV infection resulted in decreased expression of intestinal tight junction protein and increased expression of proinflammatory cytokines, which may cause increased intestinal permeability and barrier dysfunction.

Basically, in our study, the transcription level of cytokines such as ZO-1, claudin-3, LPS, IL-22, IFN- $\gamma$ , IL-6, IFN- $\alpha$ , MHC-I, MHC-II, and CD8 $\alpha$  showed significant difference between different ages in control ducks, which was associated with the development of the gut immune barrier. T and natural killer cells after activation produce the IL-22 that further activates the innate immune responses against bacterial pathogens (particularly epithelial cells of the respiratory system and intestine) and plays a unique role in the coordinated response of adaptive innate immune systems, autoimmunity, and tissue regeneration (Nikoopour et al., 2015). IFN- $\gamma$  is an important activator of macrophages which induce MHC-II molecule expression (Giroux et al., 2003), while the IFN- $\alpha$  and IFN- $\beta$ , or type I interferon, promote the expression of MHC-I molecule (Jinushi et al., 2003), which play a key role in innate and adaptive immunity of poultry against viral and some bacterial infections (Santhakumar et al., 2017). The study showed that D-GPV significantly downregulated the expression of IFN- $\gamma$ , type I interferon (mainly IFN- $\beta$ ), MHC-I, and MHC-II, with an increase of proinflammatory cytokine IL-6 suggesting the disorder of intestinal immune function. Humoral and cellular immune responses induced by viruses require recognition of antigen by T cells. Therefore, the downregulated expression of MHC-I class molecules resulted in impaired function of cytotoxic function mediated by  $CD8^+$  T lymphocytes, which play a key antivirus role in D-GPV infections. Conclusively, D-GPV inhibits the intestinal mucosal immune system during persistent viral infection at the level of gene expression.

In this study, we reported a detailed and comprehensive relationship between D-GPV infection and ileum microbiota of Cherry Valley ducks. The structure and development of Cherry Valley ducks during a typical industrial growth period were studied by analyzing the microbial community composed of ileum contents and mucosal adhesion bacteria. Furthermore, the influence of D-GPV on bacteria is revealed. We compared the differences in microbial community structure and possible developmental changes between the uninfected and D-GPV-infected groups, looking for the presence and abundance of differential bacteria in the microbial community profile. The sequencing data were processed for taxonomic distribution, and  $\alpha$  and  $\beta$  diversity indexes were evaluated. In our study, significant changes were observed in the microbiome associated with the age of ducks and D-GPV infection environment, while no significant associations were observed with other factors, such as sex of ducks.

The major bacterial phyla in ileum microbiota of Cherry Valley ducks are *Bacteroidetes*, *Firmicutes*, and Proteobacteria. Based on our preliminary analysis of  $\alpha$  diversity and  $\beta$  diversity, it was found that the diversity of each indicator microbial population increased significantly with the maturity of ducks. However, D-GPV infection restricted the colonization of normal commensal microbiome and changed the structure and composition of microbiota. Particularly, the developmental progression has been characterized by some major transitions at different infection times after D-GPV infection, such as the increase of Tenericutes after 6 d of infection, while the decrease of Firmi*cutes* and the increase of *Actinobacteria* were reported after 15 d of infection. This alteration may be due to the maturation of intestinal barrier and immune function. It was noteworthy that the reduced relative

abundance of genera, including *Streptococcus*, *Sutterella*, *Prevotella*, and *Ruminonococcus*, was positively associated with MHC-I, which has a correlation with immune disruption, as observed in previous reports (Seksik et al., 2003; Gophna et al., 2006; Dillon et al., 2014). Previous studies have been reported that the ileum and jejunum mucosa of Peking ducks showed significant morphological changes in the first 7 d after hatching, including increase in villus height and crypt depth (Applegate et al., 1999; Applegate et al., 2005), with drastic changes of the cecal microbiome (Best et al., 2016).

Normal intestinal function prevents parasitic bacteria from invading host tissues through the establishment and maintenance of the mucosal epithelial barrier. Recent studies have shown that increasing the survival of intestinal epithelial cells is accomplished by certain symbiotic bacteria inhabiting the proapoptotic pathway of epithelial cells related to pathogenic bacteria (Ohland and Macnaughton, 2010). The intestinal symbiotic flora also participates in the maintenance of barrier function by inducing the proliferation of epithelial cells and improving the integrity of the intestinal epithelium, transporting tight junction proteins and upregulating genes involved in desmosome maintenance (Sharma et al., 2010; Ashida et al., 2011). In contrast, severe intestinal mucosal damage can be caused by the absence of intestinal commensal bacteria (Rakoffnahoum et al., 2004). Briefly, enteral colonization of normal intestinal flora is necessary to maintain the intestinal barrier function. In pathological conditions, there have been always community effects involving the gain and loss of bacterial populations and change of general metabolic functions (Turnbaugh et al., 2006; Cho and Blaser, 2012). The *Firmicutes* is the main phylum in the intestines or feces of many animals and contributes toward the digestibility of roughage (Mohd Shaufi et al., 2015). The core reason behind the growth retardation of D-GPVinfected Cherry Valley ducks was the unavailability of Streptococcus, Ruminococcus, Lactobacillus, Bulleidia, and Faecalibacterium of Firmicutes, which resulted in the reduction of nutrient digestion and absorption. SCFAs are the important end products of bacterial fermentation in the intestinal lumen and considered as key molecules that mediate the regulatory effect of normal intestinal microbiota on mucosal immune system and the establishment and maintenance of the intestinal mucosal barrier based on the previous in vitro studies (Sanderson, 2004). Acetic acid is the main source of energy provided by bacteria to the host, while butyric acid is mainly used by epithelial cells to enhance the intestinal barrier function by activating adenosine 5'-monophosphate-activated protein kinase and promoting the assembly of tight junctions (Peng et al., 2009). Therefore, this decrease of butyric acid after D-GPV infection was an incentive of intestinal barrier injury.

Differences in the composition of D-GPV infectionrelated bacterial communities in Cherry Valley ducklings were not a transient change in the ileum microbiome. The decrease of *Streptococcus* and the increase of Unclassified S24-7 were the main characteristics of D-GPV infection. The present study analyzed the correlation between the composition of ileum bacteria, immune, and inflammatory parameters and found that the decrease of *Streptococcus* has a positive association with the downregulation of MHC-I, while the increase of Unclassified S24-7 was negatively related to the downregulation of  $INF-\gamma$ . Furthermore, the aforementioned relationships were confirmed at both 6 dpi and 15 dpi. The *Streptococcus* is a unique human pathogen that causes a wide range of diseases, although it is a common symbiotic flora in poultry intestinal microbes. Streptococcus has the ability to regulate innate and adaptive immune responses and has direct or indirect effects on the immune system by secreting a cysteine protease (**SpeB**). Previous studies have shown that SpeB can degrade cytokines, chemokines, complement components, extracellular matrix, immunoglobulins, and serum protease inhibitors (Nelson et al., 2011). Interestingly, the Unclassified S24-7 genomes of S24-7 family also contain a homolog of SpeB which have been found in other intestinal bacteria, including *Bacteroides fragilis* and Bacteroides thetaiotaomicron, as well as the periodontal pathogen Prevotella intermedia in which the homolog interpain A is involved in suppression of the immune response through complement degradation (Michal et al., 2009). The variety existence of potential immune evasion peptidases within members of "S24-7" does not rule out a typically commensal relationship with the host. However, they may provide the ability to opportunistic infection under the appropriate conditions (Thornton et al., 2012). Furthergram-negative more. Unclassified S24-7, а bacterium, increased the plasma LPS-indicated translocation of bacterium, promoting the inflammatory response and inducing the immune disorder (Dillon et al., 2014).

Collectively, the dysfunction of intestinal barrier and immunologic function in D-GPV infection may be related to the altered gut microbiota, as the *Unclassified\_S24-7* and *Streptococcus* undergo significant changes during infection. This research provides new perspective for the research of the molecular mechanism of D-GPV and a theoretical basis for pathogenesis of this disease.

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The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence the work and that there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "An altered gut microbiota in duck-origin parvovirus infection on Cherry Valley ducklings is associated with mucosal barrier dysfunction".

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